Development 135, 2993 (2008) doi:10.1242/dev.027763

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There was an error published in *Development* **135**, 2615-2625.

In the Materials and methods section, the subheading should be

***neuregulin 1* and *neuregulin 2* gene cloning**

The authors apologise to readers for this mistake.

Development 135, 2615-2625 (2008) doi:10.1242/dev.022178

Neuregulin-mediated ErbB3 signaling is required for formation of zebrafish dorsal root ganglion neurons

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Dorsal root ganglia (DRGs) arise from trunk neural crest cells that emerge from the dorsal neuroepithelium and coalesce into segmental streams that migrate ventrally along the developing somites. Proper formation of DRGs involves not only normal trunk neural crest migration, but also the ability of DRG progenitors to pause at a particular target location where they can receive DRG-promoting signals. In mammalian embryos, a receptor tyrosine kinase proto-oncogene, ErbB3, is required for proper trunk neural crest migration. Here, we show that in zebrafish mutants lacking ErbB3 function, neural crest cells do not pause at the location where DRGs normally form and DRG neurons are not generated. We also show that these mutants lack trunk neural crest-derived sympathetic neurons, but that cranial neural crest-derived enteric neurons appear normal. We isolated three genes encoding neuregulins, ErbB3 ligands, and show that two neuregulins function together in zebrafish trunk neural crest cell migration and in DRG formation. Together, our results suggest that ErbB3 signaling is required for normal migration of trunk, but not cranial, neural crest cells.

KEY WORDS: ErbB2, ErbB3, Neuregulin, Dorsal root ganglion, Neural crest migration, Zebrafish

INTRODUCTION

Segmentally reiterated dorsal root ganglia (DRGs) are required for vertebrates to sense and respond to many environmental and proprioceptive signals (Scott, 1992). DRGs are located adjacent to the spinal cord, and their component neurons and glia arise from trunk neural crest (NC) cells (Le Douarin and Kalcheim, 1999; Kalcheim, 2000; Eisen and Weston, 1993). Neural crest is a transient, embryonic cell population that emigrates from the dorsal neuroepithelium, migrates along specific pathways and generates a variety of progeny, including neurons and glia of the peripheral nervous system, pigment cells and craniofacial cartilages. Trunk NC cells migrate on two pathways: a ventral (also called medial) pathway between the somites and neural tube; and a dorsolateral (also called lateral) pathway between the somites and overlying ectodermal epithelium (Weston and Butler, 1966; Serbedzija et al., 1989; Serbedzija et al., 1990; Erickson et al., 1992; Erickson and Weston, 1983; Raible et al., 1992). The ventral or medial migration pathway is restricted to a particular region of the somites (Le Douarin and Kalcheim, 1999; Kalcheim, 2000); in zebrafish, this pathway is restricted to the middle of the medial surface of each somite, half way between adjacent somite boundaries (Honjo and Eisen, 2005; Raible et al., 1992). Thus, zebrafish trunk NC cells migrate in a pattern of 'streams'; a single stream underlies the middle of each somite. Zebrafish DRG neurons are derived from among the earliest NC cells to migrate on the medial pathway (Raible and Eisen, 1994). However, whether DRGs arise from a specified NC subpopulation, how the fates of individual NC cells are regulated during migration, and how NC cells recognize specific target locations appropriate for their eventual differentiation are currently not well understood.

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Accepted 11 June 2008

In amniote embryos, ErbB3 is one factor that has been implicated in NC migration. ErbB3 is a receptor tyrosine kinase proto-oncogene member of the epidermal growth factor (EGF) receptor family. Although ErbB3 can form homodimers, active receptors require heterodimerization with ErbB2 (Guy et al., 1994; Tzahar et al., 1996; Graus-Porta et al., 1997; Kim et al., 1998; Olayioye et al., 2000; Holbro et al., 2003). ErbB2/ErbB3 heterodimers bind to neuregulin (Nrg) 1 or Nrg2, members of the EGF-like polypeptide growth factor superfamily (Riese et al., 1995; Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Olayioye et al., 2000; Holbro et al., 2003). Nrg1 signaling via ErbB3 is important for several developmental processes, including glial migration and development (Riethmacher et al., 1997; Erickson et al., 1997; Garratt et al., 2000; Lyons et al., 2005), development of myelination (Chen et al., 2006), muscle acetylcholine receptor expression (Morris et al., 1999; Woldeyesus et al., 1999; Lin et al., 2000; Fall, 2003), and neural migration (Anton et al., 1997; Rio et al., 1997; Olayioye et al., 2000).

The initial suggestion of a role for ErbB3 in NC migration came from mice with targeted mutations in *Erbb2*, *Erbb3* or *Nrg1* (Britsch et al., 1998). These mice lack sympathetic ganglia, presumably because without ErbB receptor-mediated neuregulin signaling, NC cells cannot migrate to the region lateral of the dorsal aorta, where sympathetic neuron differentiation occurs (Britsch et al., 1998). By contrast, DRGs, the progenitors of which do not have to migrate any significant distance after emigrating from the neural folds (Teillet et al., 1987), initially appear normal in *Erbb3* mutant mice (Britsch et al., 1998). However, although NC cells form an ectopic cluster around the DRGs in *Erbb3* mutant mice, these ectopic NC cells do not differentiate into DRG neurons or glia (Britsch et al., 1998); later, most DRG neurons reportedly die (Riethmacher et al., 1997). *Erbb2* mutant mice die of cardiac defects too early to determine whether ErbB2 is required for DRG formation (Morris et al., 1999; Woldeyesus et al., 1999). Thus, the role of ErbB signaling in DRG formation remains unresolved.

Here, we show that zebrafish mutants lacking function of either ErbB3b or ErbB2 do not form trunk NC-derived DRG or sympathetic neurons. By contrast, cranial NC-derived enteric neurons appear

normal. Previous studies have shown that zebrafish *erbb3b* is expressed in NC cells and that *erbb3b* mutants have defects in glial migration and in myelinating Schwann cell terminal differentiation (Lyons et al., 2005), similar to mouse *Erbb3* mutants (Lee et al., 1995; Meyer and Birchmeier, 1995; Erickson et al., 1997; Meyer et al., 1997; Riethmacher et al., 1997). We provide evidence that in zebrafish *erbb3b* mutants, migrating trunk NC cells do not pause in the position where DRGs normally form. Our results suggest that ErbB receptor signaling is required during specific periods when NC-derived cells normally pause at the location where DRGs form. Thus, we suggest that the absence of DRGs in *erbb3b* mutants is a consequence of inappropriate trunk NC cell migration resulting from the inability of mutant NC cells to recognize a specific target location. We further hypothesize that the absence of sympathetic ganglia also results from failure of correct trunk NC cell migration. By contrast, the apparently normal formation of enteric neurons in *erbb3b* mutants suggests that at least some cranial NC migrates appropriately in the absence of ErbB receptor signaling. To learn which ligands affect trunk NC migration, we isolated genes encoding three different zebrafish neuregulins. We knocked these down using morpholino antisense oligonucleotides (MOs) and found that two of the neuregulins act in concert to regulate trunk NC migration and DRG formation.

MATERIALS AND METHODS

Animals

Embryos were obtained from natural spawnings of a wild-type (AB) colony or crosses of identified carriers heterozygous for the *erbb3b*^{st48} and *erbb2*^{st60} mutations (Lyons et al., 2005). Fish were maintained in the University of Oregon Zebrafish Facility on a 14-hour light/10-hour dark cycle at 28.5ºC. Embryos and larvae were staged according to Kimmel et al. (Kimmel et al., 1995) by number of somites, by hours post fertilization at 28.5°C (hpf), or by days post fertilization at 28.5°C (dpf). Mutant embryos were generated by crossing two heterozygous adult carriers. We also used the *Tg(–4.9sox10:EGFP)*^{ba2} transgenic line [hereafter referred to as *Tg(sox10:GFP)*] in which GFP is driven by the *sox10* promoter (Wada et al., 2005; Carney et al., 2006). Homozygous *Tg(sox10:GFP)* fish have a mutant phenotype; therefore, to obtain *erbb3b* mutants carrying this transgene, heterozygous *erbb3b* mutant adults were crossed with *erbb3b; Tg(sox10:GFP)* fish.

