

Zebrafish Dorsal Root Ganglia Neural Precursor Cells Adopt a Glial Fate in the Absence of *Neurogenin 1*

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The proneural transcription factor *neurogenin 1* (*neurog1*) has been shown to be a key regulator of dorsal root ganglion (DRG) neuron development. Here we use a novel transgenic zebrafish line to demonstrate that the neural crest population that gives rise to DRG neurons becomes fate restricted to a neuronal/glial precursor before the onset of *neurog1* function. We generated a stable transgenic zebrafish line that carries a modified bacterial artificial chromosome that expresses green fluorescent protein (GFP) under the control of the *neurog1* promoter [*Tg(neurog1:EGFP)*]. In contrast to previously described *neurog1* transgenic lines, *Tg(neurog1:EGFP)* expresses GFP in DRG neuronal precursor cells as they migrate ventrally and after their initial differentiation as neurons. Using this line, we are able to track the fate of DRG neuronal precursor cells during their specification. When *Neurog1* function is blocked, either by *neurog1* morpholino antisense oligonucleotide injection or in *neurog1* mutants, GFP expression initiates in neural crest cells, although they fail to form DRG neurons. Rather, these cells take on a glial-like morphology, retain proliferative capacity, and express glial markers and become associated with the ventral motor root. These results suggest that, within the zebrafish neural crest, there is a fate-restricted lineage that is limited to form either sensory neurons or glia in the developing DRG. *Neurog1* acts as the key factor in this lineage to direct the formation of sensory neurons.

Key words: dorsal root ganglia; neural crest; fate restriction; *neurogenin 1*; sensory neuron; glia

Introduction

The neural crest has proved a rich system in which to study how an initially homogeneous population of cells is directed to adopt diverse fates (Le Douarin and Kalcheim, 1999). Although some neural crest cells (NCCs) are pluripotent, other fate-restricted lineages give rise to specific cell types, such as neurons, glia, or melanocytes (Raible and Eisen, 1994; Henion and Weston, 1997; Henion et al., 2000; Selleck and Bronner-Fraser, 2000; Luo et al., 2003). Within the sublineage of neuronal and glial cells, there is a population of cells that form sensory neurons of the dorsal root ganglia (DRG) (Sieber-Blum, 1989; Greenwood et al., 1999). The DRG contain the sensory neurons of the peripheral nervous system that convey sensation from the body, such as touch, perception of pain (nociception), temperature, and sense of limb movement and position (proprioception) to the CNS. DRG neurons and associated glial cells arise from ventrally migrating trunk NCCs (Kalcheim, 1996; Anderson, 2000; Raible and Ungos, 2006).

In avian and mammalian embryos, the proneural transcrip-

tion factors *neurogenin 1* (*neurog1*) and *neurogenin 2* (*neurog2*) are expressed in migrating NCCs that have not undergone neuronal differentiation but will form sensory neurons (Greenwood et al., 1999; Ma et al., 1999; Perez et al., 1999). In mouse embryos, when both *neurogenin* genes are functionally inactivated, DRG neurons are completely absent (Ma et al., 1999). In zebrafish, blocking the function of a single *neurogenin* gene, *neurog1*, with morpholino antisense oligonucleotides (MOs) leads to complete loss of DRG neurons as well as all neurons of the cranial ganglia (Andermann et al., 2002; Cornell and Eisen, 2002). Coupled with the fact that each DRG in the zebrafish arises from an initial population of two to five neurons (Raible et al., 1992), this genetic simplicity makes zebrafish an ideal system in which to study mechanisms of cell fate choice during neural crest specification.

Here we provide evidence that, in zebrafish, the sensory neuron-restricted lineage of neural crest in the absence of *neurog1* gives rise to glial cells. We generated a transgenic zebrafish line that carries a bacterial artificial chromosome (BAC) clone modified to express the fluorescent reporter enhanced green fluorescent protein (EGFP) under the control of the regulatory elements of *neurog1*, *Tg(neurog1:EGFP)*. Using this line, we visualize DRG precursor cells in living and fixed embryos. We see expression of *Tg(neurog1:EGFP)* in a subset of migrating trunk NCCs before overt formation of DRGs and later in DRG neurons. When we block *neurog1* function, we continue to observe *Tg(neurog1:EGFP)*-positive migrating NCCs. These cells fail to differentiate as neurons but instead show a glial cell-like morphology and express glial-specific markers. We hypothesize that *neurog1* expression directs this restricted lineage to make a binary fate choice: cells that express *neurog1* form the sensory neurons of the

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DRG, but, in the absence of *neurog1* function, these cells persist and take on a glial fate.

Materials and Methods

Generation of Tg(*neurog1:EGFP*) transgenic line. We modified a *neurog1*-containing BAC clone by *Escherichia coli*-based homologous recombination (Zhang et al., 1998). BAC DanioKey clone DKEY-91L18 contains 17 kb of sequence upstream and 60 kb downstream of *neurog1* (http://www.sanger.ac.uk/Projects/D_zebrafish/mapping.shtml). After recombination, the modified BAC clone contained an *EGFP* gene positioned at the endogenous start site of *neurog1*. The accuracy of recombination was evaluated by PCR, sequencing, and analysis of transient expression. To obtain a zebrafish line with germ-line incorporation, we microinjected 20 μ g/ml linearized BAC DNA into zebrafish embryos, raised injected fish to adulthood, and screened their progeny for reporter gene expression. The germ-line transmission rate was 5%. The Tg(*neurog1:EGFP*)⁶¹ strain has been outcrossed for six generations and transmits the transgene in a Mendelian manner.

Fish husbandry. Embryos were obtained from natural spawning of adult zebrafish and staged according to Kimmel et al. (1995). Embryos were raised at 28.5°C in embryo medium (EM) (Westerfield, 1994). The *neurog1* (*neuroD3*^{hi1089}) mutant line (hereafter referred to as *neurog1*^{-/-}), generated in a retroviral insertion screen (Golling et al., 2002), was subsequently crossed to the Tg(*neurog1:EGFP*) line and selected for *neurog1*^{+/-} carriers of the Tg(*neurog1:EGFP*) transgene. The *colorless* (*cls*^{t3}) line contains a null mutation in the *sox10* gene (Dutton et al., 2001); this line was also crossed with the Tg(*neurog1:EGFP*) line and selected for Tg(*neurog1:EGFP*); *cls*^{+/-} carriers. The Tg(-3.1*neurog1:GFP*) transgenic line was described previously (Blader et al., 2003). The University of Washington Institutional Animal Care and Use Committee approved all procedures.

Whole-mount immunohistochemistry and RNA in situ hybridization. For immunohistochemistry, embryos were collected at stages indicated, killed in MS-222 (3-amino benzoate methanesulfonic acid) (10 mg/ml in buffered embryo medium), and fixed in 4% paraformaldehyde in PBS for 2 h at room temperature. Antibody labeling was performed as described previously (Ungos et al., 2003). In brief, embryos were washed in PBS with 0.1% Triton X-100 (PBT) and blocked with the addition of 2% goat serum unless otherwise noted. Before blocking, embryos older than 48 h postfertilization (hpf) were made permeable with three 30-min washes in distilled water. Embryos were incubated in primary antibodies diluted in blocking solution overnight at room temperature (RT). Primary antibodies used were anti-GFP (1:700; rabbit or mouse anti-GFP; Invitrogen), anti-Elavl1 (1:700; mAb 16A11, also called anti-HuC/D; Invitrogen), anti-Sox10 (1:1000) (Park et al., 2005), anti-myelin basic protein (MBP) (1:50) (Lyons et al., 2005), anti-5-bromo-2-deoxyuridine (BrdU) (1:500; Becton Dickinson), anti-phosphohistone H3 (pH3, 1:300; Upstate Cell Signaling Solutions), and anti-FoxD3 (1:1000 in blocking solution containing 20% goat serum) (Lister et al., 2006). Embryos were incubated in Alexa-488- or Alexa-568-conjugated secondary antibodies (Invitrogen) overnight at room temperature, rinsed in PBT, and then stored in 50% glycerol/PBS for imaging.

RNA in situ hybridization was performed as described previously (Andermann et al., 2002). Digoxigenin-labeled antisense probe was generated for *neurog1* by enzyme digestion with *XhoI* and synthesis with RNA polymerase T7 (Blader et al., 1997) and for *neuroD* probe by enzyme digestion using *NotI* and synthesis with T3 (Blader et al., 1997). Hybridization was performed overnight at 60°C. After development using 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium, embryos were washed in PBT and then taken through immunohistochemistry with anti-GFP antibody to enhance the signal of Tg(*neurog1:EGFP*). Embryos were stored in 50% glycerol/PBS for imaging.

Data collection and time-lapse imaging. Before imaging, embryos were devalked and mounted on bridged coverslips in 50% glycerol/PBS. Images and cell counts were obtained using a Zeiss LSM-5.0 Pascal confocal microscope or a Zeiss Axioplan 2 microscope and a Spot CCD camera (Diagnostic Instruments), and whole images were processed for brightness and contrast using Adobe Photoshop (Adobe Systems). For individual cell counts, the five rostralmost DRG were analyzed in each condi-

tion. Non-neuronal cells were defined as associated with the DRG if they were in direct contact with labeled DRG neuronal cell bodies. Statistical significance was determined using a Student's *t* test or ANOVA depending on the experiment. To generate time-lapse movies, Tg(*neurog1:EGFP*) and Tg(*neurog1:EGFP*);*neurog1*^{-/-} embryos were anesthetized in MS-222 (10 mg/ml in embryo medium), embedded in 1.5% agarose in EM, covered in EM containing MS-222, and imaged from 26 hpf to 36 or 39 hpf.

