ERRATUM

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In the printed version of this article, the acknowledgements section is incorrect. The correct version is shown below.

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Delta/Notch signaling promotes formation of zebrafish neural crest by repressing Neurogenin 1 function

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SUMMARY

In zebrafish, cells at the lateral edge of the neural plate become Rohon-Beard primary sensory neurons or neural crest. Delta/Notch signaling is required for neural crest formation. *ngn1* is expressed in primary neurons; inhibiting Ngn1 activity prevents Rohon-Beard cell formation but not formation of other primary neurons. Reducing Ngn1 activity in embryos lacking Delta/Notch signaling restores neural crest formation, indicating Delta/Notch signaling inhibits neurogenesis without actively promoting neural crest. Ngn1 activity is also required for later development of dorsal root ganglion sensory neurons; however, Rohon-Beard neurons and dorsal root ganglion neurons are not necessarily derived from the same precursor cell. We propose that temporally distinct episodes of Ngn1 activity in the same precursor population specify these two different types of sensory neurons.

Key words: Neural crest, Zebrafish, Delta/Notch signaling, Rohon-Beard neuron, bHLH, Neurogenin, Proneural genes, Neurogenic genes

INTRODUCTION

Although the molecular machinery that regulates neurogenesis is largely conserved between insects and vertebrates, the precise functions of neurogenic and proneural genes during vertebrate neurogenesis remain unresolved. During neurogenesis in the fly central nervous system, a cluster of ectodermal cells express proneural genes (PNG), that encode basic helix-loop-helix (bHLH) transcription factors, at a low level. Subsequently, the actions of neurogenic genes, including the ligand Delta and its receptor Notch, repress PNG expression in all but one cell of the cluster (Campos-Ortega, 1995). This cell expresses the PNG at high levels, becoming a neuronal precursor that delaminates from the ectodermal epithelium and undergoes a specific number of divisions ultimately yielding a defined number of neurons (Campos-Ortega, 1995).

Vertebrate homologs of proneural and neurogenic genes appear to function similarly to their insect counterparts. Thus, misexpression of vertebrate neural bHLH genes leads to ectopic neurogenesis, and loss of neural bHLH function leads to failure of formation or differentiation of subsets of neurons (reviewed by Kageyama and Nakanishi, 1997; Lee, 1997). Moreover, activation of Delta/Notch signaling suppresses neural bHLH gene expression in mice and other vertebrates (Kageyama and Ohtsuka, 1999), and loss of Delta/Notch signaling upregulates bHLH gene expression and leads to formation of supernumerary neurons (reviewed by Chan and Jan, 1999).

There are still unresolved questions about how vertebrate proneural and neurogenic gene homologs function during neurogenesis. For example, fly PNGs appear both to select neuronal precursors and to specify the type of neurons they generate. It is unclear whether both or perhaps just the first of these functions are carried out by vertebrate PNG homologs (reviewed by Brunet and Ghysen, 1999; Hassan and Bellen, 2000). Secondly, in both the central and peripheral nervous systems, fly PNGs specify neuronal precursor cells, which frequently undergo one or more rounds of cell division before differentiating. It is unknown whether vertebrate PNG homologs specify neuronal precursors or neurons themselves. Finally, recent studies have led to a new interpretation of the role of Delta/Notch signaling in vertebrate cell fate decisions. The prevailing model has been that Delta/Notch signaling prevents differentiation of all cell fates (Coffman et al., 1993; Struhl et al., 1993). However, several recent studies suggest that Delta/Notch signaling can promote gliogenesis in cultured mammalian neural crest cells (Morrison et al., 2000b), mammalian and zebrafish retina (Furukawa et al., 2000; Scheer et al., 2001), mammalian forebrain (Gaiano et al., 2000), and avian dorsal root gangalia (DRG) (Wakamatsu et al., 2000). It is important to determine whether Delta/Notch is actively promoting specific cell fates in other situations where it is employed.

PNG and neurogenic genes have been characterized in zebrafish. Homologs of fly neurogenic genes (*Delta*, *Notch*, *Suppressor of Hairless* and genes of the *Enhancer of split* complex) appear to mediate lateral inhibition during zebrafish

neurogenesis (Appel and Eisen, 1998; Bierkamp and Campos-Ortega, 1993; Cornell and Eisen, 2000; Dornseifer et al., 1997; Haddon et al., 1998a; Takke et al., 1999; Westin and Lardelli, 1997). A PNG homolog, neurogenin 1 (*ngn1*; *neurod3* – Zebrafish Information Network) is expressed in neural plate regions where a class of early-born neurons, called primary neurons, arises (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998). Primary neurons include Rohon-Beard spinal sensory neurons (RBs), in the lateral neural plate, and primary interneurons (INs) and motoneurons (PMNs), in the intermediate and medial neural plates, respectively (Kimmel et al., 1991). Misexpression of *ngn1* RNA results in ectopic neurons that have gene expression profiles resembling those of primary neurons. The regulatory relationship between neurogenic and proneural genes also seems to be conserved in zebrafish; for example, activation of the Delta/Notch pathway represses *ngn1* expression (Blader et al., 1997).

We have focussed on the role of *ngn1* in the zebrafish lateral neural plate to address some of the issues raised above. This region contains an equivalence domain of cells that can become RBs, but when exposed to Delta/Notch signaling, become trunk neural crest instead (Cornell and Eisen, 2000). We now show that *ngn1* is expressed early in RBs and later in neural crest-derived dorsal root ganglion neurons, and that Ngn1 function is required for formation of both of these cell types, but not for PMNs or autonomic neurons. These data provide evidence that Ngn1 function is required specifically for development of sensory neurons. We additionally show that although both RBs and DRG neurons depend on Ngn1, there is no obligate lineage relationship between these two cell types, suggesting that, consistent with the temporal expression pattern, distinct episodes of Ngn1 activity first specify RBs and later specify DRG neurons. Finally, we show that reducing Ngn1 function restores trunk neural crest in embryos that lack Delta/Notch signaling, suggesting that although Delta/Notch signaling inhibits the RB fate, it does not actively promote the neural crest fate.

MATERIALS AND METHODS

Fish and embryo rearing

Fish were cared for in the University of Oregon Zebrafish Facility. Embryos were reared as previously described (Westerfield, 1993) and staged by hours post fertilization at 28°C (h) (Kimmel et al., 1995). *mib*^{ta52b} mutant embryos were generated by crossing two heterozygous adult carriers. This allele of *mib* was initially called *whitetail* (Jiang et al., 1996).