RNA in situ hybridization and immunohistochemistry

RNA in situ hybridization was performed as described previously (Appel and Eisen, 1998). Antisense riboprobes were detected by NBT/BCIP (Roche Diagnostics). DIG-labeled antisense RNA probes (Roche Diagnostics) for RNA in situ hybridization were generated from plasmids as follows: *neurogenin 1* (*neurog1*) plasmid (Blader et al., 1997) was cut with *Bam*HI and transcribed with T7 polymerase; *neurod* plasmid (Blader et al., 1997) was cut with *Not*I and transcribed with T3; *crestin* plasmid (Luo et al., 2001) was cut with *Eco*RI and transcribed with T7; *sox10* plasmid (Dutton et al., 2001) was cut with *Sal*I and transcribed with T7. Anti-Elavl antibody (16A11; previously known as anti-HuC/D) was used at 1:1000 (Marusich et al., 1994; Henion et al., 1996) and anti-tyrosine hydroxylase (TH) antibody (Pel-Freeze) was used at 1:100 (An et al., 2002). Alexa-488 conjugated goat anti-rabbit polyclonal antibody was used as secondary antibody. Frozen sectioning was performed after RNA in situ hybridization.

Time-lapse confocal microscopy

Migration of NC expressing GFP was visualized using a confocal laser scanning microscope (Zeiss LSM5 Pascal). Embryos were mounted in 0.2% agar containing dilute tricaine. Images were taken from around 24-72 hpf. For identifying mutants, embryos were fixed after imaging and genotyped by PCR as described by Lyons et al. (Lyons et al., 2005).

AG1478 treatment

AG1478 [4-(3-chloroanilino)-6,7-dimethoxyquinazoline; Calbiochem] was used at 4 μM in 0.4% DMSO as described by Lyons et al. (Lyons et al., 2005). As a control, we used 0.4% DMSO alone. All embryos were dechorionated before treatment. DRG axial level was scored at 4 dpf in AG1478-treated embryos by counting somite number at this stage from the most posterior Elavl-positive cranial ganglia (ganglia of vagus and posterior lateral line nerves) (Raible and Kruse, 2000). The most anterior somite, which is the one closest to these ganglia, was designated as somite one, consistent with our previous studies showing that the most anterior five somites remain present through at least 12 dpf (Morin-Kensicki et al., 2002). We counted somites, rather than DRGs, because we found that DRGs were variably present medial to the first two somites in 4 dpf wild-type embryos.

neurogenin 1 and *neurogenin 2* gene cloning

Primers for *nrg1* cloning were designed based on EST clones found by using murine *Nrg1* sequence to search the zebrafish Ensemble database (http://www.ensembl.org/Danio_rerio/index.html). For *nrg2,* the Fugu Ensemble database (http://www.ensembl.org/Takifugu_rubripes/index.html) was first searched by using murine sequences and then the zebrafish database was searched using Fugu sequence. Two high homology sequences were found on chromosome (Chr) 14 and Chr 21. Primers were designed based on EST clones and amplified by PCR. 5′- or 3′-RACE were performed using SMART RACE cDNA Amplification kit (Clontech). Although we found an EST clone (clone ID810694; BC139893) that is *nrg2a* by sequence comparison, our clone from RT-PCR and 5′, 3′ RACE is different in the 3′ region (see Fig. S2 in the supplementary material). We have never been able to isolate exactly the same clone as the EST described above. This might be because of background strain or tissue sample differences.

RT-PCR

Splice-blocking and mismatch control MO-injected embryos were collected and RNA extracted with TRI reagent (MRC). RT-PCR was performed with 2 or 3 μg of each RNA using SuperscriptIII (Invitrogen). For MO confirmation, gene-specific primers were used; an oligo dT primer was used at each stage to examine gene expression. Subsequently, fragments were amplified by PCR. Primers sequences used for RT-PCR are as follows: *nrg1* (MO) forward primer, 5′-ctgttgatacggaaatccac-3′; *nrg1* (MO) reverse primer, 5′-tcgctctcgtaaactctgcc-3′; *nrg1* (type I) forward primer, 5′-atggctgaggtgaaagcaggcaa-3′; *nrg1* (type II) forward primer, 5′-atggcgattctgccaggacgca-3′; *nrg1* (type III) forward primer, 5′-gagatgaagtcggaggcggcgga-3′, *nrg1* (all types) reverse primer, 5′-cagtattccttctcgctctcgttg-3′; *nrg2a* forward primer, 5′-ggcggcgactgttactacat-3′; *nrg2a* reverse primer, 5′-cccattggccaggttgcgatt-3′; nrg2b forward primer, 5′-ctacacctgtgtggtggagaa-3′; nrg2b reverse primer, 5′-tgcctccatacacgccgggctactgc-3′; β-actin forward primer, 5′-tggcatcacaccttctac-3′; β-actin reverse primer, 5′-agaccatcaccagagttc-3′.

Morpholino microinjection

Embryos were injected with 2-5 nl of MOs into the yolk at the one-cell stage. *nrg1* MO [100 μM-1 mM; MO sequences reported by Milan et al. (Milan et al., 2006)], *nrg2a* MO (75 μM for single MO injections, 100 μM for co-injection with *nrg1* MO; 5′-tgacagaggagaaactcacttgcag-3′) and *nrg2b* MO (100 μM; 5′-ttgacaatgtgtaacttacttgcag-3′) were injected into wild-type embryos. Mismatch MOs were used as a control for each MO. Pipettes were pulled on a Sutter Instruments Micropipette puller (Model P-2000). Injections were performed with an air injection apparatus (ASI). The efficacy of the *nrg1* MO varied from injection to injection. Therefore, we performed an RT-PCR for each injection and compared fish that had similar levels of mis-spliced transcript, rather than comparing fish that received similar MO concentrations.

RESULTS

erbb3b and *erbb2* mutants lack DRG and sympathetic neurons but have enteric neurons

We investigated development of peripheral neurons in *erbb3b* mutants using an antibody to Elavl3 and Elavl4 (previously called HuC and HuD; hereafter referred to as Elavl), a pan-neuronal marker previously shown to be robustly expressed by DRG (Marusich et al., 1994; Henion et al., 1996; Honjo and Eisen, 2005), sympathetic (An