Morpholino injection. MOs (Gene Tools) were diluted in RNase-free water, and 1–2 nl was pressure injected into embryos at the one- to two-cell stage. The *neurog1* MO 5'-CCATATCGGAGTATACGATCTCCAT-3' was designed against the *neurog1* start codon and injected at 7 ng/ml, to block translation of native *neurog1* message but not the Tg(*neurog1:EGFP*) transgene. This *neurog1* MO showed a phenotype identical to that seen using previously reported *neurog1* morpholinos and to the *neurog1* mutant (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Andermann et al., 2002; Cornell and Eisen, 2002).

Cyclopamine treatment. Tg(*neurog1:EGFP*) embryos were treated at 22 hpf with cyclopamine prepared as described by Ungos et al. (2003). In brief, cyclopamine (Toronto Research Chemicals) was diluted to 15 μ g/ml in embryo medium from a stock of 4 mg/ml cyclopamine dissolved in 45% (w/v) 2-hydroxypropyl- β -cyclodextrin (HBC) (Sigma) and PBS. Embryos were incubated at 28.5°C in cyclopamine continuously beginning at indicated time points until they were collected for processing. Control embryos were treated with comparable concentrations of HBC in embryo medium and were indistinguishable from untreated embryos.

Cell cycle analysis. To determine the number of cells in S-phase in the DRG, both Tg(*neurog1:EGFP*) control and Tg(*neurog1:EGFP*);*neurog1* MO-injected embryos were exposed to an overnight pulse of 10 mM BrdU (Sigma) dissolved in 1% dimethylsulfoxide (DMSO) in EM beginning at 30 or 50 hpf and then collected for processing the following day. After fixation and immunolabeling for anti-GFP antibody, embryos were then refixed for 20 min at RT and washed three times for 10 min in PBS with 1% DMSO and 0.1% Tween 20 (PBDTw). BrdU incorporation was detected using a modified protocol developed in zebrafish (Harris et al., 2003). Embryos were dehydrated in a series of steps to 100% methanol, incubated at -20°C for 1 h, then rehydrated in a stepwise manner, and washed in PBDTw two times for 10 min. Embryos were permeabilized with 10 μ l/mg Proteinase K (Invitrogen) in PBDTw for 20 min, refixed for 20 min in 4% paraformaldehyde, and washed three times for 10 min in PBDTw. Embryos were incubated in 1N HCl for 1 h at RT and then washed three times for 10 min in PBDTw. After washing, embryos were blocked in 10% goat serum in PBDTw for at least 1 h at RT and then incubated overnight at RT in mouse anti-BrdU IgG in blocking solution (1:100; Becton Dickinson). Embryos were washed three times for 30 min in PBDTw and incubated in Alexa-568 antibody for 6 h at RT, then washed in PBDTw, and stored in 50% glycerol/PBS until visualized as described above. Individual cells were counted in the five rostralmost DRG.

Cells in M-phase at 2 and 3 d postfertilization (dpf) were identified with the anti-pH3 antibody using the immunohistochemistry conditions described above. Because of the transient nature of pH3 expression, antibody labeling is infrequent. Therefore, all of the DRG along the length of the embryo were analyzed for pH3 labeling, and the percentage of DRG that contained at least one pH3-positive (pH3⁺) cell was quantified.

Results

Generation of a recombinant *neurog1* BAC transgenic zebrafish line

To generate a reporter line that allowed us to track DRG development, we screened the DanioKey BAC library (Koch et al., 2004) for a BAC that contained the coding sequence of *neurog1*; we identified the zebrafish BAC clone DKEY-91L18 (zK91L18). Mapping sequenced BAC ends onto the zebrafish genome reveals that the BAC contains 17 kb of sequence upstream and 60 kb downstream of the *neurog1* coding sequence. Using bacterial recombination techniques (Zhang et al., 1998), the single exon *neu-*

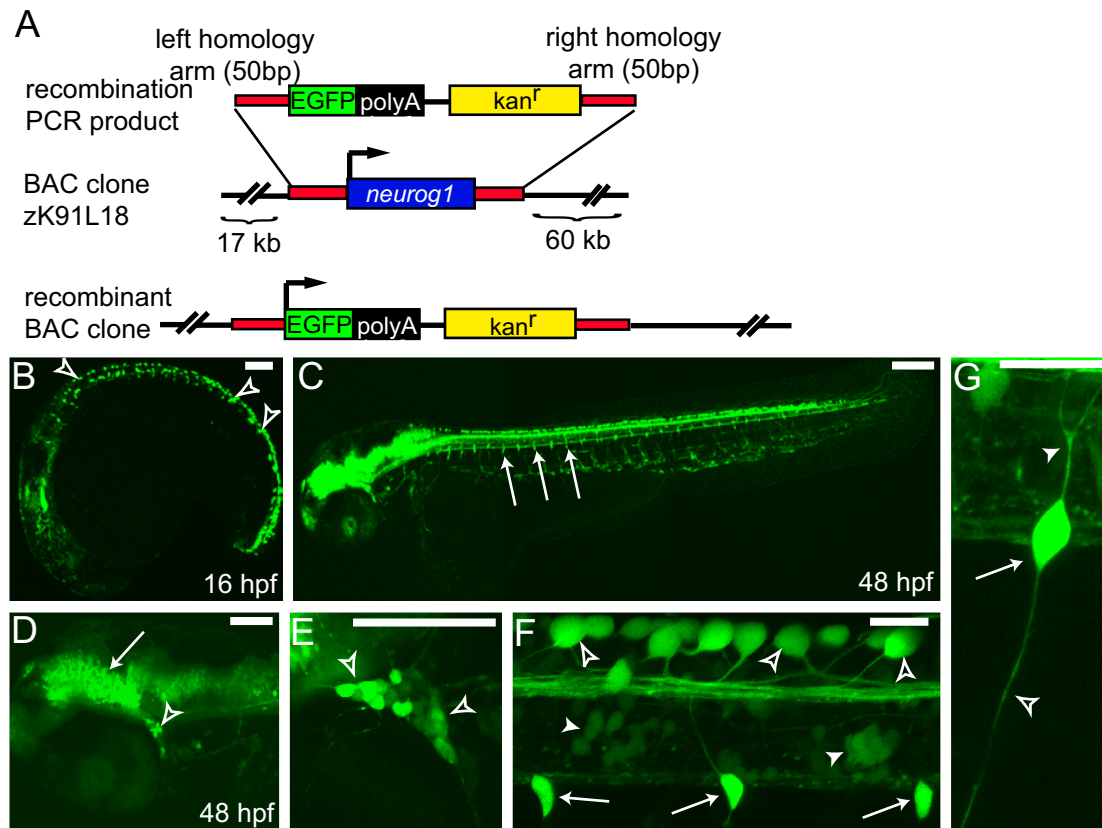


Figure 1. Using BAC recombination to produce a *Tg(neurog1:EGFP)* transgenic line. **A**, Schematic showing the strategy for BAC recombination. In the BAC clone DKEY-91L18 (zK91L18) from the DanioKey library, the single coding exon of the *neurog1* gene (blue bar) was replaced with an EGFP/kanamycin resistance cassette (green and black bars) by recombination using flanking homologous sequences (red bars). The EGFP gene is placed in-frame at the *neurog1* start site. Schematic is not to scale. **B–F**, Confocal projections of live transgenic *Tg(neurog1:EGFP)* embryos. **B**, At 16 hpf, *Tg(neurog1:EGFP)* is expressed in developing brain, spinal neurons, and RB neurons (open arrowheads). **C**, By 48 hpf, *Tg(neurog1:EGFP)* is strongly expressed in the CNS, and DRG (arrows) neurons are visible ventral to the spinal cord along the trunk. **D**, In the head at 48 hpf, several regions in the brain are intensely GFP positive, including the midbrain–hindbrain boundary (arrow). A subset of cells in the trigeminal ganglia (open arrowhead) expresses *Tg(neurog1:EGFP)*. **E**, GFP⁺ trigeminal ganglia neurons (open arrowheads). **F**, Close-up image showing GFP-expressing RB cells in the dorsal spinal cord and their associated axons (open arrowhead), cells in the spinal cord (arrowhead), and DRGs ventral to the spinal cord (arrow). **G**, DRGs containing a single GFP-expressing neuron (arrow) showing a centrally projecting axon (arrowhead) and a peripherally projecting axon (open arrowhead). Scale bars: **B**, **D**, 100 μ m; **C**, 200 μ m; **E–G**, 20 μ m.

rog1 coding sequence in DKEY-91L18 was replaced with that of EGFP along with a kanamycin resistance cassette (Fig. 1A). The modified BAC was used to create a stable transgenic zebrafish line *Tg(neurog1:EGFP)*. The expression pattern of GFP in this line is similar but not identical to that seen in previously reported *neurog1* transgenic lines (Blader et al., 2003). In the *Tg(neurog1:EGFP)* line, GFP expression is visible in live embryos at 16 hpf in Rohon Beard (RB) sensory neurons and the developing CNS (Fig. 1B). At 48 hpf, GFP expression can be seen in the developing CNS in subsets of neurons in the spinal cord, the trigeminal ganglia, and RB cells (Fig. 1C–F), as well as DRG neurons arrayed along the trunk of the developing embryo (Fig. 1F, G). The central and peripheral axons of the DRG are also labeled with GFP (Fig. 1G). The BAC clone DKEY-91L18 does not contain all of the regulatory elements required for endogenous *neurog1* expression; for example, with the exception of the trigeminal ganglion, all of the cranial ganglia that express *neurog1* mRNA lack GFP expression (supplemental Fig. S2A, available at www.jneurosci.org as supplemental material) (Andermann et al., 2002). Ectopic GFP expression is also visible in areas in which *neurog1* mRNA is not detected, including the vessels of the vascular system (supplemental Fig. S2B, available at www.jneurosci.org as supplemental material), indicating that this construct may lack some inhibitory as well as positive regulatory elements.