Composition of morpholinos

Morpholino (MO) antisense oligonucleotides (Gene Tools, Corvallis OR) were designed to complement the *ngn1* cDNA. Positions where different GenBank submissions of this sequence (Accession numbers AF 017301, AF036149, U94588 and AF024535) varied were avoided. Base positions listed below refer to GenBank Accession Number AF 017301 (Blader et al., 1997). These morpholinos do not complement the only other published *ngn1* ortholog *ngn3* (*neurog3* – Zebrafish Information Network) (Wang, 2001) or any of the expressed sequence tags homologous to neurogenin published to date by the Washington University Zebrafish Genome Resources Project.

ngn1^{AUG} MO and *ngn1* MO (complementing bases 222-246, straddles start codon): 5′-TATACGATCTCCATTGTTGATAACC-3′

Epitope-tagged *ngn1* MO contains a carboxyfluorescein modification at the 3′ end (Gene Tools, Corvallis OR)

ngn1^{mismatch} MO (lower case letters indicate bases that do not complement *ngn1* cDNA sequence): 5′-TATtCGAaCTCCATTGT-TcATAtCC-3′

ngn1$^{5'UTR}$ MO (complementing bases 94-118): 5′-ACCTTATTG-GTGGGCTGGGAGATGC-3′

Injection protocols for widespread or mosaic distribution

Morpholinos were reconstituted in Danieau's media [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca $(NO_3)_2$, 5 mM Hepes, pH 7.6) (Nasevicius and Ekker, 2000)] at 25 mg/ml and stored at –20°C. Immediately before injection, fresh dilutions were made in 0.2 M KCl to 0.6 mg/ml, except for the initial titration of each morpholino, in which case a series of concentrations from 0.1 mg/ml to 7.5 mg/ml were tested. For widespread distribution of morpholinos, one-cell stage embryos in their chorions were injected with 2-3 nl diluted morpholino into the yolk just beneath the nascent cells before the four-cell stage. Drop sizes from a given pipette appeared to be consistent over the course of an experiment, so drop volume was calculated by dividing the volume loaded into the pipette by the number of injections required to empty the pipette. Pipettes were pulled on Sutter Instruments Micropipette puller (Model P-2000). Injections were performed with an air injection apparatus (ASI).

For mosaic distribution of morpholinos or rhodamine-dextran (10,000 M_r; Molecular Probes), dechorionated 32-cell-stage embryos were placed in agarose-coated dishes. The pipette tip was positioned against an animal pole blastomere such that the surface of the cell dimpled inwards. The injection apparatus was then gently tapped until the pipette tip entered the cell. For 32-cell-stage blastomeres, ~1 nl of epitope-tagged morpholino at 0.3 mg/ml, or rhodamine-dextran at approximately 2% concentration, was injected.

RNA in situ hybridization, β-galacactosidase staining and immunohistochemical staining

DIG-labeled antisense RNA probes (Roche Diagnostics) for in situ hybridization were generated from plasmids as follows: *crestin* plasmid (gift of Marnie Halpern) was cut with *Eco*RI and transcribed with T7 polymerase; *fkd6* plasmid (*foxd3* – Zebrafish Information Network) (gift of Joerg Odenthal and Christine Nuesslein-Volhard) was cut with *Bam*HI and transcribed with T7 polymerase; *isl2* plasmid was cut with *Eco*RI and transcribed with T7 polymerase. RNA in situ hybridization was performed as described previously (Appel and Eisen, 1998). Embryos processed to reveal fluorescein-labeled morpholino were first processed for RNA in situ hybridization, then stripped of anti-Dig by three 10 minute incubations in 0.2 M glycine (pH 1.2), reblocked and incubated in 1:10,000 dilution of anti-FITC, alkaline phosphatase-conjugated (Roche) in block solution for 12 hours at 4°C, then rinsed and developed in Sigma Fast Fast Red (Sigma product F-4648).

Embryos injected with *lacZ* RNA were developed for β-galactosidase activity as previously described (Cornell and Eisen, 2000), post-fixed overnight at 4°C, then processed for *fkd6* RNA in situ hybridization.

Monoclonal antibodies were used at the following dilutions: zn12, 1:4000; zn1, 1:200; znp1, 1:1000 ['zn' antibodies described previously (Trevarrow et al., 1990)]; anti-HU [monoclonal antibody 16A11 (Marusich et al., 1994)], 1:100; and anti-acetylated tubulin (Sigma), 1:500. Samples were developed as described elsewhere (Cornell and Eisen, 2000). Polyclonal anti-tyrosine hydroxylase (Pel-Freez Biologicals) was used at 1:100 dilution and developed as described elsewhere (Cornell and Eisen, 2000).

RNA for microinjection

Capped RNAs from dominant negative *Xenopus laevis* Suppressor of Hairless plasmid [*X-Su(H)*^{DBM}] (a gift from Daniel Wettstein and Chris Kintner) and SP64T-*lacZ* plasmid (a gift from Marnie Halpern)

were synthesized as previously described (Cornell and Eisen, 2000). Capped RNAs were mixed and injected at a final concentration of approximately 0.3 mg/ml each into one cell at the two-cell stage.

RESULTS

ngn1 is expressed in RBs and DRG neurons

ngn1 is expressed broadly in neurogenic regions of the zebrafish neural plate; some cells express high levels and others low levels (Blader et al., 1997; Kim et al., 1997; Korzh et al., 1998). To establish whether RBs expressed *ngn1*, we examined anti-Islet immunoreactivity, an early marker of primary neurons (including RBs) (Korzh et al., 1993), together with *ngn1* mRNA expression in neural plate stage embryos. We found that in caudal neural plate, RBs and most surrounding cells expressed *ngn1* (Fig. 1A′), while in more rostral, developmentally older neural plate, RBs but not surrounding cells expressed high levels of *ngn1* (Fig. 1A″). The pattern of expression of *ngn1* thus resembles that of proneural genes in *Drosophila melanogaster* neurogenic ectoderm, where initial broad expression is restricted to the subset of cells that will become neuroblasts [e.g. scute (Ruiz-Gomez and Ghysen, 1993)]. Furthermore, as some of the cells that surround RBs are premigratory neural crest cells (Cornell and Eisen, 2000), these observations provide evidence that, in contrast to RBs, premigratory neural crest cells express *ngn1* only transiently. However, we did detect high levels of expression of *ngn1* later in the position of nascent DRG neurons (Fig. 1B,C), which derive from neural crest. Together, these data suggest that the precursor population that arises in the lateral neural plate expresses high levels *ngn1* at two distinct periods: first in the subset of cells that becomes RBs and later in the subset of neural crest cells that becomes DRG neurons.

Consistent with expression of mouse neurogenin homologs in cranial ganglia (Fode et al., 1998; Ma et al., 1998), we also detected *ngn1* expression in the position of nascent cranial ganglia (Fig. 1D).

Reduction of *ngn1* function eliminates RB neurons

To test the role of Ngn1 in zebrafish neuronal development, we depleted embryos of Ngn1 protein by injecting a morpholino antisense oligonucleotide (hereafter referred to as *ngn1* MO). We processed embryos injected with *ngn1* MO to reveal *islet2* (*isl2*) mRNA expression, a marker of RBs and PMNs (Fig. 2A) (Appel et al., 1995; Tokumoto et al., 1995). RB expression of *isl2* was highly reduced or absent in embryos injected with *ngn1* MO, while PMN expression was unperturbed (Fig. 1B). Expression of *isl2* in trigeminal ganglia was also highly reduced (not shown). Injection of a second, non-overlapping morpholino complementary to the *ngn1* transcript gave the same result (Fig. 2C), arguing that these morpholinos targeted the *ngn1* mRNA, and not a spurious target with fortuitous sequence identity (Nasevicius and Ekker, 2000). For the remaining experiments, we injected *ngn1* MO.