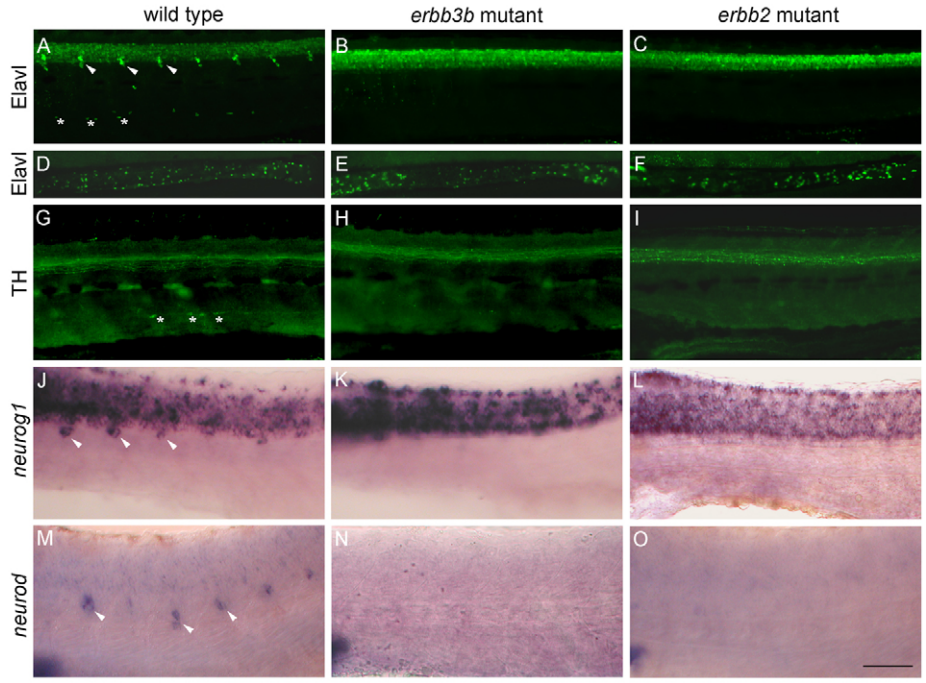

Fig. 1. *erbb3b* and *erbb2* mutants lack DRG and sympathetic neurons. (**A-C**) Elavl antibody labeling reveals DRG (arrowheads) and sympathetic neurons (asterisks). DRGs are segmentally arranged, whereas at this stage, sympathetics are not segmental. DRG and sympathetic neurons are present in wild-type embryos (A) but are absent from *erbb3b* (B) and *erbb2* (C) mutants at 7 dpf. (**D-F**) Elavl-positive enteric neurons appear normal in *erbb3b* (E) and *erbb2* (F) mutants compared with wild-type embryos (D). (**G-I**) TH antibody labeling reveals sympathetic neurons. Whole-mount labeling of 8 dpf larvae showing that sympathetic neurons (asterisks) are present in wild type (G) but absent from *erbb3b* (H) and *erbb2* (I) mutants. (**J-L**) Whole-mount labeling of 31 hpf embryos with a *neurog1* riboprobe reveals nascent DRG neurons. Nascent DRG neurons (arrowheads) are segmental in wild-type embryos (J) but absent from *erbb3b* (K) and *erbb2* (L) mutants. (**M-O**) A *neurod* riboprobe reveals DRG neurons at 36 hpf. DRG neurons (arrowheads) are segmental in wild-type embryos (M) but absent from *erbb3b* (N) and *erbb2* (O) mutants. Lateral views; anterior towards the left. Scale bar: 20 μm.

et al., 2002; Stewart et al., 2004) and enteric (Kelsh and Eisen, 2000; Kuhlman and Eisen, 2007) neurons. At 7 dpf, wild-type embryos have Elavl-positive DRG neurons in every segment, except they are occasionally absent from the first segment. By contrast, *erbb3b* mutants lack DRG neurons (Fig. 1). To determine whether DRG neurons formed earlier, we examined expression of *neurod* and *neurog1*, the earliest-known markers of nascent DRG neurons (Cornell and Eisen, 2002; Ungos et al., 2003). These markers were never expressed in the DRG region of *erbb3b* mutants (Fig. 1), suggesting that they never form DRG neurons. DRG neurons are derived from trunk NC, which also generates sympathetic neurons (Raible and Eisen, 1994); *erbb3b* mutants lack sympathetic neurons at 7 dpf (Fig. 1). In contrast to DRG and sympathetic neurons, enteric neurons are derived from cranial NC (Tobin et al., 2008; Reichenbach et al., 2008); enteric neurons appeared normal in *erbb3b* mutants (Fig. 1).

ErbB3 receptors work as heterodimers and preferentially heterodimerize with ErbB2 receptors. To determine whether ErbB2 also plays a role in zebrafish DRG neuron development, we examined *erbb2* mutants. Similar to *erbb3b* mutants, in *erbb2* mutants, expression of *neurod*, *neurog1* and Elavl were absent from the DRG region, expression of Elavl and TH were absent from the sympathetic ganglion region, and Elavl was expressed normally in enteric neurons (Fig. 1). These data show that zebrafish *erbb3b* and *erbb2* mutants do not form DRG or sympathetic neurons, consistent with the idea that ErbB3 and ErbB2 work as a heterodimer in zebrafish, as they do in other organisms. Our finding that *erbb3b* and *erbb2* mutants lack trunk NC-derived peripheral neurons but have cranial NC-derived enteric neurons raises the possibility that ErbB2/ErbB3 signaling may play different roles in trunk and cranial NC; we return to this point in the Discussion.

ErbB2 and ErbB3 are required for migrating trunk neural crest cells to pause at the location where DRGs normally form

The absence of DRG and sympathetic neurons in *erbb3b* and *erbb2* mutants might result from trunk NC depletion. To test this possibility, we labeled *erbb3b* (Fig. 2) and *erbb2* (not shown) mutants with riboprobes for two genes expressed in migrating NC cells, *crestin* (Luo et al., 2001) and *sox10* (Dutton et al., 2001); the number of NC cells and their migratory pattern appeared normal at 24 hpf. At 27 hpf, a subset of NC cells normally clusters at the dorsal aspect of the notochord, where DRGs form in wild type. A subset of NC cells remains clustered at the dorsal and ventral aspects of the notochord at 30 hpf, when many NC cells have already migrated to a position ventral of the notochord. NC cell clustering at the dorsal aspect of the notochord was never seen in *erbb3b* and *erbb2* mutants, and the region ventral of the notochord appeared depleted of NC cells (Fig. 2 and data not shown). This diminution in ventral NC cells did not result from delayed migration, because the region dorsal of the notochord had approximately the same number of NC cells in wild type, *erbb3b* and *erbb2* mutants (Fig. 2), suggesting some other alteration of NC migration in the mutants.

To understand migration of NC cells, we performed time-lapse confocal microscopy using the *Tg(sox10:GFP)* transgenic line (Wada et al., 2005; Carney et al., 2006). In wild type, as NC cells migrate

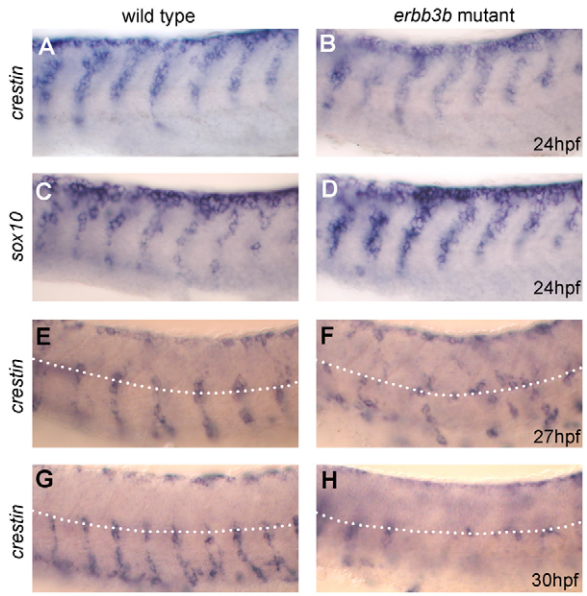

Fig. 2. Neural crest cell migration is initially normal but later disrupted in *erbb3b* mutants. (**A-H**) Whole-mount labeling using *crestin* (A,B,E-H) or *sox10* (C,D) riboprobes of wild-type embryos (A,C,E,G) and *erbb3b* mutants (B,D,F,H). At 24 hpf, both probes show that NC cell migration appears normal in *erbb3b* mutants (B,D) and in wild type (A,C). At 27 (E,F) and 30 (G,H) hpf, NC cells form segmental streams ventral of the notochord in wild type (E,G). By contrast, in *erbb3b* mutants (F,H) there are no streams at these stages, and NC cell migration is disrupted. Broken lines represent dorsal aspect of the notochord. Lateral views, anterior towards the left. Scale bar: 20 μm.

along the medial pathway, some of them pause at the dorsal and ventral aspects of the notochord, often for more than 24 hours (Fig. 3; average pause=27 hours; *n*=3 segments in each of three embryos). Interestingly, these NC cells contact one another, as previously described for migrating NC cells (Fig. 3) (Krull et al., 1995; Kasemeier-Kulesa et al., 2005). Later, between about 50-70 hpf, these paused NC cells become undetectable at mid-trunk levels, presumably because *sox10* is downregulated. In *erbb3b* mutants, very few NC cells paused at the dorsal and ventral aspects of the notochord, and instead they continuously migrated away (Fig. 3). The small number of NC cells paused at the dorsal aspect of the notochord in *erbb3b* mutants did so only briefly compared with wild type (average pause=4.8 hours; *n*=3 segments in each of three embryos). These data are consistent with results from fixed embryos, and together suggest that ErbB3b is required for migrating NC cells to pause just dorsal of the notochord, in the location where DRGs normally form.