The *Tg(neurog1:EGFP)* line marks DRG precursors and nascent DRG neurons

The *Tg(neurog1:EGFP)* transgenic line marks DRG precursors early in their development, in contrast to previously described *neurog1* transgenic lines (Blader et al., 2003). At 22 hpf, segmental streams of neural crest cells are identifiable with anti-Sox10 antibody as they begin their ventral migration from the dorsal neural tube (Fig. 2A). Beginning at 24 hpf, a subset of the Sox10-positive neural crest cells begin to coexpress GFP, and we hypothesize that these GFP-positive cells are the precursors of the sensory neurons of the DRG (Fig. 2B). Beginning at 28 hpf, the GFP-positive cells begin to coalesce to form rounded cell bodies (Fig. 2C), and, by 36 hpf, a subset of GFP-positive nascent DRG neurons have begun to send out axons centrally to the spinal cord and out to peripheral targets; at this stage, Sox10 has begun to be downregulated in some, but not all, GFP⁺ DRG cells (Fig. 2D). By 48 hpf, most GFP-positive cells have downregulated Sox10, whereas cells surrounding the DRG continue to express Sox10 (Fig. 2E). These Sox10-positive cells may represent undifferentiated neural crest cells or satellite glial cells; at the present time, no markers have been identified in zebrafish that allow definitive identification of these cells. At this stage, Sox10-negative (Sox10⁻)/GFP-positive cells express the Elavl antigen (Fig. 2F)

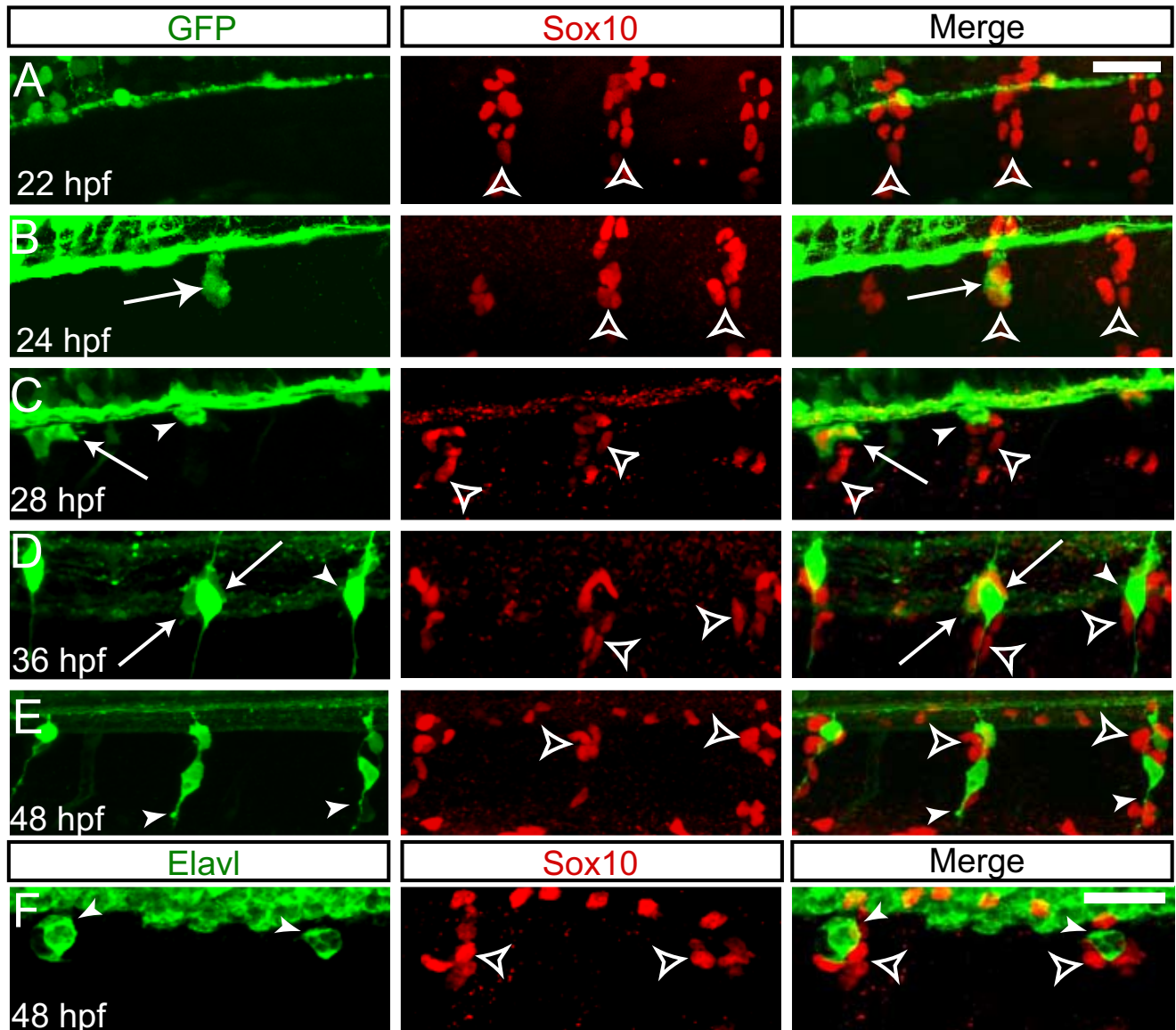


Figure 2. *Tg(neurog1:EGFP)* is expressed in migrating DRG precursor cells. Formation of DRG anlagen after neural crest migration. **A–E**, Immunofluorescent detection of Sox10 in neural crest cells and glia (red) and GFP in developing DRG neurons (green). All images are confocal z-projections of the second to fourth rostralmost DRG. **A**, At 22 hpf, Sox10⁺ neural crest cells migrate ventrally in segmented streams (open arrowheads). **B**, A subset of Sox10⁺ neural crest cells begins to express *Tg(neurog1:EGFP)* at 24 hpf (arrow). **C**, By 28 hpf, GFP⁺ cells have begun to coalesce into rounded cells bodies, the majority of which coexpress Sox10 (arrows), although some GFP⁺ cells have downregulated Sox10 (arrowhead). Sox10⁺ glial cells surround the DRG neuronal precursor cells (open arrowhead). **D**, As the GFP⁺ cells differentiate as neurons at 36 hpf, they in turn downregulate Sox10 (arrowheads), whereas a subset of GFP⁺ cells continue to express Sox10 (arrow). **E**, By 48 hpf, DRG neurons have begun to send axons to both the periphery and centrally to the spinal cord (arrowheads). Sox10 is expressed in glial cells that surround the DRG neurons (open arrowheads). **F**, Anti-Elavl (green) and anti-Sox10 labeling in a 48 hpf *Tg(neurog1:EGFP)* embryo. DRG neurons are Elavl⁺ (arrowheads) but Sox10⁻, whereas glial cells continue to be labeled by Sox10 (open arrowheads). Scale bars, 20 μ m.

To confirm that the GFP-positive cells form the neurons of the DRG, we used live time-lapse imaging of three *Tg(neurog1:EGFP)* transgenic embryos followed by immunohistochemical labeling with the anti-Elavl antibody to mark differentiated neurons. A representative *Tg(neurog1:EGFP)* embryo beginning at 26 hpf and continuing for 14 h is shown in Figure 3A (supplemental Movie 1, available at www.jneurosci.org as supplemental material). During this period GFP-positive cells can be seen to coalesce at the ventral edge of the spinal cord. During the later stages of development, these cells begin to extend ventral processes. After time-lapse image, these GFP-positive cells can be clearly identified by anti-Elavl antibody (Fig. 3B).

Previous studies examining *neurog1* expression during the development of the zebrafish DRG did not analyze time points before 30 hpf (Andermann et al., 2002; Cornell and Eisen, 2002; Ungos et al., 2003). As we see expression of the *Tg(neurog1:EGFP)* in the forming DRG before this period, we set out to correlate the *Tg(neurog1:EGFP)* with *neurog1* RNA expression by *in situ* hybridization (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). The rostralmost DRG precursors showed *neurog1* expression beginning at 26 hpf and continuing through 36 hpf. By 48 hpf, only occasional cells in the DRG express *neurog1*. Although transgene expression in combination with immunohistochemistry may allow visualization of DRG

precursors slightly earlier than *neurog1* *in situ* hybridization (24 vs 26 hpf), for the most part, *Tg(neurog1:EGFP)* shows reasonable fidelity to endogenous *neurog1* expression.