Ngn1 is required for formation of RBs but not PMNs

Distinct vertebrate neural bHLH genes regulate specification or differentiation of neurons (reviewed by Chan and Jan, 1999). To learn whether Ngn1 was required for specification or differentiation of RBs, we examined the earliest markers of the RB fate in embryos injected with *ngn1* MO. *isl1* and *HuC* (*elavl3* – Zebrafish Information Network) are both expressed in primary neurons at neural plate stages (Appel et al., 1995; Inoue et al., 1994; Kim et al., 1996). Embryos injected with *ngn1* MO had reduced or absent expression of these markers in RBs (Fig. 3A,B and not shown), while PMN expression appeared normal. *neurod* is expressed at an early stage in RBs (Blader et al., 1997; Korzh et al., 1998). This expression, as well as expression in trigeminal ganglia, was lost in embryos injected with *ngn1* MO (Fig. 3C,D). *deltaA* (*dla*) is expressed at low levels in regions that give rise to all three classes of primary neurons, but at high levels in individual neuronal precursors within these regions (Appel and Eisen, 1998; Haddon et al., 1998b). In embryos injected with *ngn1* MO, *dla* expression was reduced in the RB domain and in the trigeminal ganglia, but

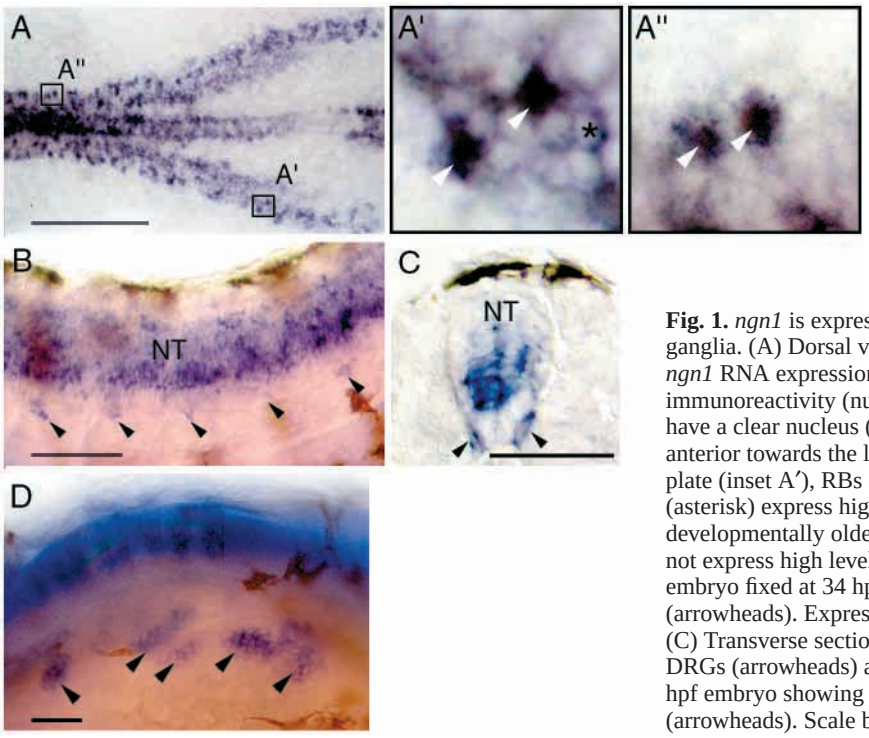

Fig. 1. *ngn1* is expressed in RB neurons and in dorsal root and cranial ganglia. (A) Dorsal view of 6-somite stage embryo, processed to reveal *ngn1* RNA expression (cytoplasm, dark blue) and anti-Islet immunoreactivity (nuclear, brown). Cells expressing *ngn1* but not Islet have a clear nucleus (asterisk, A′). In all figures, embryos are shown with anterior towards the left, except in transverse sections. In caudal neural plate (inset A′), RBs (white arrowheads) and a few cells adjacent to RBs (asterisk) express high levels of *ngn1*. By contrast, more rostrally, in developmentally older neural plate (inset A″), cells surrounding RBs do not express high levels of *ngn1*. (B) Lateral view of somites 5-12 of an embryo fixed at 34 hpf and processed to reveal *ngn1* expression in DRGs (arrowheads). Expression is also visible in neural tube (NT). (C) Transverse section of the embryo in A showing *ngn1* expression in DRGs (arrowheads) and neural tube (NT). (D) Lateral head view of 34 hpf embryo showing *ngn1* expression in nascent cranial ganglia (arrowheads). Scale bars: 50 μm.

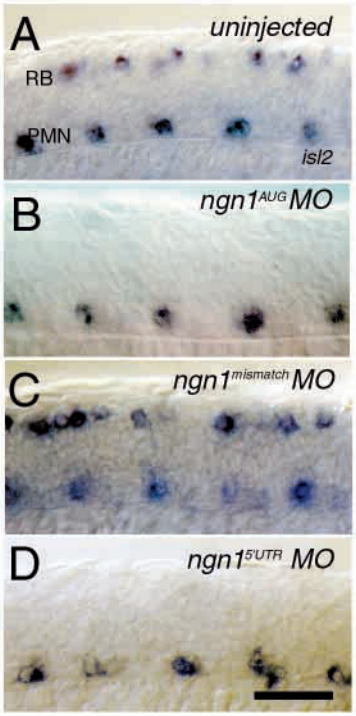

Fig. 2. RBs but not PMNs are eliminated by *ngn1* morpholinos. (A-D) Lateral views of 18 hpf wild-type embryos at somites 15-20, processed to reveal *isl2* RNA expression. In this and subsequent figures, the portrayed phenotype was observed in at least 80% of injected embryos. (A) Uninjected embryo. Expression of *isl2* can be seen in RBs in dorsal neural tube and in a subset of primary motoneurons (PMN) in ventral neural tube. (B) Embryo injected at the two-cell stage with approximately 1.5 ng of *ngn1* MO that straddles the start AUG of the *ngn1* transcript. As revealed by *isl2* expression, RBs were absent or highly reduced, whereas PMNs were normal (40 embryos scored). Note: in embryos injected with 7.5 ng of *ngn1* MO, PMNs in the tail were reduced in some embryos; however, it is unclear whether this phenotype resulted from targeting of the *ngn1* transcript, because target specificity may not be maintained at this concentration. (C) Embryo injected at two-cell stage with approximately 3.0 ng of $ngn1^{mismatch}$ MO, which is identical to *ngn1* MO except at four base positions (28 embryos scored). RBs and PMNs are normal, indicating that sequence complementarity is essential for protein knockdown at this morpholino concentration. At 6.0 ng of injected $ngn1^{mismatch}$ MO, embryos resembled *ngn1* MO-injected embryos (20 embryos scored), suggesting that at this high dose, the *ngn1* mismatch MO targets the *ngn1* transcript, despite the lack of complementarity at four out of 25 positions. We therefore ignored effects of morpholinos at this high dose, because of the uncertainty of the transcript being targeted. (D) Embryo injected at two-cell stage with approximately 1.2 ng of $ngn1^{5'UTR}$ MO, that complements an upstream region of the 5′UTR. The phenotype appeared identical to that in *ngn1* MO-injected embryos at this dose (25 embryos scored). At 3.6 ng and higher, embryos showed patchy necrosis in the CNS. This phenotype was presumed to result from toxicity of the morpholino at this dose, as opposed to further reduction of Ngn1 function. Scale bar: 50 μm.

intermediate and medial domains of neural plate expression were relatively unaffected (Fig. 3E,F).