NC cells also failed to pause at the ventral aspect of the notochord in *erbb3b* mutants. Cells that normally pause in this region in wild-type embryos may be sympathetic ganglion precursors. However, because early markers such as *sox10* are downregulated by the beginning of the third day of development and later markers such as Elavl and TH are not expressed until several days later (An et al., 2002), there are no detailed studies linking trunk NC migration and trunk sympathetic ganglion formation in zebrafish. Zebrafish trunk sympathetic ganglia are not segmentally organized for at least the first week of development (Fig. 1; Y.H. and J.S.E., unpublished). This is consistent with studies from other species showing that sympathetic ganglion segmentation appears to arise by rostral or caudal migration of cells, rather than being imposed by the segmental migration of trunk NC (Young et al., 2004; Kasemeier-Kulesa et al., 2005). Thus, to learn how ErbB2/ErbB3 signaling affects NC migration and formation of trunk ganglia, we focused on DRG formation.

To test directly when ErbB signaling is required for migrating NC cells in DRG neuron formation, we blocked ErbB signaling conditionally with a pharmacological inhibitor, AG1478. Previous studies have shown that AG1478 inhibits signaling through all ErbB receptors (Busse et al., 2000; Levitzki and Gazit, 1995); Lyons et al. (Lyons et al., 2005) showed that in zebrafish, AG1478 treatment replicates the phenotype of *erbb3b* mutants. We treated zebrafish embryos with 4 μM AG1478 starting at 20 hpf and followed migrating NC cells by time-lapse confocal microscopy, as we had done with wild type and *erbb3b* mutants (see above). The NC migration phenotype of AG1478-treated embryos was essentially the same as the NC migration phenotype of *erbb3b* mutants; NC cells failed to pause at the dorsal and ventral aspects of the notochord, and instead migrated away (Fig. 3; average pause=7.65 hours; *n*=3 segments in each of three embryos).

After showing that AG1478 treatment mimics the *erbb3b* mutant phenotype, we treated embryos at various time periods to learn when ErbB receptors are required for DRG formation (Fig. 4). We performed two series of experiments. In one series we treated embryos from 8 hpf until various later time points. In the other series we treated embryos from various early time points until 30 hpf. DRGs formed medial to the anterior-most five somites even with the earliest treatment, which started at 8 hpf; thus, we only analyzed DRGs in segments posterior of the fifth somite. When we treated embryos from 8 to 14 or 18 hpf, most DRG neurons were missing from segments 6-18, but many DRG neurons formed in more posterior segments (Fig. 4A,B,G). Embryos treated from 8 to 30 hpf lacked DRG neurons from segments 6-30; embryos treated from 8 hpf to times between 18 and 30 hpf had intermediate phenotypes in which the axial level at which DRGs did not form was progressively more posterior for later treatments (Fig. 4G). Embryos treated beginning at 8, 14 or 18 hpf through 30 hpf lacked DRG neurons from segments 6-30 (Fig. 4H). Embryos treated beginning at 22, 24, or 26 hpf to 30 hpf formed DRGs neurons in anterior segments, but not in more posterior ones. Based on these results, we conclude that ErbB signaling is required between 18-30 hpf for DRG neuron formation in segments 6-30, consistent with the period when migrating NC cells pause at the location where DRGs normally form.

neuregulin 1 alone is not required for DRG neuron formation

Mice with targeted mutations in *Erbb3* or *Erbb2* have NC cell migration defects (Britsch et al., 1998). Mice with a targeted mutation in the ErbB2/ErbB3 ligand *Nrg1* show the same NC cell defects as *Erbb3* and *Erbb2* mutant mice, suggesting that in mouse Nrg1 is required for normal NC cell migration. To determine whether Nrg1 affects NC cell migration or DRG formation in zebrafish, we isolated zebrafish *nrg1* and found that there are five splice variants that conform to the three splice variant types described in mouse and human (see Fig. S1 in the supplementary material) (Falls, 2003). To determine the temporal expression pattern of each *nrg1* isoform, we performed RT-PCR at various stages from 50% epiboly to 72 hpf, and in adult brain (Fig. 5). The type I isoform is expressed throughout almost all developmental stages, except 36 hpf, and is only very weakly expressed in adult brain. The type II isoform is strongly expressed during epiboly, but is then downregulated and upregulated at several developmental stages. It is more strongly expressed in adult brain than is the type I isoform. The type III isoform is strongly

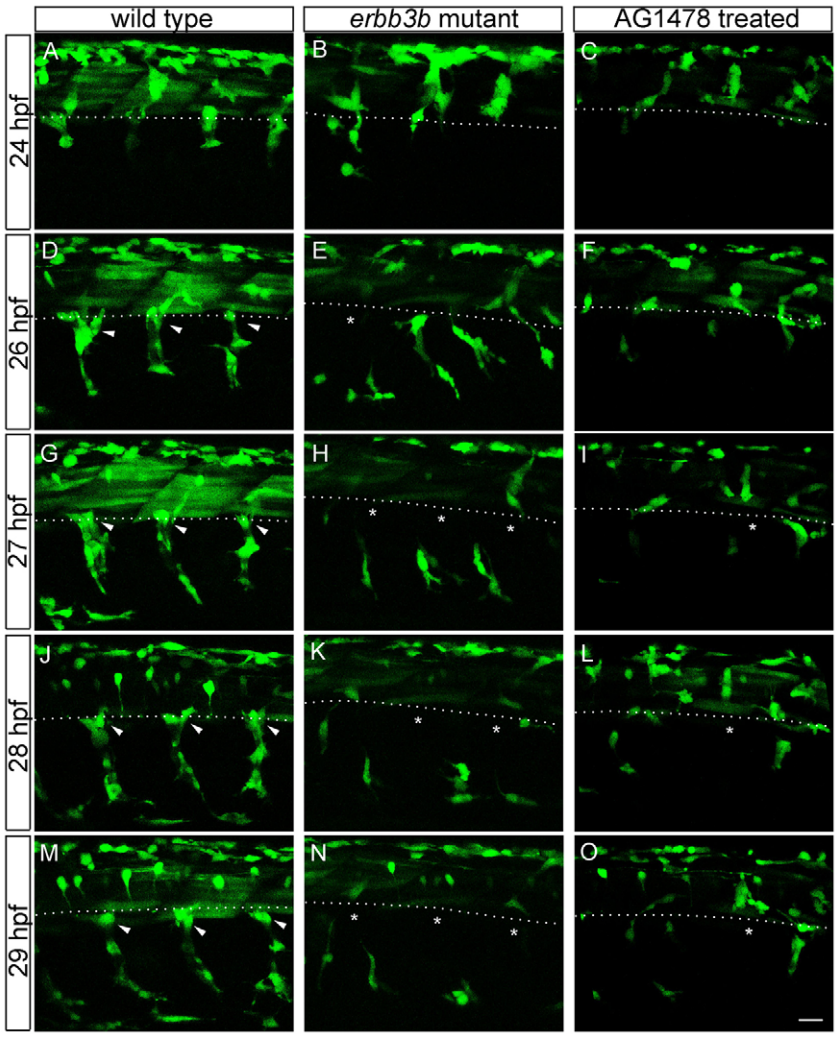

Fig. 3. In *erbb3b* mutants, neural crest cells fail to pause in the location where DRGs normally form. Single frames taken from confocal time-lapse movies of 22-70 hpf *Tg(sox10:GFP)* zebrafish. In wild-type embryos (**A**,**D**,**G**,**J**,**M**), NC cells pause at the dorsal and ventral aspects of the notochord for more than 24 hours. By contrast, in *erbb3b* mutants (**B**,**E**,**H**,**K**,**N**) and in AG1478-treated wild type (**C**,**F**,**I**,**L**,**O**), NC cells did not pause and abnormally migrated away. Arrowheads indicate pausing NC cells; asterisks indicate places where pausing NC cells are missing. Broken lines indicate dorsal aspect of notochord. Scale bar: 20 μm.