Initial addition of neurons to the DRG

We followed the time course of neuronal addition in DRG during the first 4 d of development by monitoring expression of the pan-neuronal marker *Elavl* in the *Tg(neurog1:EGFP)* transgenic line. At 36 hpf, DRGs contain an average of two GFP-positive cells per ganglion, and, on average, one of these cells also expresses *Elavl* (Fig. 4A). Both GFP-positive and *Elavl*-positive neurons continue to be added to the DRG as the embryo develops (Fig. 4B); by 72 hpf, a subset of *Elavl*-positive cells has begun to downregulate *Tg(neurog1:EGFP)* expression (Fig. 4C). By 96 hpf, almost all GFP-positive cells also express *Elavl*, whereas the number of *Elavl*⁺ cells that have downregulated GFP expression has grown (Fig. 4D). A small number of cells express GFP alone, perhaps indicating that these are newly added cells that will give rise to neurons. Quantification of neuronal addition is shown in Figure 4E. Together, these results suggest that neural crest cells first turn on *neurog1* and then downregulate *Sox10* and express *Elavl* as they differentiate into DRG neurons, suggesting that *neurog1* expression is the earliest step in sensory neuron specification. We note that GFP expression in our transgenic line is only a surrogate for endogenous *Neurog1* expression, and we use it here to document the dynamics of DRG neuron addition.

The expression of the *Tg(neurog1:EGFP)* transgene in migrating neural crest cells contrasts with the expression of a previously described transgenic zebrafish line in which GFP is controlled by -3.1 kb of the upstream *neurog1* regulatory sequence [*Tg(-3.1neurog1:GFP)*] (Blader et al., 2003). In the *Tg(-3.1neurog1:GFP)* line, GFP is not expressed in DRG neuronal precursor cells (supplemental Fig. S4A, available at www.jneurosci.org as supplemental material). GFP expression is not seen in the DRG until 36 hpf, at a stage when nascent DRG neurons have begun to express the transcription factor *NeuroD* (Ungos et al., 2003) and continues through 48 hpf (supplemental Fig S4C,E, available at www.jneurosci.org as supplemental material). GFP-positive cells are *Sox10*-negative, confirming that transgene expression commences with neuronal differentiation in the *Tg(-3.1neurog1:gfp)* line. Inactivation of *neurog1* function using antisense morpholino oligonucleotides abolishes *Tg(-3.1neurog1:gfp)* expression in the DRG. These results suggest that there are positive autoregulatory elements in the *neurog1* gene, as have been described previously for the related basic helix–loop–helix (bHLH) transcription factor *ato1* (Helms et al., 2000), although regulation might not be direct.

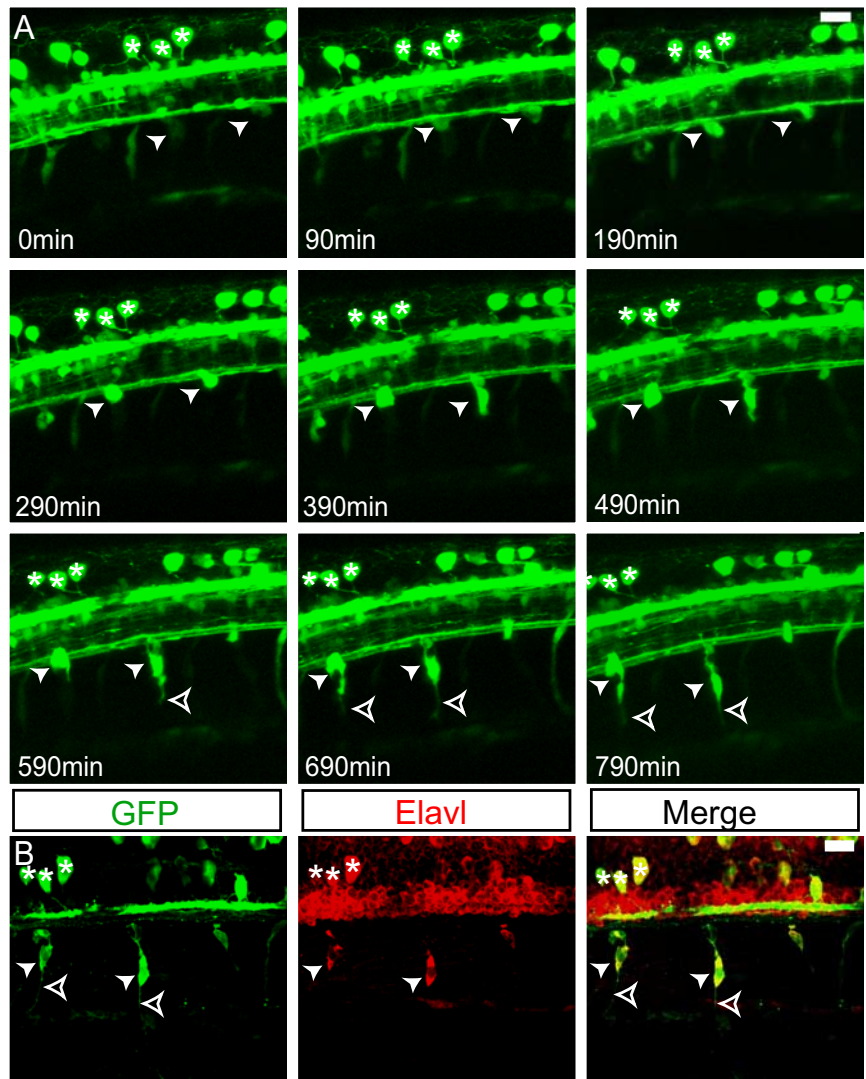


Figure 3. GFP-positive cells become DRG neurons. *A*, Individual frames of z-projections of a confocal time-lapse movie of a *Tg(neurog1:EGFP)* from 26 to 39 hpf. GFP-positive DRG precursor cells become visible at the ventral edge of the spinal cord (arrowheads). The same embryo is shown in supplemental Movie 1 (available at www.jneurosci.org as supplemental material). As the cells mature, they send out projections (open arrowheads). A group of RB neurons is maintained throughout the duration of the imaging (asterisks) and serves as a landmark for registration. *B*, Immunolabeling of the embryo at 40 hpf. The DRG cells from *A* are labeled with both anti-GFP and anti-*Elavl* antibodies, indicating that they have differentiated as neurons (arrowheads). Axons extend ventrally from the DRG neurons (open arrowheads). The group of RB neurons remains identifiable (asterisks). Scale bars, 20 μ m.

Following precursor formation in embryos with disrupted DRG development

Previous work suggests that Hedgehog (Hh) signaling pathway is required for the formation of zebrafish DRG neurons and is required near the onset of *neurog1* expression for proper sensory neuron development (Ungos et al., 2003). We treated *Tg(neurog1:EGFP)* embryos with the Hh antagonist cyclopamine or the vehicle HBC. Embryos treated with cyclopamine beginning at 22 hpf until 48 hpf lack the vast majority of GFP-positive DRG precursor cells, with only an occasional GFP-positive cell in the developing DRG (Fig. 5A,B). Although there is a significant reduction in the number of GFP⁺ cells after cyclopamine treatment, *Sox10*-positive cells remain present in treated embryos (Fig. 5C). These results support the idea that Hh signaling is required for the initiation of *neurog1* expression and, by extension, specifica-

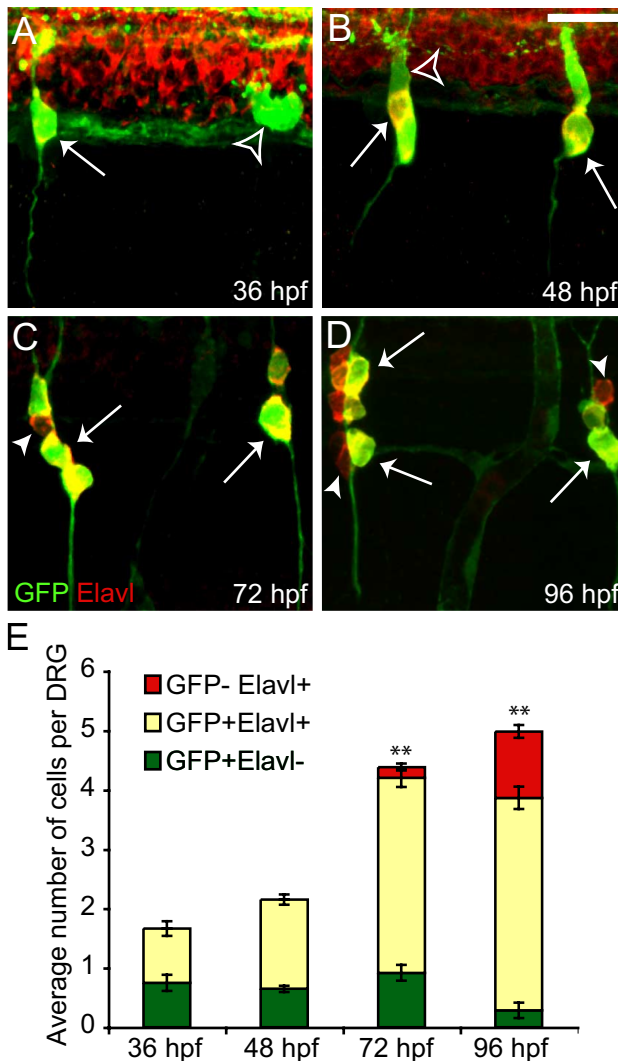


Figure 4. *Tg(neurog1:EGFP)* is transiently expressed in nascent DRG neurons. **A–D**, Immunofluorescent labeling of *Tg(neurog1:EGFP)* embryos between 36 and 96 hpf showing GFP (green) and Elavl (red). **A**, The DRGs initially form from one or two GFP⁺ cells per ganglion (open arrowhead), and a subset are also Elavl⁺ (arrow) at 36 hpf. **B**, At 48 hpf, all cells in the DRG are GFP⁺ (open arrowhead) and an increasing number of cells are also Elavl⁺ (arrow). **C**, By 72 hpf, a small number of Elavl⁺ DRG neurons have begun to downregulate GFP (arrowhead), although the majority of neurons colabel with GFP and Elavl (arrow), a trend that continues through 96 hpf as more cells become Elavl⁺ and GFP[−] (**D**). **E**, Quantification (mean ± SEM) of the contribution of GFP⁺ and Elavl⁺ cells to the DRG (cells from the 5 rostralmost DRGs were counted in each embryo; $n = 12$ embryos for 36 and 48 hpf; $n = 11$ for 72 and 96 hpf). The bar represent the average total number of cells in each DRG divided into those that only express GFP (green bars), those that express both GFP and Elavl (yellow bars), and those that only express Elavl (red bars). There is a significant increase in the total number of cells in the DRG (** $p < 0.0001$, ANOVA). The number of cells that express GFP alone significantly decrease over the same period ($p = 0.004$, ANOVA). Scale bars, 20 μm .