In addition to these early markers of RB identity, we examined zn12 antibody immunoreactivity, which labels differentiated RBs (Metcalfe et al., 1990), and zn1/znp1 antibody immunoreactivity, which labels motoneurons (Melancon et al., 1997; Trevarrow et al., 1990). Consistent with the effects on *isl2* expression in RBs, zn12 labeling was extremely reduced in *ngn1* MO-injected embryos (Fig. 2G,H), while zn1/znp1 labeling appeared normal (Fig. 2I,J). Together these data suggest *ngn1* activity is required for a very early step of RB development, perhaps their specification, but that Ngn1 is not required for any step in PMN development.

Reducing Ngn1 activity restores trunk neural crest in embryos with reduced Delta/Notch signaling

Because neural crest and RBs are alternative fates of precursor cells in the lateral neural plate (Cornell and Eisen, 2000), we reasoned that if Ngn1 function were required for specification of RBs, reduction of Ngn1 might cause cells that would have become RBs to become neural crest instead. *fkd6* is expressed in presumptive premigratory neural crest in lateral neural plate (see Fig. 5D) (Odenthal and Nusslein-Volhard, 1998). *fkd6* expression appeared normal in *ngn1* MO-injected wild-type embryos (not shown). However, as the ratio of RBs to premigratory neural crest cells in wild-type embryos is quite small, even if all the RBs converted to neural crest cells it would be difficult to detect. To make it easier to recognize such a cell fate conversion, we made use of a mutant, *mindbomb* (*mib*), that has a large excess of RBs at the expense of neural crest, apparently resulting from disrupted Delta/Notch signaling (Haddon et al., 1998a; Jiang et al., 1996; Schier et al., 1996).

mib mutants have supernumerary RBs and PMNs relative to wild-type embryos (Jiang et al., 1996; Schier et al., 1996), as revealed by excess *isl2* expression (Fig. 4A,B). In *ngn1* MO-injected *mib* mutants, recognizable as mib^{-} by the PMN phenotype, RBs were nearly absent (Fig. 4C). This double loss-of-function phenotype suggests that the *mib* gene, and hence Delta/Notch signaling, acts upstream of *ngn1*. *mib* mutants also lack *fkd6* expression in the trunk (Fig. 5E), consistent with all lateral neural plate precursor cells adopting the RB fate because of reduced Delta/Notch signaling (Cornell and Eisen, 2000). By contrast, trunk neural crest was restored in *mib* mutants injected with *ngn1* MO (Fig. 4F). We also examined a marker of migratory neural crest, *crestin* (Rubinstein et al., 2000). *mib* mutants have a strong reduction of *crestin* expression in the trunk relative to wild-type embryos (Fig. 4G,H). By contrast, *mib* mutants injected with *ngn1* MO, still recognizable as mutants by their abnormal somites and curved tails, had extensive *crestin*-positive neural crest in the trunk and tail (Fig. 4I). At 3 days postfertilization (dpf), *mib* mutants lack pigment cells and other trunk neural crest derivatives (Fig. 4J,K) (Jiang et al., 1996; Schier et al., 1996) (R. A. C. and J. S. E., unpublished), whereas *ngn1* MO-injected *mib* mutants had melanophores throughout the trunk (Fig. 4L). Xanthophores, a separate pigment cell type that are yellow in color, were also restored (Fig. 4M-O). Together these results are consistent with the model that cells that cannot become RBs in *mib* mutants instead differentiate as neural crest derivatives following suppression of Ngn1 function.

The *ngn1 MO* might rescue neural crest in *mib* mutants by cell-autonomously causing precursor cells to become neural crest, or by killing these cells, thereby allowing neighboring cells to be exposed to neural crest-inducing signals. To distinguish between these possibilities, we injected single cells of *mib* mutants at the 16- or 32-cell stage with epitope-tagged *ngn1* MO. This occasionally resulted in dispersed clones of morpholino-

containing cells in the lateral neural plate. In such cases, small groups of cells were seen to express *fkd6*, and these cells always contained the morpholino, whereas neighboring cells that did not contain morpholino also did not express *fkd6* (Fig. 4P). This result provides evidence that RB precursors inheriting the *ngn1* MO themselves adopted the neural crest fate, a cell-autonomous event.

Although *mib* mutants appear to have a disruption of Delta/Notch signaling, the mutated gene has not yet been molecularly identified. We considered using a *dla* mutant (dla^{dx2}) (Appel et al., 1999); however, as the phenotype of this allele is incompletely penetrant, a reversal of its phenotype would be difficult to quantitate. Instead, to test the effect of *ngn1* MO in embryos with a known disruption of Delta/Notch signaling, we injected embryos with RNA encoding dominant negative *X. laevis* Supressor of Hairless [$X\text{-}Su(H)^{DBM}$], which disrupts an effector of Delta/Notch signaling (Wettstein et al., 1997). Like *mib* mutants, $X\text{-}Su(H)^{DBM}$-injected embryos also have excess RBs and a loss of trunk neural crest (Fig. 4Q) (Cornell and Eisen, 2000). Co-injection of *ngn1* MO restored *fkd6*-positive trunk neural crest in these embryos (Fig. 4R), supporting our interpretation of the effect of disrupting Ngn1 in *mib* mutants.