expressed at all stages, with the exception of 36 hpf. We then designed isoform-specific riboprobes (Fig. 5). A type I-specific riboprobe showed weak expression in the somites along the NC medial migration pathway. A type II-specific riboprobe showed similar somite expression, and also showed expression in clusters of spinal cord cells in the position of motoneurons. A type III-specific riboprobe showed no somite expression, but similar to type II, was expressed in clusters of ventral spinal cord cells. Considering that somites are important for NC cell migration (Honjo and Eisen, 2005) and that ErbB3 signaling is required from 18-30 hpf for DRG neuron formation, the type I isoform is the most likely candidate ErbB2/ErbB3 receptor ligand for DRG neuron formation.

To determine whether Nrg1 is important for DRG formation, we blocked its function using a previously published splice-blocking MO (Milan et al., 2006) that targeted the EGF-like domain and knocked down all Nrg1 isoforms. Knockdown of Nrg1 resulted in occasional mislocalization or loss of DRG neurons (Fig. 5; Table 1). However, the phenotype was not as severe as *erbb3b* or *erbb2* mutants, suggesting that other ErbB3 ligands are also important for DRG formation.

Zebrafish has two *neuregulin 2* genes

The ErbB2/ErbB3 receptor complex has two known ligands, Nrg1 and Nrg2 (Riese et al., 1995; Busfield et al., 1997; Carraway et al., 1997; Chang et al., 1997; Olayioye et al., 2000; Holbro et al., 2003; Lin and Winer, 2004). To examine whether Nrg2 is important for zebrafish DRG neuron development, we isolated *nrg2* and found that zebrafish has two *nrg2* genes (*nrg2a* and *nrg2b*; see Fig. S2 in the supplementary material). *nrg2a* is located on chromosome 21 and *nrg2b* is located on chromosome 14. Both of these genes have the typical neuregulin gene structure (see Fig. S2 in the supplementary material).

nrg2a has four splice variants, whereas *nrg2b* has only a single splice variant. We performed RT-PCR to examine expression of the two *nrg2* genes during development and in adult brain. *nrg2a* is first expressed during epiboly and continues to be expressed until 72 hpf; it is also expressed in adult brain. By contrast, *nrg2b* is first expressed at 16 hpf. Like *nrg1* type II, *nrg2b* is downregulated and upregulated during development; it is also expressed in adult brain. We designed riboprobes specific to either *nrg2a* or *nrg2b* and found that *nrg2a* is expressed in somites, whereas *nrg2b* appears to be expressed primarily in ventral spinal cord at 24 hpf (Fig. 6). The distinct expression patterns of these genes suggest that they have different roles in zebrafish development.

Neuregulin 2a is important for DRG development

To test whether either of the two Nrg2 proteins affects DRG development, we knocked them down individually using splice-blocking MOs designed against the EGF-encoding domain of each gene. Like Nrg1, the EGF domain is crucial for binding ErbB

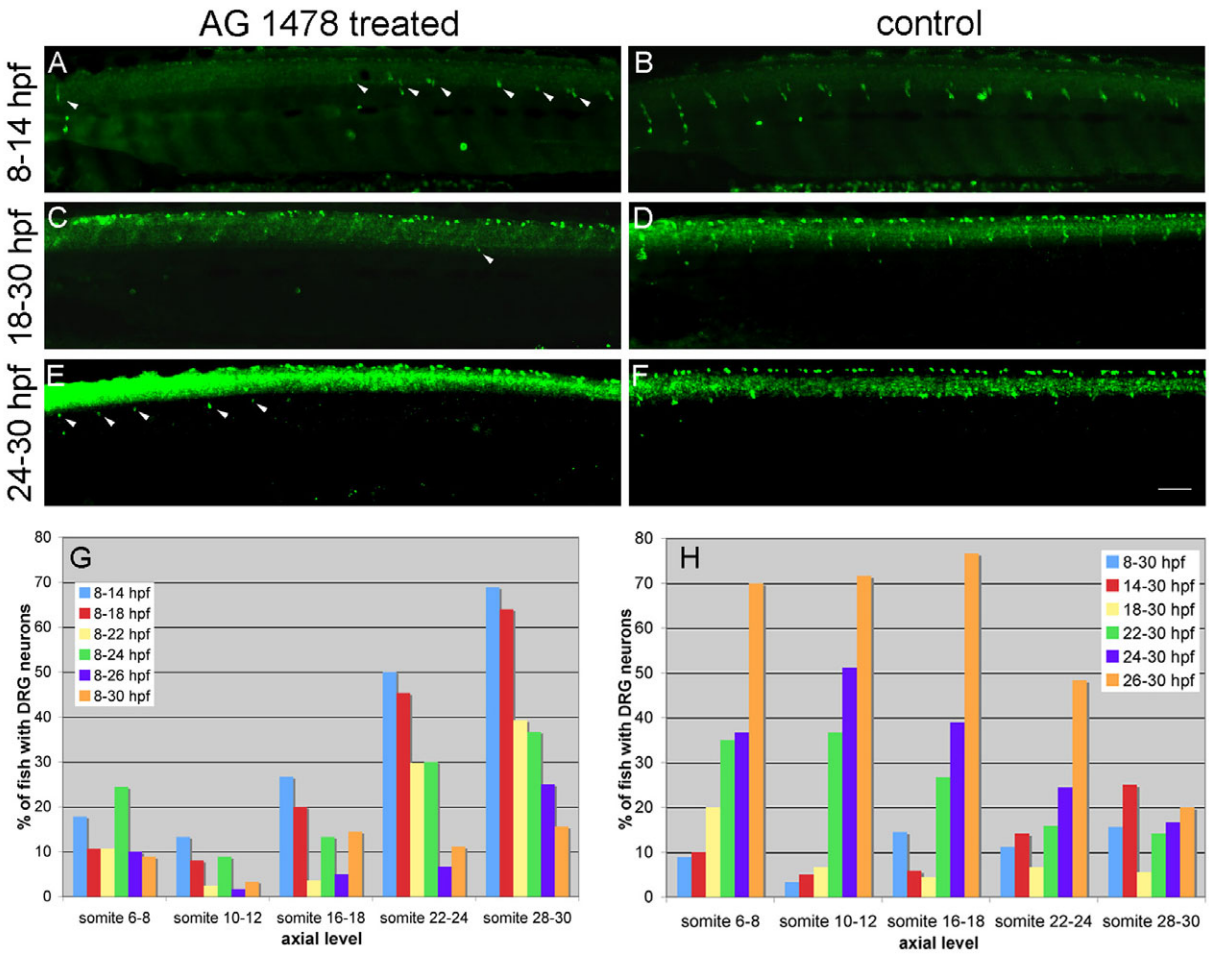

Fig. 4. ErbB signaling is required during neural crest migration. Control embryos and embryos treated with AG1478 for various time periods were labeled with Elavl antibody at 4 dpf to examine DRG formation. (**A-F**) Somites 4-18 of inhibitor-treated (A,C,E) and control (B,D,F) embryos. DRG neurons were missing between somites 5 and 11 when embryos were treated from 8-14 hpf (A) and DRG neurons were missing throughout the body when embryos were treated from 18-30 hpf (C). DRG neurons were present anteriorly but missing posterior of somite 13 when embryos were treated from 24-30 hpf (E). DRG neurons were present in all segments in control embryos. Arrowheads in A, C and E indicate DRG neurons. (**G**,**H**) Graphs showing average percentage of fish with DRG neurons at each of three axial levels when embryos were treated for various time periods. In control embryos, DRG neurons were present in 90-100% of fish for all experiments. At least 30 embryos were counted in each experiment. Scale bar: 50 μm.