tion of DRG neuronal precursors but acts downstream of neural crest specification and migration. Although Sox10⁺ cells remain in the position of the DRG, it is not possible to distinguish between undifferentiated Sox10-positive neural crest cells and Sox10-positive satellite glia. These results contrast with those seen after mutation of *erbb3*, in which no neural-crest-derived cells appear to remain in the position of the DRG (Honjo et al., 2008).

The transcription factors FoxD3 and Sox10 have been described to act upstream of *neurog1* expression in the developing

DRG, with their loss of function leading to a loss of most but not all DRG neurons (Carney et al., 2006; Lister et al., 2006). Additionally, these factors are also required for the formation of neural-crest-derived glial cells and continue to be expressed in this lineage (Britsch et al., 2001; Dutton et al., 2001; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006). We monitored the development of DRG sensory neuron precursor cells after disruption of Foxd3 or Sox10 expression using the *Tg(neurog1:EGFP)* transgenic line. At 48 hpf, embryos lacking FoxD3 activity after *foxd3* MO injection showed a loss of most DRG neurons as marked by *Tg(neurog1:EGFP)* as well as a loss of the vast majority of Sox10-positive cells (Fig. 5*D,E,H*). When DRG development is analyzed in the zebrafish *sox10* mutant *colorless* (*cls*) carrying the *Tg(neurog1:EGFP)* transgene, we found that most DRG neurons were absent, and many that remain show disorganized process growth (Fig. 5*F–H*), probably because of the lack of glial cells described previously in *sox10* mutants (Britsch et al., 2001; Dutton et al., 2001; Carney et al., 2006). Although we observed occasional Foxd3-positive cells in *sox10* mutants, these cells were GFP negative. Together, these results provide additional support for the idea that Hedgehog signaling, Sox10, and Foxd3 all act upstream of *neurog1* expression to specify DRG precursors.

Cell fate changes in the absence of *neurog1* function

We reasoned that the early expression of the *Tg(neurog1:EGFP)* BAC transgene in migrating DRG neuron precursors might allow us to follow the fates of cells in the absence of *neurog1* function. For these studies, we followed GFP expression in animals injected with *neurog1* MO (Andermann et al., 2002; Cornell et al., 2002) or in animals with a retrovirus insertion into the *neurog1* gene that disrupts *neurog1* function (Golling et al., 2002). These *neurog1* mutant animals have the same loss of cranial ganglia (supplemental Fig. S1*A–C*, available at www.jneurosci.org as supplemental material) and RB neuron phenotypes as *neurog1* MO-injected animals (supplemental Fig. S1*D–G*, available at www.jneurosci.org as supplemental material). The loss of cranial ganglia neurons in the *neurog1* mutant has been described in greater detail by Caron et al. (2008). Both the *neurog1* MO and *neurog1* mutant embryos fail to express *neuroD* at 36 hpf (supplemental Fig. S1*H–J*, available at www.jneurosci.org as supplemental material).

We used live time-lapse imaging of *Tg(neurog1:EGFP)* embryos to track the development of DRG neurons. Imaging began at 26 hpf during the period of late neural crest migration, after GFP has begun to be expressed in DRG precursor cells but before their coalescence at the ventral edge of the spinal cord (Fig. 2*B*). GFP-positive cells are found in similar locations with similar elongated morphologies of migrating neural crest cells in both control (Fig. 6*A*) and *neurog1*^{−/−} embryos (Fig. 6*B*). By 36 hpf in control embryos, most of the GFP-positive neural crest cells have formed nascent DRGs (Fig. 6*C*). In contrast, in *neurog1*^{−/−} embryos, GFP-positive cells are still visible, but rather than coalescing at the edge of the spinal cord, they remain elongated (Fig. 6*D*). By 48 hpf, most of the GFP-positive cells in the DRG of control embryos express the neuronal marker Elavl and have sent out central and peripheral axons (Fig. 6*G*). In contrast, GFP-positive cells in either *neurog1* morphant or *neurog1*^{−/−} embryos do not express the Elavl antigen and fail to send out axons (Fig. 6*H,I*). When the total number of segments that contain GFP-positive cells along the trunk of the embryo are quantified, the numbers in *neurog1* MO and *neurog1*^{−/−} do not differ signifi-

icantly from controls. In the absence of *neurog1* function, very few GFP⁺ cells express *Elavl*, indicating that they have not differentiated as neurons (Fig. 6J). In addition, GFP-positive neural crest cells also change their location in the absence of *neurog1* function. Unlike the GFP-positive DRG neurons in control embryos that reside dorsolateral to the ventral motor roots of the spinal cord (Fig. 6E), GFP-positive cells in *neurog1*^{-/-} embryos become associated with the ventral roots and in some instances enwrap them (Fig. 6F). These results lead us to hypothesize that these cells have switched to a glial fate.

To determine whether cells that would normally differentiate as DRG neurons have instead formed glial cells in the absence of *neurog1* function, we examined whether *Tg(neurog1:EGFP)*-expressing cells displayed glial markers. Although Sox10 and FoxD3 are initially expressed in all trunk neural crest cells, including those that will give rise to neurons or glia, they are later expressed only in glial cells (Carney et al., 2006; Lister et al., 2006). Foxd3 is downregulated in neural crest cells before initial expression of *Tg(neurog1:EGFP)*. FoxD3 continues to be expressed in cells surrounding the DRG, which are either unspecified neural crest cells or satellite glial cells (Fig. 7A, B). In *neurog1* mutant animals, GFP-positive cells coexpress Foxd3 (Fig. 7B). Sox10 is coexpressed with GFP at the earliest stages of DRG formation (Fig. 2C–E) but is then downregulated in DRG neurons and expressed in cells surrounding the DRG (Fig. 7C). In contrast, Sox10 expression is maintained with loss of *neurog1* function (Fig. 7D). Although the total number of cells that comprise the DRG shows a small but significant decrease in *neurog1* MO and *neurog1*^{-/-} embryos compared with controls, the number of cells that coexpress Sox10 and GFP is greatly increased in embryos lacking *neurog1* function (Fig. 7E).

Because both FoxD3 and Sox10 are markers of both neural crest cells and glial cells and it has not been established when the transition from neural crest to glia occurs in the zebrafish DRG, we used an additional glial marker. To determine whether GFP/Sox10-positive cells in embryos lacking *neurog1* function simply remain undifferentiated or whether they instead become Schwann cells, we examined expression of MBP (Lyons et al., 2005). At 5 dpf, MBP marks the Schwann cells that are associated with the ventral motor roots (Brosamle and Halpern, 2002). In control embryos, the GFP-positive axons of the DRG run parallel to, but are not associated with, the ventral roots and are not labeled with MBP (Fig. 7F). In *neurog1*^{-/-} embryos, GFP-positive cells that have become associated with the ventral roots coexpress MBP, suggesting that at least some of the GFP-positive cells have become Schwann cells (Fig. 7G).