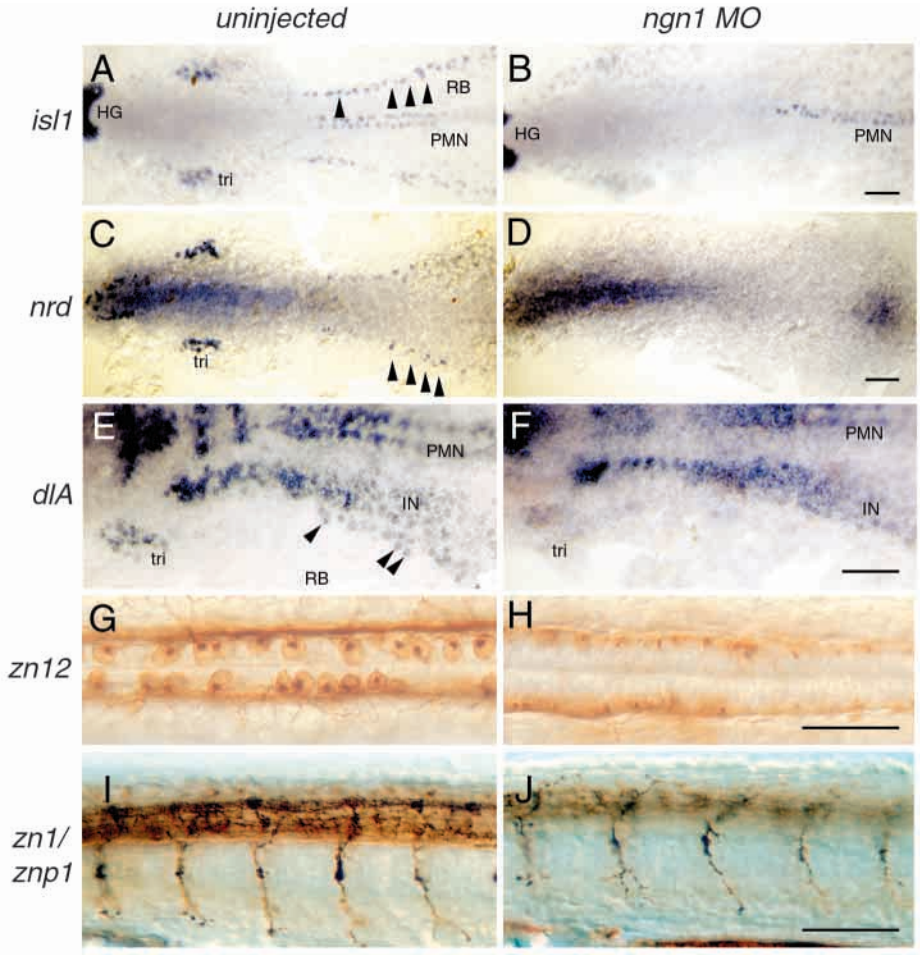

Fig. 3. Early markers of RBs are eliminated by *ngn1* MO. (A,C,E,G,I) Uninjected embryos. (B,D,F,H,J) Embryos injected at the one- to two-cell stage with 1.5 ng of *ngn1* MO. (A,B) Embryos fixed at the five-somite stage (about 12 hpf), processed to reveal *isl1* RNA expression. RBs (arrowheads, RB) and trigeminal ganglion precursors (tri) were highly reduced, whereas PMNs and hatching gland (HG) were still present in injected embryos (25 embryos scored). (C,D) Embryos fixed at the five-somite stage, processed to reveal *neurod* (*nrd*) RNA expression. RBs (arrowheads) and trigeminal ganglion precursors (tri) were highly reduced in injected embryos (24 embryos scored). (E,F) Embryos fixed at the three-somite stage (about 11 hpf), processed to reveal *dla* expression (left half of neural plate is shown). High level expression, identifying RB precursors (arrowheads) and trigeminal ganglia (tri) was eliminated, whereas low level expression in lateral neural plate and trigeminal ganglia was still present in injected embryos. Medial neural plate expression at high and low levels was present in injected embryos (43 embryos scored). (G,H) Embryos fixed at 24 hpf and processed to reveal zn12 antibody immunoreactivity in RBs. RBs were highly reduced or completely absent from injected embryos, although residual labeling was still visible in unidentified cell bodies and axon tracts within the intermediate spinal cord and hindbrain (28 embryos scored). (I,J) Embryos fixed at 24 hpf and processed to reveal zn1 and znp1 antibody immunoreactivity. PMN axons are labeled and appear normal in number in injected embryos (26 embryos scored). Scale bars: 100 μm in A-F; 50 μm in G-J.

Ngn1 function is required for development of peripheral sensory neurons

Neurogenin homologs are required for development of mouse peripheral sensory but not autonomic neurons (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999). To learn what peripheral neurons depend on Ngn1 in zebrafish, we examined peripheral neurons in embryos injected with *ngn1* MO. *ngn1* MO-injected embryos were touch insensitive at 3 dpf, suggesting a problem with sensory neurons, although they retained the ability to swim, suggesting motoneurons were still functional. Embryos injected with *ngn1* MO had dramatically fewer neurons in the DRG relative to uninjected embryos (Fig. 5A,B). Cranial sensory neurons were also highly reduced or absent in *ngn1* MO-injected embryos (Fig. 5C,D). By contrast, enteric neurons (Fig. 5E,F), cranial motoneurons (not shown) and sympathetic neurons (Fig. 5G,H) were all grossly normal in number and distribution in *ngn1* MO-injected embryos. Thus, consistent with results in mouse (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999), zebrafish that lack Ngn1 activity were deficient in sensory neurons but had normal autonomic neurons.

Interestingly, another phenotype of *ngn1* MO-injected embryos is a permanently open mouth (Fig. 5I,J). This phenotype perhaps results from disrupted sensory feedback from the jaw.

RB and DRG neurons have no determinate lineage relationship

Two models for the role of Ngn1 could explain the observation that both RBs and DRG neurons were absent from *ngn1* MO-