receptors and thus for Nrg2 function (Jones et al., 1999; Falls, 2003). Embryos injected with *nrg2a* MO lacked most DRG neurons at 2 dpf, however, many DRG neurons were present at 4 dpf (Fig. 7), although some were mislocalized (Table 1). This recovery might occur because MO efficacy decreased over time; thus, we performed RT-PCR to examine *nrg2a* splicing from 1-4 dpf. RT-PCR showed that the MOs affected EGF-like domain splicing through the first 2 days of development. However, at later stages, both full-length and incorrectly spliced mRNA were present (Fig. 7). As we described above, our data from pharmacological inhibition revealed that the crucial period of ErbB signaling for DRG formation is between 18-30 hpf (Fig. 4). Thus, we would not expect diminution of MO efficacy at 3 or 4 dpf to affect DRG neurons. Therefore, our results suggest that Nrg2a plays a role in DRG neuron development in zebrafish, but that other factors must also be involved.

To learn whether Nrg2b also contributed to DRG neuron development, we knocked it down with a splice-blocking MO and confirmed by RT-PCR that the mRNA was incorrectly spliced (Fig. 7). *nrg2b* MO-injected embryos had normal DRGs at both 2 dpf and 4 dpf (Fig. 7), consistent with expression of *nrg2b*, which appears to be primarily in ventral spinal cord, rather than in somites, and is strongest after 24 hpf, a stage when most NC cells have completed migration on the medial pathway. Furthermore, injection of both *nrg2a* and *nrg2b* MOs simultaneously did not enhance the phenotype of *nrg2a* MO alone. Thus, our results suggest that Nrg2b is unnecessary for DRG neuron formation.

Our results showed a partial requirement for both Nrg1 and Nrg2a in DRG neuron development. To learn whether DRGs are present in the absence of both of these neuregulins, we knocked them down together by injecting *nrg1* and *nrg2a* MOs simultaneously and confirmed by RT-PCR that both genes were incorrectly spliced through 2 dpf. Embryos injected with *nrg1* plus *nrg2a* MOs lacked DRG neurons at 4 dpf (Fig. 8; Table1), suggesting that Nrg1 and Nrg2a both act during DRG neuron formation.

To learn whether the absence of DRG neurons in *nrg1* plus *nrg2a* MO-injected embryos resulted from abnormal NC migration, as it did in *erbb3b* or *erbb2* mutants, we labeled double MO-injected

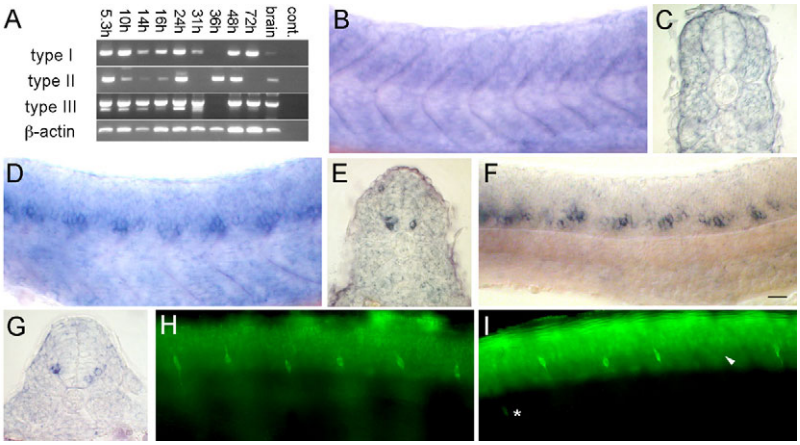

Fig. 5. *nrg1* alone is not required for DRG formation. (**A**) RT-PCR showing expression of each *nrg1* isoform at various developmental stages and in the adult brain. (B,D,F) Expression patterns of each isoform at 24 hpf shown in whole-mount. (C,E,G) Cross section of expression pattern of each isoform respectively. (**B**,**C**) Type I is expressed weakly in somites. (**D**,**E**) Type II and (**F**,**G**) type III are expressed strongly in ventral spinal cord neurons. (E,F) Elavl labeling at 2 dpf. (**H**,**I**) Control MO-injected embryos (H) had segmental DRG neurons. *nrg1* MO-injected embryos (I) had only a slight decreased in DRG neuron number; arrowhead indicates absent DRG, asterisk shows mislocalized DRG. Scale bar: 40 μm.

embryos with either *crestin* or *sox10* riboprobe. As in the mutants, the number of NC cells and their initial migration appeared normal at 24 hpf (Fig. 8). However, at 27 and 30 hpf there were fewer NC cells ventral of the notochord than in wild type (Fig. 8), consistent with the idea that Nrg1 and Nrg2a both act as ligands for ErbB2/ErbB3 receptors in DRG neuron formation and are important for migrating NC cells to pause at the location where DRGs normally form.

DISCUSSION

In this paper, we present three key findings: first, we show that ErbB2/ErbB3 signaling is required during a specific time window for trunk NC cells migrating along the medial pathway to pause at the location where DRGs normally form; second, we show that zebrafish have three neuregulins, two of which participate in activating the ErbB2/ErbB3 signaling necessary for DRG neuron formation; third, we show that ErbB2/ErbB3 signaling is required for sympathetic ganglion formation, but not for enteric neuron formation. We discuss each of these finding below and we propose that ErbB2/ErbB3 signaling may play different roles in migration of cranial and trunk neural crest.

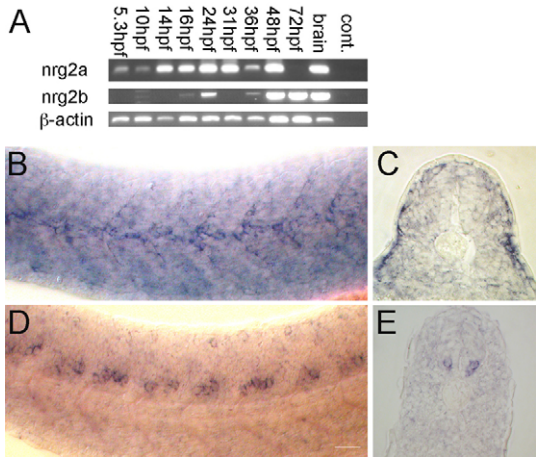

Fig. 6. Zebrafish has two *nrg2* genes. (**A**) RT-PCR showing expression of *nrg2a* and *nrg2b* at various developmental stages and in the adult brain. Whole-mount labeling of wild-type embryos showing *nrg2a* expression in somites (**B**, cross section in **C**) and *nrg2b* expression in spinal neurons at 24 hpf (**D**, cross section in **E**). Scale bar: 20 μm.