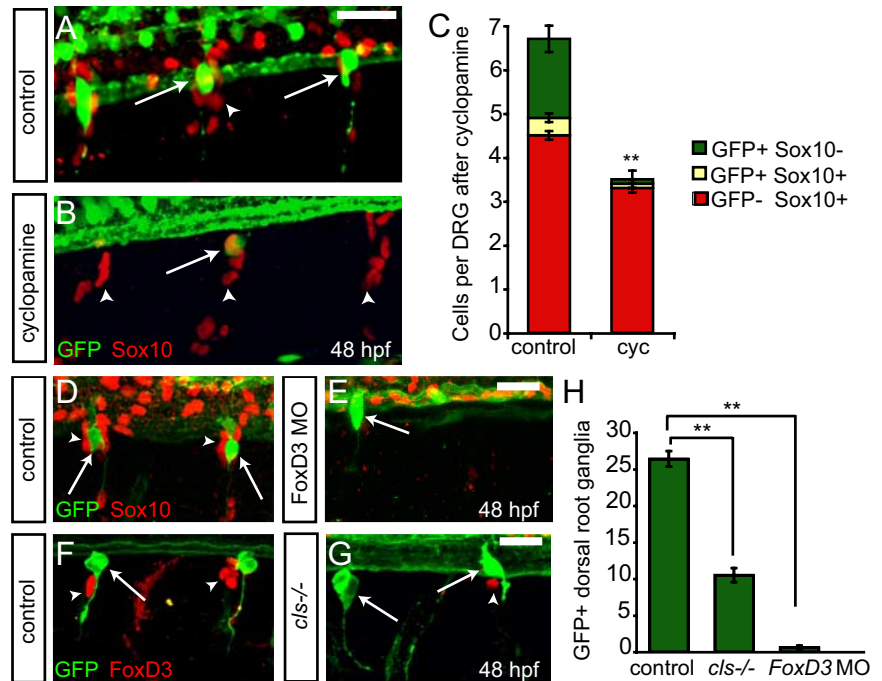


Figure 5. Factors that act upstream of *neurog1* block *Tg(neurog1:EGFP)* cells in the DRG. **A, B**, *Tg(neurog1:EGFP)* embryos at 48 hpf treated with cyclopamine or HBC at 22 hpf and labeled with anti-GFP (green) and anti-Sox10 (red). **A**, An HBC-treated control embryo showing GFP-labeled DRG neurons and Sox10⁺ glia (arrow). **B**, Cyclopamine treatment starting at 22 hpf leads to a loss of nearly all GFP expression in the DRG (arrow), although glial cells remain present (arrowhead). **C**, Quantification (\pm SEM) of the GFP⁺ neurons and Sox10⁺ glial cells in the DRG. There is a significant decrease in the number of GFP⁺ cells in cyclopamine-treated embryos. ($n = 11$; $**p < 0.0001$, Student's *t* test). **D, E**, Confocal *z*-projections of 48 hpf *Tg(neurog1:EGFP)* embryos injected with *foxd3* MO and labeled with anti-GFP (green) and anti-Sox10 (red). **D**, Control uninjected embryo with GFP⁺ DRG neurons (arrows) and Sox10⁺ glia (arrowhead). **E**, *foxd3* morphant embryo with a rare GFP⁺ DRG neuron (arrow). No Sox10⁺ glial cells are associated with the DRG. **F, G**, Confocal images of 48 hpf *cls*^{-/-}; *Tg(neurog1:EGFP)* and control *Tg(neurog1:EGFP)* embryos labeled with anti-GFP (green) and anti-Foxd3 (red). **F**, Control embryo showing GFP⁺ DRG neurons (arrow) and Foxd3⁺ glia (red). **G**, The rostralmost GFP⁺ DRG neurons are present in *cls*^{-/-} embryos (arrow), whereas the vast majority of Foxd3⁺ cells are absent (arrowhead). **H**, Comparison of the number of GFP⁺ DRG (\pm SEM) in control, *foxd3* MO, and *cls*^{-/-} embryos, showing the loss of most DRG in both experimental conditions ($n = 10$; $**p < 0.0001$, Student's *t* test). Scale bars, 20 μ m.

Changes in proliferation in the absence of *neurog1* function

Differentiated sensory neurons in the zebrafish DRG show a very low level of cellular proliferation as the embryo develops (An et al., 2002). In contrast, zebrafish peripheral glial cells are derived from larger neural crest cell clones (Raible and Eisen, 1994), and, in other organisms, peripheral glial cells are typically highly proliferative (Jessen and Mirsky, 2005). To determine whether GFP-positive cells alter their rates of proliferation in embryos lacking *neurog1* function, we assayed BrdU incorporation that marks cells in S-phase and expression of anti-pH3 antibody that marks cells in M-phase. We performed a BrdU pulse during two stages: during day 2 of development (30–48 hpf), beginning before most DRG precursor cells differentiate as neurons, and during day 3 (50–72 hpf), when most DRG neurons have begun to express the neuronal marker *Elavl*. In control embryos, some GFP⁺ cells were derived from cells that proliferate during the first period of BrdU treatment (Fig. 8A); an average of one cell of the approximately three that make up the DRG has incorporated BrdU (Fig. 8E). GFP⁺ cells had rarely incorporated BrdU during the later period (Fig. 8C, E). In contrast, embryos injected with the *neurog1* MO show a significantly higher level of BrdU incorporation on both days (Fig. 8B, E), including robust incorporation on 3 dpf. These findings are supported by analysis using anti-pH3 antibody. Although pH3-positive cells are rare, there is still a signifi-

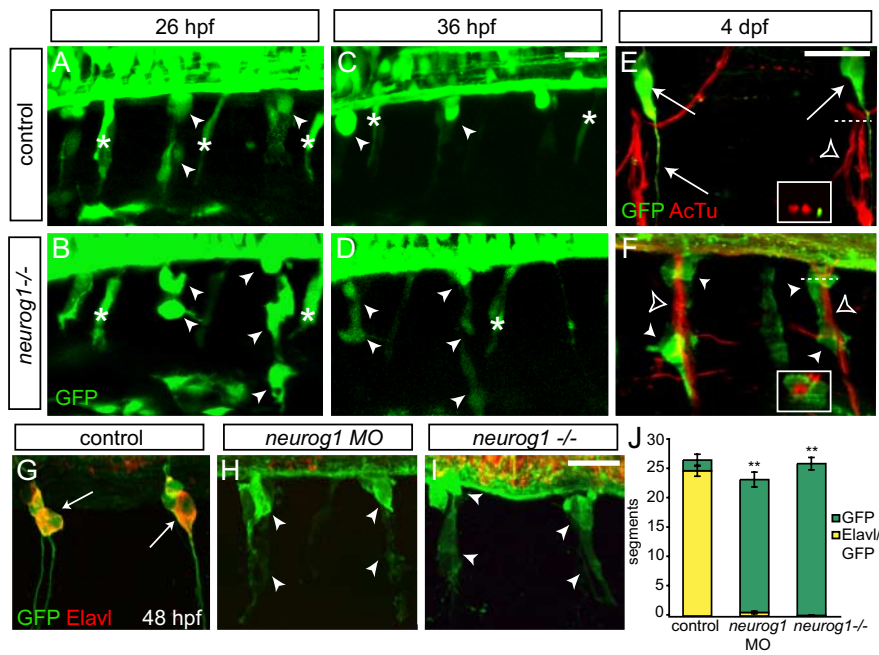


Figure 6. *Tg(neurog1:EGFP)*-expressing cells persist in the absence of *neurog1* function. **A–D**, Live time-lapse imaging of the forming rostralmost DRG in *Tg(neurog1:EGFP)* embryos from 26 to 36 hpf in the presence and absence of *neurog1* function. At 26 hpf, GFP⁺ DRG precursors migrate ventrally (arrowhead) in both control (**A**) and *neurog1*^{-/-} (**B**) embryos. By 36 hpf, GFP⁺ DRG cells have coalesced at the ventral edge of the spinal cord (**C**). In contrast, GFP⁺ cells in the *neurog1*^{-/-} embryo remain elongated (**D**). **E, F**, Confocal z-projections of 4 dpf *Tg(neurog1:EGFP)* and *neurog1*^{-/-} embryos labeled with anti-GFP (green) to label DRG and anti-acetylated tubulin to label ventral motor roots (red). **E**, In control embryos, GFP⁺ DRG neurons and their axons (arrows) are located adjacent to the ventral roots (open arrowhead). Inset, Cross-section of the ventral root taken at the level denoted by the dashed line showing the proximity of DRG axons to the motor axons. **F**, In contrast, GFP⁺ cells (arrowheads) in *neurog1*^{-/-} embryos become tightly associated with the ventral root (open arrowhead). Inset, Cross-section of the ventral root taken at the level denoted by the dashed line indicating that, in the *neurog1* mutant, the GFP⁺ cells unwrap the ventral root. **G, H**, Confocal images of 48 hpf *Tg(neurog1:EGFP)* embryos in the presence or absence of *neurog1* function label with anti-GFP (green) and anti-Elavl (red). **G**, In control embryos, the majority of GFP⁺ DRG neurons are also labeled with Elavl (arrow). In both *neurog1* morphant embryos (**H**) and *neurog1*^{-/-} embryos (**I**), GFP⁺ cells are present (arrowheads) but have an elongated morphology compared with control and are not labeled with Elavl. **J**, Quantification (\pm SEM) of the total number of segments along the trunk of the embryo that contain GFP⁺ (green bars) and Elavl⁺/GFP⁺ (yellow bars) clusters in the position of the DRG in control, *neurog1* MO, and *neurog1*^{-/-} embryos ($n = 10$ in all conditions). Although there is only a minor decrease in the number of GFP⁺ segments after loss of *neurog1* function, there is a dramatic decrease in the number of Elavl⁺/GFP⁺ segments compared with control (** $p < 0.0001$, ANOVA). Scale bars, 20 μ m.

cant increase in labeling when *neurog1* function is blocked. We examined the total number of DRG that contained at least one pH3-labeled cell. Approximately 3% of DRG in controls show pH3 labeling, whereas 7.8% of DRG in *neurog1* MO embryos are labeled with pH3 ($p < 0.01$). Considered together, these data indicate that the GFP-positive glial-like cells that form in the absence of *neurog1* function act like satellite glia during early DRG development, and later at least a subset become associated with the ventral motor root, much like myelinating Schwann cells.