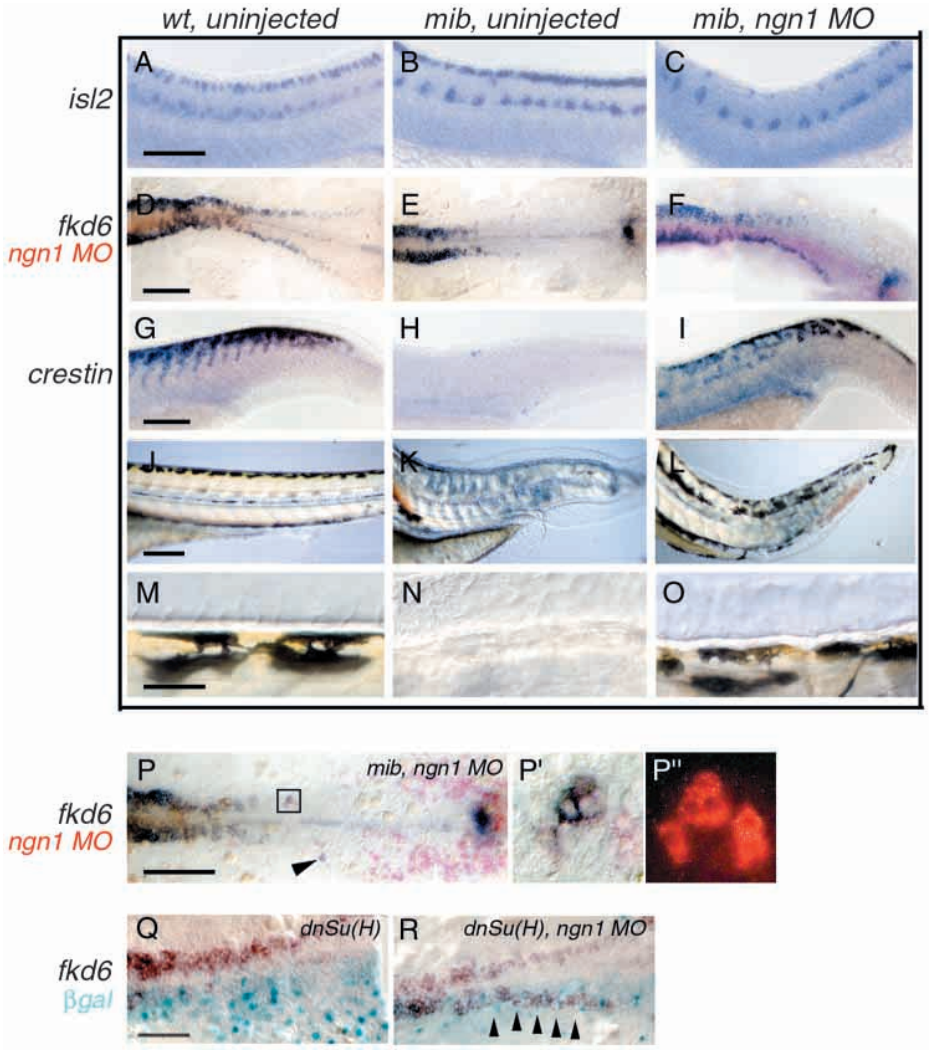

Fig. 4. Injection of *ngn1* MO rescues trunk neural crest in embryos with reduced Delta/Notch signaling. (A,D,G,J,M) Wild-type embryos; (B,E,H,K,N) *mib* mutants; (C,F,I,L,O) *mib* mutants injected with 1.5 ng *ngn1* MO. (A-C) Lateral views of 20 hpf embryos processed to reveal *isl2* expression. (A) In wild types, *isl2* expression is visible in RBs and a subset of PMNs. (B) In *mib* mutants, there are supernumerary RBs and PMNs. (C) In *ngn1* MO-injected *mib* mutants, RBs are highly reduced but supernumerary PMN are still present (nine mutant embryos). (D-F) Dorsal views of flat-mounted, 11 hpf embryos processed to reveal *fkd6* expression (black) and distribution of epitope-tagged *ngn1* MO (red). (D) In wild types, *fkd6* expression is visible in premigratory neural crest in head and trunk. (E) In *mib* mutants, *fkd6* expression is highly reduced in the trunk domain. (F) In *ngn1 MO*-injected *mib* mutants, *fkd6* expression is restored on the injected side (an epitope-tagged morpholino was used in this experiment, see Materials and Methods) (five *mib* mutants with morpholino in lateral neural plate scored). (G-I) Lateral trunk views of 20 hpf embryos processed to reveal *crestin* expression. (G) In wild types, *crestin* expression is visible in premigratory and migratory neural crest. (H) In *mib* mutants, crestin expression is all but absent from the trunk. (I) In *ngn1* MO-injected *mib* mutants, *crestin* expression is restored, although in an abnormal pattern. (J-L) Lateral trunk views of live 4 dpf embryos. (J) In wild types, neural-crest-derived black melanophores are visible in dorsal, intermediate and ventral stripes. (K) In *mib* mutants, melanophores are virtually absent from the trunk and tail. (L) In *ngn1* MO-injected *mib* mutants, melanophores are restored in the trunk and tail. Melanophores are abnormally distributed, however, often present between the spinal cord and somites (12 *mib* mutants scored). (M-O) Higher magnification lateral views of the embryos shown in J-L, respectively. Neural crest-derived yellow xanthophores are present in wild-type embryos (M), are highly reduced in *mib* mutants (N) and are restored in *ngn1* MO-injected *mib* mutants (O) (12 *mib* mutant embryos scored). (P) Dorsal view of a flat-mounted, 11 hpf *mib* mutant embryo processed to reveal *fkd6* expression (black) and distribution of epitope-tagged *ngn1* MO (red). *ngn1* MO was injected into one cell at the 32-cell stage. A few cells in trunk lateral neural plate express *fkd6* (box and arrowhead), and these all contain the *ngn1* MO, suggesting a cell autonomous of conversion of RBs to neural crest by the morpholino (four *mib* mutants with dispersed clones of morpholino-containing cells scored). (P′,P″) Higher-magnification view of boxed area in P in bright field (P′) to show *fkd6* expression, and under fluorescent optics (P″) to show distribution of the morpholino. (Q,R) Dorsal view of flat mounted, 11 hpf embryos processed to reveal *fkd6* expression (blue) and β-gal activity (turquoise). (Q) Embryos co-injected with RNAs encoding dominant negative *X. laevis* Supressor of Hairless [*dnSu(H)*] and β-gal have reduced *fkd6* expression in the trunk neural plate (10/85 injected embryos had X-gal stain in the trunk neural plate, all of these had highly reduced *fkd6* expression). (R) Embryos injected with these RNAs in addition to 1.5 ng *ngn1* MO have restored *fkd6* expression in this domain (arrowheads) (six out of six embryos with co-localization of β-gal activity and *ngn1* MO in lateral neural plate). Scale bars: in A, 100 μm in A-C; in D, 200 μm in D-F; in G, 100 μm in G-I; in J, 100 μm in J-L; in M, 50 μm in M-O; in P, 200 μm; in Q, 200 μm in Q,R.

injected embryos. One is that an early period of *ngn1* activity specifies some cells to become RBs and a later period of activity specifies other cells to become DRG neurons. However, in the fly central nervous system, proneural genes specify precursor cells that often give rise to many neurons in temporally separate waves (Campos-Ortega, 1995). Because RBs and neural crest both arise in the lateral neural plate, another model is that Ngn1 specified a lateral neural plate precursor cell that first generated an RB and later generated a DRG neuron. To test whether RBs and DRG neurons are derived from a single precursor cell, we injected rhodamine-dextran into one cell at the 32-cell stage. This resulted in 10 embryos with labeled RBs, only one of which had a labeled DRG neuron (Fig. 6). This result reveals that although RBs and DRG neurons can both derive from the same 32-cell-stage blastomere, they do not invariably do so. This result negates the possibility that RBs and DRG neurons at this stage invariably arise from a single *ngn1*-dependent precursor cell in the neural plate.

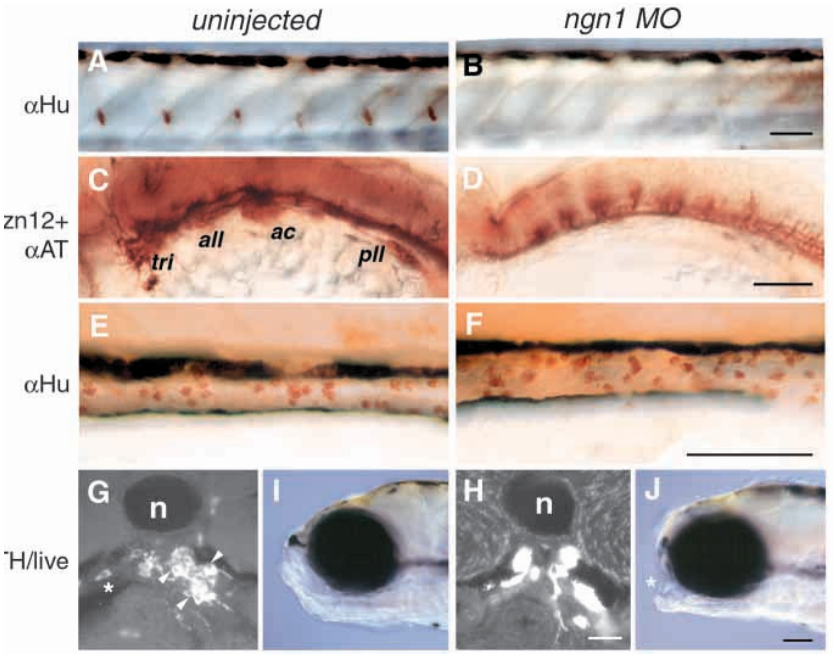

Fig. 5. Sensory neurons, but not autonomic neurons, are eliminated by *ngn1* MO. (A,C,E,G,I) Uninjected, wild-type embryos; (B,D,F,H,J) embryos injected with 1.5 ng of *ngn1* MO. (A,B) Lateral trunk views of 4 dpf embryos, processed to reveal anti-Hu immunoreactivity. Embryos have been deliberately under-developed to avoid strong staining in spinal cord underlying DRG neurons. Hu-labeled DRG neurons are absent from injected embryos, although spinal cord stain is beginning to become visible. (C,D) Lateral view just posterior to eye of 28 hpf embryos processed to reveal zn12 and anti-acetylated tubulin immunoreactivity. Trigeminal (tri), anterior lateral line (all), acoustic (ac) and posterior lateral line (pll) cranial ganglia are all highly reduced in injected embryos (28 embryos scored). (E,F) Lateral views of gut lumen of 4 dpf embryos processed to reveal anti-Hu immunoreactivity. Enteric neurons are present in both untreated and injected embryos. (G,H) Transverse sections, near axial level of pectoral fins, of 5 dpf embryos processed to reveal anti-tyrosine hydroxylase immunoreactivity. Label ventral to notochord (n) is in sympathetic neurons (arrowheads), which are present in both untreated and injected embryos. Asterisk indicates autofluorescence in a blood cell. (I,J) Lateral head view of live embryos at 5 dpf. Asterisk indicates permanently open mouth of injected embryos. Scale bars: in B, 50 μm in A,B; in D, 50 μm in C,D; in F, 50 μm in E,F; in H, 50 μm in G,H; in J, 100 μm in I,J.