ErbB2/ErbB3 signaling is required during a specific time window of trunk neural crest migration for DRG neuron formation

In the absence of ErbB2/ErbB3 signaling in zebrafish, migrating NC cells fail to pause at the location where DRGs normally form, and DRG neurons are not produced. Our time-lapse observations of *Tg(sox10:GFP)* fish suggest that DRG neurons do not form in the mutants because their progenitors continue migrating ventrally rather than pausing. Consistent with this idea, our experiments using the AG1478 inhibitor show that ErbB signaling is required between 18 and 30 hpf, precisely when DRG progenitors reach the dorsal aspect of notochord and pause. For example, trunk NC starts to migrate around 16 hpf at the level of somite 6 and around 19 hpf at the level of somite 17 (Raible et al., 1992), and it takes about 3-5 hours of migration for the first cell to reach the dorsal aspect of the notochord (Y.H. and J.S.E., unpublished). Consistent with this timing, starting inhibitor treatment at 18 hpf prevented DRG neuron formation beginning just posterior of somite 6, and starting treatment at 24 hpf prevented DRG neuron formation just posterior of somite 20. Interestingly, earlier treatment, from 8-14 hpf inhibited DRG neuron formation anteriorly, possibly because the inhibitor takes some time to wash out. However, consistent with the idea that ErbB signaling is required around the time NC reaches the position where it normally pauses, DRG neurons formed posteriorly in this treatment protocol. Together, these observations argue that ErbB signaling is required for DRG progenitors to pause in a location where they receive a DRG neuron differentiation-promoting signaling, and that in the absence of ErbB signaling, the progenitors migrate through this location, thus they fail to receive the signal and cannot generate DRG neurons.

Mice with targeted mutations in the *Erbb3* and *Erbb2* genes also have defects in trunk NC migration (Britsch et al., 1998). Our observations and those of Budi and colleagues (Budi et al., 2008) provide evidence that zebrafish trunk NC cells migrate past their normal targets in *erbb3b* mutants. By contrast, in mouse *ErbB3*, *ErbB2* and *Nrg1* mutants, trunk NC cells stop migrating immediately after emigrating from the neural tube. This apparent difference between zebrafish and mouse might result from differences in the migration pathway of DRG progenitors. In zebrafish, DRG progenitors migrate ventrally until they reach the dorsal aspect of notochord. By contrast, in mouse, DRGs are formed very close to the region where trunk NC cells emigrate from the neural tube. Thus, DRG progenitors do not migrate any significant distance in mouse, whereas in zebrafish they need both to migrate a significant distance and to recognize a specific location at which to

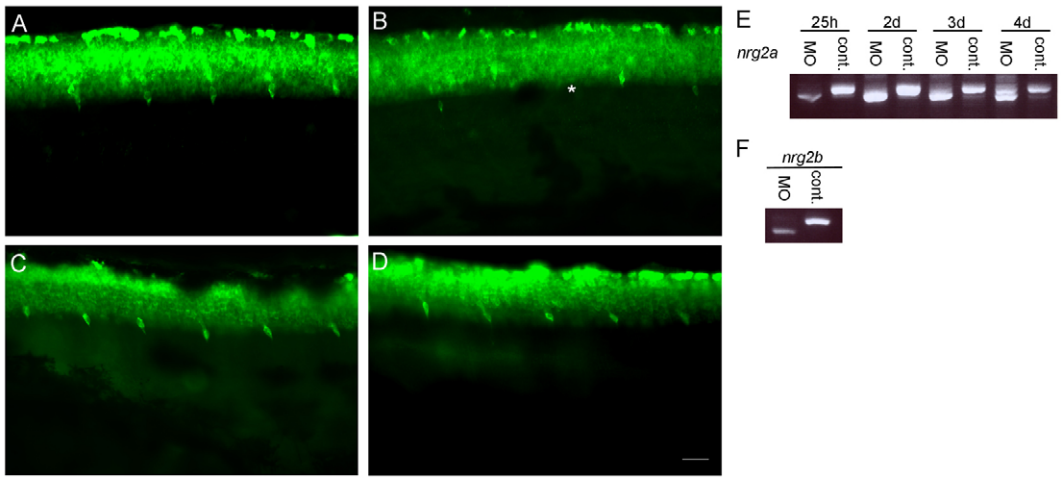

Fig. 7. *nrg2a* alone is unnecessary for DRG neuron formation. DRG neurons labeled with Elavl antibody at 4 dpf. DRG neurons in controls (**A**,**C**). DRG neurons are missing from some segments of *nrg2a* MO-injected embryos (**B**) but appear normal in *nrg2b* MO-injected embryos (**D**). Asterisks indicate absent DRG neurons in B. (**E**) RT-PCR showing that *nrg2a* MOs cause incorrect mRNA splicing through 3 dpf, but by 4 dpf, both incorrectly-spliced and correctly-spliced transcripts are present. (**F**) RT-PCR showing that both the *nrg2a* and *nrg2b* MOs cause incorrect splicing at 25 hpf. Scale bar: 20 μm.

stop migrating. ErbB receptors have previously been shown to regulate expression of adhesion molecules (D'Souza and Taylor-Papadimitriou, 1994; Sweeney et al., 2001; Bertucci et al., 2004; Amin et al., 2005; Way and Lin, 2005; Yasmeen et al., 2006). We predict that there will be species-specific differences in this regulation, based on the different distances that DRG progenitors migrate in mouse and zebrafish.

Another apparent difference between zebrafish and mouse is that DRGs are absent from *erbb3b* and *erbb2* mutant zebrafish, but are reported to form in *Erbb3* mutant mice (Britsch et al., 1998); *Erbb2* mutant mice die of cardiac defects too early to assay DRGs (Morris et al., 1999; Woldeyesus et al., 1999). Most DRG neurons die in *Erbb3* mutant mice (Riethmacher et al., 1997) and rescue of the cardiac defects in *Erbb2* mutant mice reveals that, as for *Erbb3* mutant mice, there is a severe loss of DRG neurons in the absence of *Erbb2* (Morris et al., 1999; Woldeyesus et al., 1999). ErbB3 is thought to function autonomously in glial cell development and non-autonomously in survival of DRG neurons in *Erbb3* mutant mice (Riethmacher et al., 1997). However, as in cardiac defect-rescued *Erbb2* mutant mice, in *Erbb3* mutant mice the loss of DRG neurons occurs very early, almost simultaneously with the loss of glia (Riethmacher et al., 1997), raising the possibility that the same mechanisms could be affecting both cell types autonomously. This possibility is consistent with the observation that ErbB3 is expressed in at least some mouse DRG neurons (Riethmacher et al., 1997; Meyer et al., 1997) and virtually all rat DRG neurons are ErbB2 and ErbB3 immunoreactive (Pearson and Carroll, 2004). Whether ErbB2/ErbB3 signaling is required autonomously in DRG neurons has not yet been tested directly.

Zebrafish has multiple neuregulins, two of which participate in DRG neuron formation

We isolated one *nrg1* gene and two *nrg2* genes in zebrafish. MO-mediated knockdown showed that Nrg1 and Nrg2a work together in zebrafish DRG neuron development, whereas Nrg2b appears to be uninvolved in this process. These results are consistent with the expression patterns of these genes, *nrg1* type I and type II and *nrg2a* are expressed in somites, whereas *nrb2b* is only expressed in ventral spinal cord neurons and is expressed more strongly after 48 hpf. In mouse, only *Nrg1* has been shown to be required for trunk NC cell migration. By contrast, *nrg1* and *nrg2a* both act during trunk NC cell migration in zebrafish. DRG development was not examined in mice with a targeted *Nrg2* deletion (Britto et al., 2004), so whether DRGs

Table 1. Quantification of DRGs in morpholino-injected embryos at 4 dpf

	Number of segments lacking DRGs	Number of segments with dislocated DRGs	Number of segments with normal DRGs
nrg1 MO (*n*=300*)	69 (23%)	72 (24%)	159 (53%)
nrg1 mismatch MO (*n*=400†)	1 (0.25%)	0 (0%)	399 (99.75%)
nrg2a MO (*n*=300*)	87 (29%)	47 (15.7%)	166 (55.3%)
nrg2a mismatch MO (*n*=300*)	2 (0.7%)	3 (1%)	295 (98.3%)
nrg2b MO (*n*=400†)	4 (1%)	12 (3%)	384 (96%)
nrg2b mismatch MO (*n*=400†)	7 (1.75%)	1 (0.25%)	392 (98%)
nrg1+2a MO (*n*=400†)	293 (73.25%)	39 (9.75%)	68 (17%)
nrg1+2a mismatch MO (*n*=400†)	14 (3.5%)	10 (2.5%)	376 (94%)
nrg2a+2b MO (*n*=200‡)	28 (14%)	11 (5.5%)	161 (80.5%)
nrg2a+2b mismatch MO (*n*=200‡)	4 (2%)	1 (0.5%)	195 (97.5%)

*30 embryos.
†40 embryos.
‡20 embryos.

were normal is still an unanswered question. Furthermore, there have been no reports about DRG development in mice with targeted deletions of both *Nrg1* and *Nrg2.* Thus, whether both Nrg1 and Nrg2 are required for DRG neuron development in mammals remains unknown.