Discussion

In this study, we show that, in zebrafish, a fate-restricted population of neural crest cells will form neurons of the DRG and that the proneural factor *neurog1* is a key player in directing cell fate decisions within this population. We used our BAC transgenic zebrafish, *Tg(neurog1:EGFP)*, to monitor the subset of migrating neural crest cells that give rise to the sensory neurons of the DRG and show that these precursor cells persist in the absence of *neurog1* function to take on the attributes of myelinating glial cells. These cells also show an increase in proliferation compared with controls, which may indicate that *neurog1* expression is required in the neural crest to restrict the fate of precursor cells to postmi-

totic neurons. Our data, together with the fact that loss of *neurog1* function does not affect other neural-crest-derived cell types, suggest that there is a subpopulation of neural crest that is restricted to a sensory neuron/glial cell lineage before the expression of *neurog1*. These results are consistent with previous research documenting that, within the population of trunk neural crest, there exists cells that are fate restricted to give rise to specific classes of progeny (Raible and Eisen, 1994; Henion and Weston, 1997; Luo et al., 2003).

The expression of *neurogenin* appears to be the critical factor to direct neural crest cells preferentially toward sensory neuron fates. Our current study and work by others has shown that loss of zebrafish *neurog1* function leads to a loss of DRG sensory neurons, whereas other neural-crest-derived neurons of the peripheral nervous system are unaffected (Andermann et al., 2002; Cornell and Eisen, 2002). A similar role for the *neurogenins* has been described in mouse and chick, indicating that *neurog1* and *neurog2* are required for the development of DRG sensory neurons from neural crest (Greenwood et al., 1999; Ma et al., 1999; Perez et al., 1999; Parras et al., 2002). Both *neurog1* and *neurog2* are expressed in a subset of migrating neural crest cells before their differentiation and later in nascent DRG neurons, suggesting that *neurogenin* expression may be acting to restrict these cells to a neuronal lineage (Ma et al., 1999; Perez et al., 1999). Overexpression studies suggest that *neurogenin* biases migrating neural crest to localize to the DRG and to express sensory neuron-specific markers (Perez et al., 1999). Moreover, ectopic expression of *neurogenins* in non-neural-crest-derived tissue elicited expression of sensory neuron-specific markers (Ma et al., 1996; Blader et al., 1997; Perez et al., 1999). In the mouse embryo, *neurog1* and *neurog2* are expressed in temporally distinct, but overlapping waves, and can be partially compensatory for one another; knockdown of both genes is required for complete loss of DRG neurons (Ma et al., 1999). Although alterations in cell fate were not investigated, an increase in terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling-positive cells was observed in *neurog2*^{-/-} mice, confirming that some cells undergo apoptosis in the absence of proneural factors (Ma et al., 1999). In contrast to this previous study, we see little cell loss (Fig. 6E); rather, cells fated to become DRGs instead became glial cells in the absence of *neurogenin* function.

A role for *neurogenin* in directing binary cell fate decisions between neurons and glia has been suggested to occur in the CNS (Bertrand et al., 2002; Ross et al., 2003; Miller and Gauthier, 2007). In the developing cortex, neurons and then glia are born from multipotent precursors in a sequential manner (Qian et al., 2000; Shen et al., 2006). Expression of *neurog1*, *neurog2*, and *mash1* play essential roles in directing cell fate decisions (Nieto et

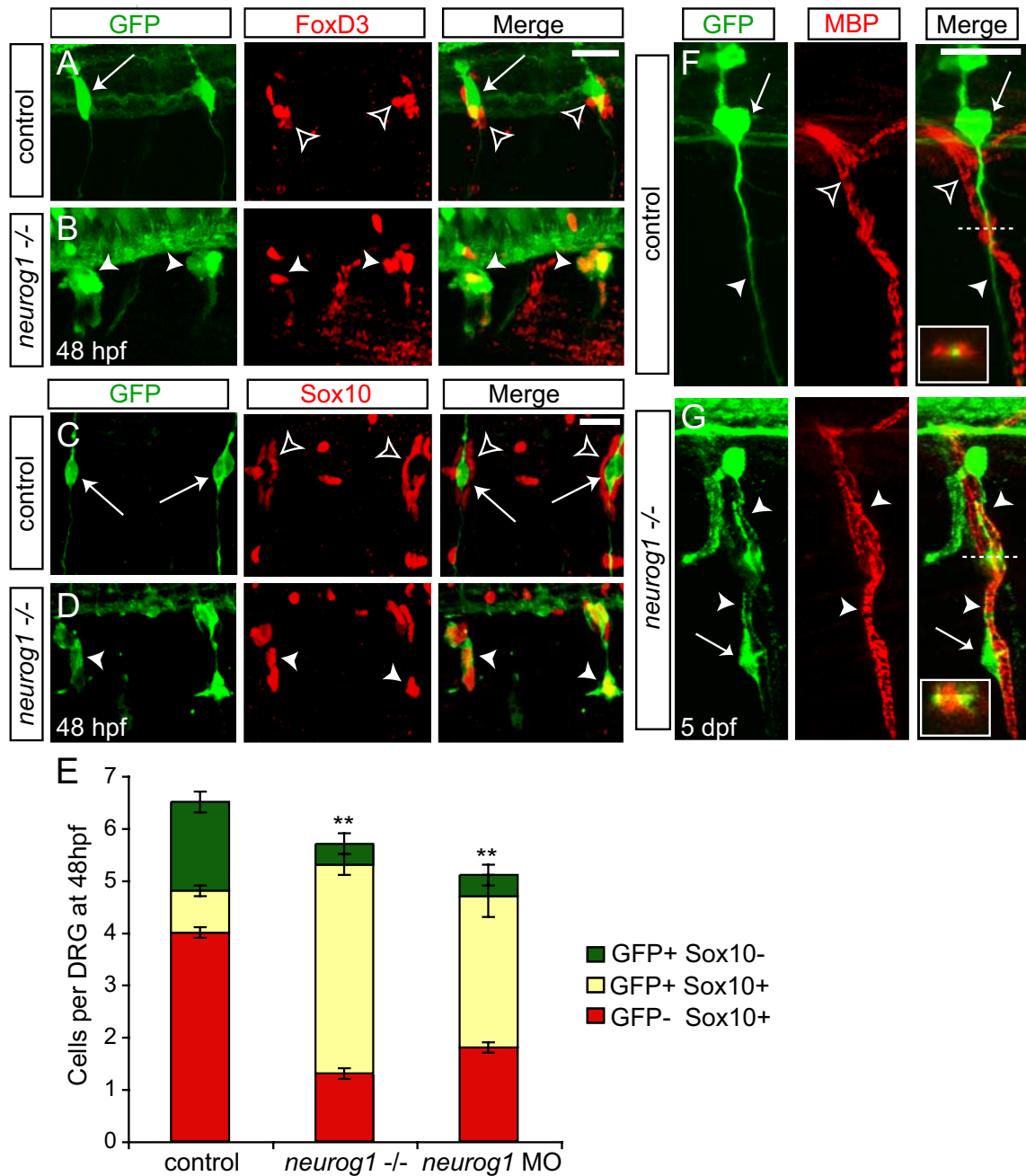


Figure 7. In the absence *neurog1* function, GFP⁺ cells express glial markers. **A, B,** Confocal images of 48 hpf *Tg(neurog1:EGFP)* embryos with or without *neurog1* MO injection and labeled with anti-GFP (green) and anti-Foxd3 (red). **A,** Foxd3 is expressed in glial cells associated with the DRG (open arrowhead) but is not coexpressed with GFP in DRG neurons in control embryos (arrow). **B,** In *neurog1* morphant embryos, Foxd3 is coexpressed with GFP (arrowhead) in the DRGs. There remains a population of Foxd3⁺ glia that is not GFP⁺ (open arrowhead). **C, D,** Confocal images of a 48 hpf *neurog1*^{-/-} embryo and a wild-type or heterozygous sibling labeled with anti-GFP (green) and anti-Sox10 (red). As with Foxd3, Sox10 marks glial cells in the DRG at 48 hpf. **C,** In control embryos, Sox10⁺ glia (open arrowheads) surround GFP⁺ DRG neurons (arrows). **D,** In contrast, GFP⁺ cells in *neurog1*^{-/-} embryos coexpress Sox10 (arrowheads). **E,** Quantification (± SEM) of GFP⁺ and Sox10⁺ cells in control embryos and in the absence of *neurog1* function at 48 hpf. Each bar represents the number of GFP⁺ (green bars) cells in the DRG, plus the number of cells that are GFP⁺/Sox10⁺ (yellow bars) and the number of Sox10⁺ cells that are in direct contact with the DRG cells (red bars). On average, the total number of cells in the DRG decreases in *neurog1* MO and *neurog1*^{-/-} embryos compared with controls ($p = 0.04$, ANOVA). There is not a significant difference in the total number of Sox10⁺ cells between *neurog1* MO, *neurog1*^{-/-}, and uninjected control embryos (control, *neurog1* MO embryos, $n = 10$; *neurog1*^{-/-}, $n = 11$). Significantly more cells are GFP⁺ and Sox10⁺ in the absence of *neurog1* function compared with controls (** $p < 0.0001$, ANOVA). **F, G,** Images of 5 dpf *neurog1* mutant and sibling embryos labeled with anti-GFP (green) and anti-MBP (red) to mark myelinating Schwann cells. Insets, Cross-sections of ventral roots taken at the level of the dashed line. **F,** A GFP⁺ DRG neuron (arrow) and its peripheral axon (arrowhead) are located adjacent to, but are not associated with, MBP⁺ Schwann cells surrounding the ventral motor root (open arrowhead). The inset shows the GFP⁺ axon in close proximity to MBP⁺ Schwann cells. **G,** In the *neurog1* mutant, GFP⁺ cells (arrows) enwrap the ventral motor root and are MBP⁺ (arrowhead). The inset shows the coexpression of GFP and MBP in the *neurog1*^{-/-} embryo. Scale bars, 20 μ m.

al., 2001; Britz et al., 2006). Using LacZ expression under the control of the *neurog2* promoter to trace the fate of cortical cells in *Mash1*^{-/-}, *neurog2*^{-/-} mice, Nieto et al. (2001) found a significant decrease in the number of cortical neurons and an in-

crease in the number of astrocytes, leading the authors to propose a model in which the proneural factors are acting to both promote a neuronal cell fate and to restrict a glial cell fate in cortical progenitor cells.