DISCUSSION

Ngn1 activity is required for sensory neuron formation

The data we present here provide evidence that Ngn1 function is required for formation of sensory neurons but not other neurons. Fly PNGs have been ascribed dual roles: to imbue a cell with the potential to become a neuronal precursor and to confer subtype identity upon the neurons that are derived from that precursor (reviewed by Bray, 2000; Brunet and Ghysen, 1999). It has been suggested that these functions have been split in vertebrate *atonal* homologs (Hassan and Bellen, 2000). *ngn1* is expressed in neural plate domains that later give rise to sensory neurons, interneurons and motoneurons (Korzh et al., 1998). Moreover, Blader et al. (Blader et al., 1997) have shown that injecting *ngn1* mRNA led not only to ectopic cells that expressed zn12 antigen and thus could be RBs, but also to ectopic cells expressing *hlx1* characteristic of a class of interneurons. They also found that co-expression of *ngn1* mRNA and *dnReg* mRNA, which mimics stimulation of the Hedgehog pathway, led to ectopic cells expressing *lim3*, characteristic of ventral spinal cord neurons. Together these data imply that, in zebrafish, Ngn1 activates a generic neuronal program, with local signals determining the particular subtype of neuron that is formed.

By contrast, our finding that blocking Ngn1 function inhibits formation of RBs but not of PMNs or autonomic neurons, suggests that Ngn1 activity is linked particularly with sensory neuron formation. We propose that induction of *lim3* and *hlx1* by misexpressing *ngn1* results from inappropriate levels of Ngn1 protein that upregulate genes normally activated by other bHLH genes. This interpretation is consistent with results from two studies in flies of misexpressed *atonal* (*ato*), a proneural gene required in

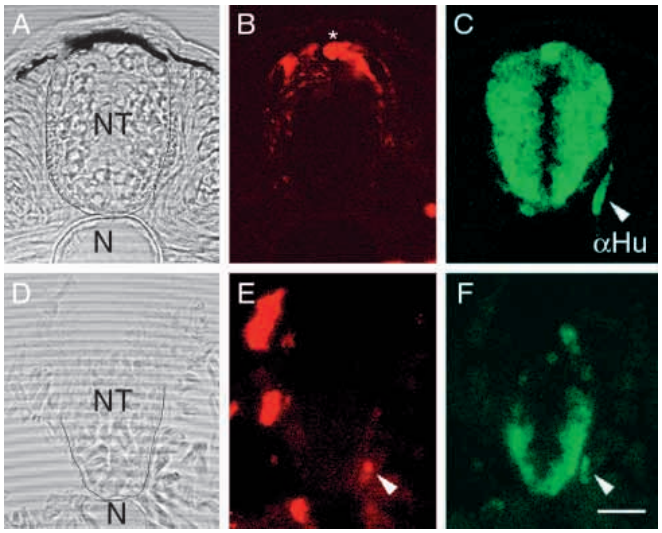

Fig. 6. RBs and DRG neurons are not necessarily derived from the same neural plate precursor cell. Embryos were injected with rhodamine-dextran into one cell at the 32-cell stage. Of 60 successfully labeled embryos, 10 had labeled RBs at 24 hpf, a frequency that is consistent with earlier experiments (Kimmel and Warga, 1987). We fixed all embryos at 48 hpf and processed them to reveal Hu-immunoreactivity. (A-F) Transverse sections near somite 10 of 2 dpf embryos. (A) Bright field image of an embryo with a labeled RB. The neural tube has been outlined to make it more obvious. (B) Lineage-tracer distribution in the section shown in A. Asterisk indicates a labeled RB, recognizable by its large cell body and dorsal position. (C) Anti-Hu immunoreactivity in the section shown in A. Although a DRG neuron (arrowhead) is present in this section, it does not contain lineage tracer, nor did any other DRG neurons in this embryo (not shown). (D) Bright field image of a different embryo that had a labeled RB at 24 hpf. This section was torn in processing. The neural tube has been outlined to make it more prominent. (E) Lineage-tracer distribution in the section shown in D. Arrowhead indicates a lineage-labeled DRG. (F) Anti-Hu immunoreactivity in lineage-labeled cell indicates that it is a neuron (arrowhead). Scale bar: 25 μm.

chordotonal organs (CHO), but not external sensory organs (ESO). Surprisingly, misexpressed *ato* driven by a heat-shock promoter led to some ectopic ESOs (Jarman et al., 1993), suggesting Ato had little or no ability to specify particular neuronal subtypes. However, later experiments where *ato* was driven in particular cells for an extended period elicited only ectopic CHOs, suggesting that under appropriate expression levels, *ato* uniquely specifies CHOs (Jarman and Ahmed, 1998).

If Ngn1 specifies sensory neurons, why is *ngn1* mRNA expressed in medial and intermediate neural plate, domains where no sensory neurons arise? Perhaps Ngn1 uniquely specifies sensory neurons in the PNS, while in the CNS it activates a generic neuronal program. If so, the apparent absence of phenotypes in medial and intermediate neural plate in embryos with reduced Ngn1 might be explained by redundant activity of other bHLH proteins found in these regions. There is precedent for redundant activity of Ngn homologs in mouse: neurons in cranial ganglia that express either Ngn1 or Ngn2 are lost in the corresponding mutant, whereas neurons in the nodose ganglion, which expresses both Ngn1 and Ngn2, are not lost in either mutant (Fode et al., 1998; Ma et al., 1998). Further gain-of-function studies of Ngn1 and analysis of PNG homologs in zebrafish will be required to determine whether Ngn1 activates a specific or generic neurogenic program.

Peripheral sensory neurons depend on Ngn1

All zebrafish peripheral sensory neurons that we examined depend on Ngn1, in apparent contrast to mouse, in which different subsets of peripheral sensory neurons depend on different neurogenin homologs. Thus, we detected no DRG neurons in 4 dpf *ngn1* MO-injected embryos. In mouse, chick and quail, there are two subsets of DRG neurons, distinguished by cell size, birth date, gene expression profile and sensory modality (Le Douarin and Kalcheim, 1999), and these subsets depend on distinct *ngn* homologs (Ma et al., 1999). The formation of zebrafish DRGs is not yet well described. If there are two classes of DRG neurons present in 4 dpf zebrafish, then one zebrafish *ngn* homolog would appear to perform the role of the two mouse homologs. This is the opposite of the prediction made based on the presence of additional copies of many genes in zebrafish resulting from a presumed genome-wide duplication event (Amores et al., 1998; Postlethwait et al., 2000). However, as many more DRG neurons are present in the adult than in the 4 day embryo, it is also possible that additional DRG neurons that arise later may depend on other *ngn* homologs.