ErbB2/ErbB3 signaling is required for sympathetic neuron formation

In the absence of ErbB2/ErbB3 signaling, sympathetic neurons do not form in mouse (Britsch et al., 1998), and we report here that this is also the case in zebrafish. Development of cervical and trunk sympathetic neurons has been described in zebrafish (An et al., 2002; Stewart et al., 2004). However, virtually nothing is known about the NC migratory processes during formation of zebrafish trunk sympathetic ganglia, because early markers such as *sox10* are downregulated several days before later markers such as Elavl and TH are expressed, leaving a window during which sympathetic ganglion formation cannot be followed. We observed that NC cells failed to pause at the ventral aspect of the notochord in *erbb3b* mutants and we hypothesize that the NC cells that normally pause in this region in wild-type embryos are sympathetic ganglion precursors. Thus, we propose that, as for DRG precursors, ErbB2/ErbB3 signaling is also required for sympathetic ganglion precursors to pause at a specific location. Sympathetic ganglion formation appears much less regular than DRG formation in zebrafish. For example, DRGs are segmentally arranged and develop in a strict rostral-to-caudal sequence. By contrast, sympathetic ganglia initially are not segmentally arranged and it is unclear whether they develop in a strict rostral-to-caudal sequence (An et al., 2002). Studies in avians suggest that sympathetic ganglion progenitors migrate rostrally and caudally during formation of sympathetic ganglia (Young et al., 2004; Kasemeier-Kulesa et al., 2005). These studies also suggest that the segmental arrangement of sympathetic ganglia arises late and is a consequence of this rostral and caudal migration, rather than of the earlier segmental NC migration that results in segmentally arranged DRGs (Young et al., 2004). Thus, we are unable to draw conclusions about when ErbB2/ErbB3 signaling is required for sympathetic neuron formation. However, sympathetic neurons were present in the AG1478 treatments in which DRGs were absent, suggesting that ErbB2/ErbB3 signaling is required either over a longer time period or over an entirely different time period for sympathetic neuron formation than for DRG formation.

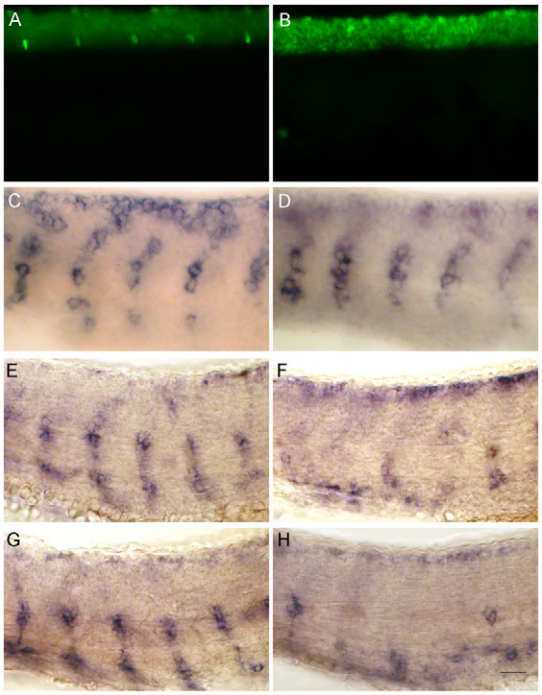

Fig. 8. *nrg1* and *nrg2a* act redundantly during DRG neuron formation. (**A**,**B**) Elav1 antibody labeling at 4 dpf. Co-injection of *nrg1* and *nrg2a* MOs resulted in absence of DRG neurons (B), whereas DRG neurons are normal in controls (A). (**C-H**) Whole-mount wild-type (C,E,G) and *nrg1* plus *nrg2a* MO-injected embryos (D,F,H) labeled with *crestin* riboprobe at 24 hpf (C,D), 27 hpf (E,F) and 30 hpf (G,H). Like *erbb3b* and *erbb2* mutants, double MO-injected embryos have normal early NC migration (C,D) but, at later stages, NC migration is disrupted (E-H). Scale bar: 20 μm.

ErbB2/ErbB3 signaling is not a general requirement for formation of peripheral neurons

ErbB2/ErbB3 signaling is required for both DRG and sympathetic neuron formation, raising the possibility of a general requirement for ErbB signaling in NC-derived peripheral neurons. However, this is apparently not the case, because ErbB2/ErbB3 signaling is unnecessary for zebrafish enteric neuron formation. Previous work suggested a role for ErbB2/ErbB3 signaling in formation of mouse enteric neurons (Erickson et al., 1997). However, studies of conditional *Erbb2* mutant mice revealed that ErbB2 is unnecessary for initial enteric neuron formation, but is required cell non-autonomously for enteric neuron survival, consistent with expression of ErbB2 in the colonic epithelium (Crone et al., 2003). Thus, in mouse (Crone et al., 2003), as in zebrafish, the cranial NC cells that generate enteric neurons are able to migrate normally into the intestine and to generate enteric neurons in the absence of ErbB2. Together these studies suggest that ErbB2/ErbB3 signaling may play distinct roles in migration of cranial and trunk NC. This is consistent with other reports suggesting that, although cranial ganglia are defective in *Nrg1*, *Erbb2* and *Erbb3* mutant mice, this likely arises not because of an effect on cranial NC migration, but because Nrg1-mediated ErbB2/ErbB3 signaling is required for survival of cranial NC-derived neurons (Meyer and Birchmeier, 1995; Erickson et al., 1997).

Although we argue that ErbB2/ErbB3 signaling is required for migrating trunk NC cells to pause in locations where they receive differentiation signals, there are other possibilities that we cannot yet rule out. For example, it is possible that ErbB2/ErbB3 signaling makes cells competent to receive differentiation signals and that failure of trunk NC cells to migrate properly in *erbb2* and *erbb3b* mutants is a secondary consequence of failure to perceive a differentiation signal. Furthermore, the failure of DRG progenitors to pause at the right place in *erbb2* and *erbb3b* mutants could cause defects in other progenitors, for example sympathetic ganglion progenitors. The lack of multiple trunk NC neuronal derivatives in *erbb2* and *erbb3b* mutants raises the possibility that ErbB2/ErbB3 signaling acts early in trunk NC cells, perhaps in specific subpopulations. Although we cannot completely rule out this possibility, our inhibitor treatment experiments show that loss of ErbB2/ErbB3 signaling later, during trunk NC migration, is sufficient to cause the DRG neuron defect. In addition, early inhibitor treatment had no effect on sympathetic neuron formation. These results suggest that lack of DRG and sympathetic neurons in *erbb2* and *erbb3b* mutants does not result from absence of specific subsets of trunk NC cells, but rather results from effects that occur to those cells during their migration.

We thank Will Talbot for *erbb2* and *erbb3b* mutants; Robert Kelsh for *Tg(sox10:GFP)* fish; Xinjun He for participation in *nrg1* cloning; Joy Murphy, Jacob Lewis and the University of Oregon Zebrafish Facility staff for animal husbandry; the University of Oregon Histology Facility for sectioning; David Parichy for illuminating discussions about ErbB receptors; and David Parichy, Monte Westerfield, Julie Kuhlman and Jim Weston for comments on earlier versions of the manuscript. Supported by NIH grants NS23915 and HD22486 and by the Medical Research Foundation of Oregon.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/15/2615/DC1

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