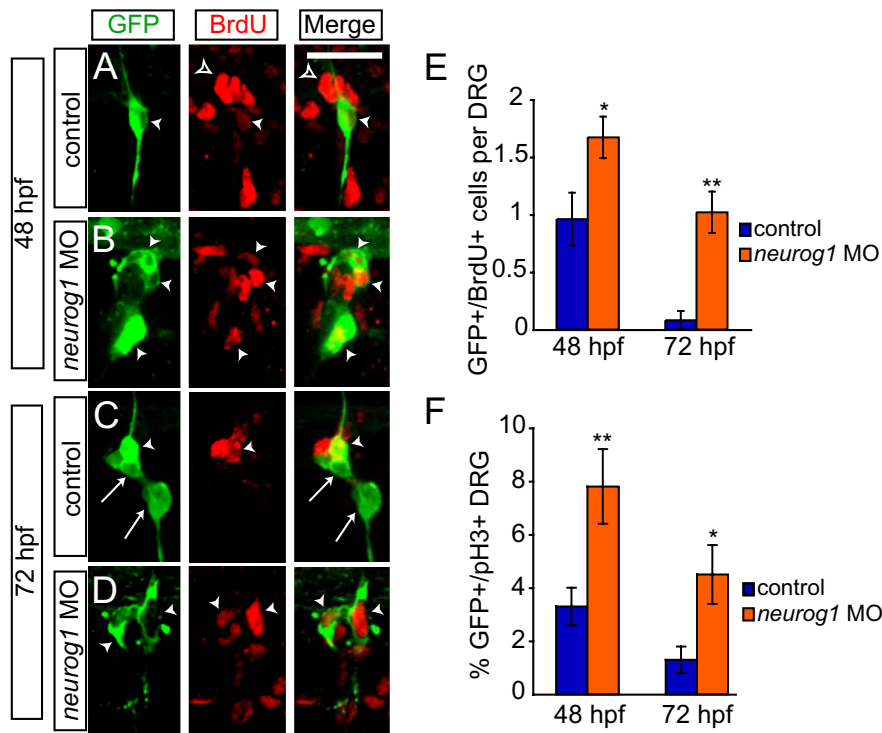


Figure 8. GFP⁺ cells are mitotically active after *neurog1* MO injection. **A–D**, *Tg(neurog1:EGFP)* embryos and *neurog1* morphants labeled with anti-GFP (green) and anti-BrdU (red) to mark proliferating cells in S-phase. **A**, At 48 hpf, BrdU labels a GFP⁺ DRG neuron (arrowhead). Several BrdU⁺/GFP[−] cells can be seen surrounding the DRGs (open arrowhead). **B**, In a *neurog1* MO embryo at 48 hpf, several GFP⁺ cells are also labeled with BrdU (arrows). **C**, At 72 hpf, a subset of GFP⁺ cells is also BrdU⁺ (arrowhead), although many GFP⁺ cells are not BrdU⁺ (arrow). **D**, At the same time point, GFP⁺/BrdU⁺ cells (arrowhead) can be seen in a *neurog1* MO embryo. **E**, Quantification (±SEM) of GFP⁺ and BrdU⁺ cells 48 and 72 hpf. At 48 hpf, there are significantly more GFP⁺/BrdU⁺ cells in *neurog1* MO-injected embryos ($n = 10$; orange bars) compared with controls ($n = 12$; blue bars; * $p = 0.02$, Student's t test). At 72 hpf, very few GFP⁺/BrdU⁺ cells are seen in control embryos ($n = 9$), whereas significantly more GFP⁺/BrdU⁺ are still seen in *neurog1* MO embryos ($n = 10$; ** $p = 0.0002$, Student's t test). **F**, Comparison of anti-GFP antibody labeled DRG that contain at least one anti-pH3-labeled cell in the DRG of control (blue bars) and *neurog1* MO (orange bars) embryos at 48 and 72 hpf ($n = 15$ embryos for all conditions). The percentage of pH3⁺/GFP⁺ DRG seen in *neurog1* MO embryos compared with control is significantly high at both 48 hpf (** $p < 0.005$, Student's t test) and at 72 hpf (* $p < 0.05$, Student's t test). Scale bars, 20 μ m.

A mechanism underlying the dual function of *neurog1* in promoting neural differentiation while inhibiting glia in multipotent progenitor cells has been described in detail for cultured rat cortical neurons (Sun et al., 2001). During neurogenesis, Neurog1 acts to turn on another proneural bHLH factor, *neuroD*, which in turn promotes neuronal differentiation (for review, see Bertrand et al., 2002). The coactivator proteins p300/CBP are recruited by Neurog1 to the *neuroD* promoter to activate its expression (Sun et al., 2001). By sequestering p300/CBP, *neurog1* also inhibits glia differentiation. The expression of glial fibrillary acid protein (GFAP) is activated by the cooperation of Smad1 and Stat1/3 proteins that in turn require p300/CBP as a coactivator. When Neurog1 sequesters available p300/CBP to the *neuroD* promoter, there is insufficient p300/CBP to activate the *GFAP* promoter. It is possible that a similar process may function during neuron versus glial cell fate decision during DRG development.

We found that loss of *neurog1* resulted in increased cell division in addition to promotion of glial differentiation. Peripheral glial cells are typically highly proliferative (Jessen and Mirsky, 2005), so changes in proliferation rates in DRG precursors may simply reflect their acquisition of glial fates. Increased proliferation may also reflect the block in neuronal differentiation, be-

cause these cells become postmitotic. Expression of *neurogenin* genes in *Xenopus* primary neurons has been suggested to promote cell cycle exit through regulation of the cyclin-dependent kinase inhibitor p27 (Vernon et al., 2003) or its activating kinase (Souopgui et al., 2002), so loss of *neurog1* would be predicted to allow cell cycle reentry. Alternatively, cells may retain characteristics of undifferentiated neural crest precursors. For example, the continued expression of Sox10 after loss of *neurogenin* function may maintain cells in a proliferative multipotent state (Kim et al., 2003). Our results suggest that, in the absence of *neurog1* function, at least a subset of cells differentiate as Schwann cells, although these observations do not exclude the possibility that others remain undifferentiated.

Our results differ somewhat from those of Zirlinger et al. (2002), who followed the fates of mouse neural crest cells using an inducible Cre recombination system to permanently mark cells that express *neurog2* at various stages during DRG formation. In that study, cells that expressed *neurog2* were significantly more likely to be found in the DRGs than in sympathetic ganglia, indicating that *neurog2* expression drives neural crest toward a sensory fate. However, cells that expressed *neurog2* were equally likely to form DRG sensory neurons or satellite glia. These results suggest that, in mouse, there is a fate-restricted neural crest lineage that is biased toward a sensory ganglion fate rather than a neuronal fate. There are several possible explanations for the differences between this study and our findings. There may be

differences in the sensitivity of the lineage tracing systems: the Cre–Lox marking system used in mice may be more sensitive and mark transiently expressing cells, whereas detectable GFP expression from the zebrafish transgene may require more prolonged gene transcription. Alternatively, expression of mouse *neurog2* may not be sufficient to direct all cells to neuronal fates without expression of additional neurogenic factors, including *neurog1*, complementary functions that are subsumed by the single zebrafish *neurogenin* gene. It is interesting to note however that, in both systems, the marked cells have fates limited to sensory neurons and glia. Together, these results suggest that signals upstream of *neurogenin* have limited neural crest cell fates to these lineages.

One crucial question that needs to be answered is how *neurog1* expression is initiated in neural crest cells to direct them to become DRG precursors. Sox10 and FoxD3 have been shown to regulate *neurog1* expression in the developing zebrafish DRG (Carney et al., 2006; Lister et al., 2006). Although these factors play important roles in mouse DRG development (Sonnenberg-Riethmacher et al., 2001; Teng et al., 2008), regulation of *neurogenin* genes has not been established. The receptor tyrosine kinases ErbB2 and ErbB3 have also been shown to be required for *neurog1* expression and for the formation of both glial cells and

DRG neurons (Honjo et al., 2008). It remains to be established whether these factors act directly to control *neurog1* expression or whether they act by regulating other potential regulators. Additionally, all of these factors act in both neurons and glia. The Hh signaling pathway is also a good candidate for a key regulator of *neurog1* during DRG development in the zebrafish and is not required for glial formation (Ungos et al., 2003). Additional research is needed to determine whether these regulatory pathways are required for sensory neurons as they are added later during DRG development. Because the zebrafish DRG continues to develop well into adulthood (An et al., 2002), it may prove to be a useful model in which to identify how *neurog1* is directly regulated in the DRG.

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