Similarly, there are two classes of cranial sensory ganglia in mouse and chick, distinguished by placode versus neural crest origin of their precursors and by proximal versus distal position of the ganglia themselves (Le Douarin and Kalcheim, 1999). For the most part, the two classes depend on distinct neurogenin homologs (Fode et al., 1998; Ma et al., 1998). Relatively little is known about the distal ganglia in zebrafish, although a new transgenic line facilitates visualizing them with GFP under control of the *isl1* promoter (Higashijima et al., 2000). It will be interesting to determine whether all zebrafish cranial sensory ganglia depend on *ngn1*, which would suggest that one gene in zebrafish serves the role of two in mouse, or whether formation of other cranial sensory neurons depend on other *ngn* homologs.

Epistasis experiments reveal that Delta and Ngn1 function in the same pathway

It appears that in vertebrates Delta/Notch signaling regulates neurogenin activity and vice versa. In fly, an essential element of lateral inhibition is that Delta/Notch signaling represses proneural gene expression in the receiving cell (Simpson, 1997). In zebrafish and mouse, misexpressed *X-Delta-1* represses neurogenin expression (Blader et al., 1997; Ma et al., 1996). Moreover, ectopic neurons induced by misexpressed *ngn1* tend to be widely dispersed (Blader et al., 1997), suggesting lateral inhibition functions upon Ngn1 activity even in ectopic locations. We provide genetic evidence that Ngn1 activity is epistatic to Delta/Notch signaling: the phenotype of reduced RBs in *ngn1* MO-injected embryos that also lack Delta/Notch signaling resembles that of *ngn1* MO-injected wild-type embryos. These data show that *ngn1* is regulated by Delta/Notch signaling.

A second essential element of the feedback inherent to lateral inhibition in flies is that proneural genes activate Delta genes (Campos-Ortega, 1995). Consistent with this activity, misexpression of *X-ngnr-1* is sufficient to promote ectopic expression of *X-Delta-1* in frog (Ma et al., 1996). In mouse, *Ngn1* (*Neurod3* – Mouse Genome Informatics) expression precedes that of Delta homolog *Dll1*, and in mice with a targeted deletion of *Ngn1*, Delta homolog expression is absent, at least in those regions that express *Ngn1* and not *Ngn2* (*Atoh4* – Mouse Genome Informatics) (Ma et al., 1998). Similarly, in our study, *dla* expression is reduced in the trigeminal ganglia and lateral neural plate domains in *ngn1* MO-injected embryos. The persistent, low level expression of *dla* in these domains may result from residual Ngn1 activity in *ngn1* MO-injected embryos, or activity of other neurogenin homologues. Alternatively, upstream neural patterning genes may directly induce *dla* expression independently of *ngn1*. Isolation of null mutants of *ngn1* will be necessary to resolve these possibilities.

Distinct episodes of Ngn1 activity specify RBs and DRG neurons

Based on the activity of PNGs in flies, it seemed possible that RBs and DRG neurons could be two derivatives of a single Ngn1-dependent precursor in the lateral neural plate. In *D. melanogaster*, neuronal precursor cells transiently express PNGs and most will subsequently generate many neurons in a specific lineal order (Schmid et al., 1999). As RBs and DRG neurons both derive from precursors in the lateral neural plate, they might be derived from a single Ngn1-dependent precursor. However, *ngn1* is expressed in the position of DRG neurons, while late derivatives of neuronal precursors in flies do not express PNGs. More significantly, our dye-labeling experiment reveals that although RBs and DRG neurons can be lineally related, there is no obligate lineage relationship between these cells, which would necessarily be the case if they both derived from a single precursor. Together, these results provide evidence for two episodes of Ngn1 activity: one that directs some lateral neural plate precursors to become RBs, and a second, presumably later one that directs some neural crest cells, which are also derived from neural plate precursors, to become DRG neurons. This second episode may begin shortly after the first.

Does Ngn1 specify DRG neurons? If so, reduction of Ngn1 activity might cause DRG neuron precursors to adopt a different neural crest fate, for example, to become pigment

cells, glia or autonomic neurons. We detected neither pigment cells nor Hu-positive neurons in the position of the DRG in *ngn1* MO-injected embryos, indicating that if the sensory neuron precursors became pigment cells or autonomic neurons, they did not remain in the DRG location. Given the evidence for Delta/Notch signaling in segregating neuronal and glial fates in chick DRG (Morrison et al., 2000a; Wakamatsu et al., 2000), it will be particularly interesting to determine if DRG neurons become glial cells in Ngn1-deprived embryos. However, the paucity of markers of differentiated glia makes it impossible at present to identify glial cells, particularly in ectopic locations. Lineage analysis may be required to determine whether DRG neuron or cranial sensory neuron precursors adopt another fate, or perhaps die, in *ngn1* MO-injected embryos.

Delta/Notch signaling promotes neural crest formation by suppressing Ngn1 activity

Several recent gain-of-function studies indicate that Notch signaling enhances differentiation of a variety of glial cell types in mice (Furukawa et al., 2000; Gaiano et al., 2000; Morrison et al., 2000b), thereby calling into question the prevailing model that Delta/Notch signaling acts by preventing all differentiation (reviewed by Wang and Barres, 2000). However, because transgenic mice deficient in Notch die before gliogenesis, it was not possible to demonstrate a requirement for Delta/Notch signaling in gliogenesis in vivo. In addition, these studies did not distinguish between the possibilities that Delta/Notch signaling actively promotes the glial fate, or that it represses specific alternative fates, allowing other environmental cues to induce the glial fate.

We have distinguished these possibilities in a cell type with a demonstrated requirement for Delta/Notch signaling: trunk neural crest. In the decision between RB and premigratory neural crest, reducing Ngn1 function is sufficient to substitute for the Delta/Notch signal. Thus, it appears that Delta/Notch signaling does not instruct neural crest formation, but rather inhibits Ngn1-mediated entry into neuronal differentiation. How does reducing Ngn1 lead to neural crest formation? One possibility is that no other inductive cues are present at the time Ngn1 normally acts, so blocking Ngn1 prevents precursors from differentiating until such time as neural crest-inducing signals appear. Alternatively, local cues may induce RBs if Ngn1 is present, or neural crest if it is not. In this scenario, Ngn1 may actively repress neural crest, a testable hypothesis that is inspired by the recent demonstration that neurogenin appears to actively suppress glial fates in cells from the rat cerebral cortex (Sun et al., 2001).

Although RBs are typically thought of as a population of cells unique to anamniotes such as fish and amphibians, there are numerous reports of similar appearing cells in amniotes including reptiles (Kappers et al., 1936), rabbit and pig (Held, 1909), hedgehog and cow (Agduhr, 1922), gopher (Antoni, 1930), sheep (Bonnet, 1907) and human (Humphrey, 1944; Humphrey, 1947; Youngstrom, 1944). It would be intriguing to learn more about these RB-like mammalian cells, because these descriptions imply that similar mechanisms may be operating during specification of trunk crest in anamniotes and amniotes, especially in light of the proposal that neural crest originated from an RB-like cell in the vertebrate precursor (Fritzsch and Northcutt, 1993).

Note added in proof

Expression of *ngn1* RNA in DRG and a reduction of sensory neurons in embryos injected with *ngn1* MO has been independently observed (J. Ungos and D. Raible, personal communication).

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