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Delta signaling mediates segregation of neural crest and spinal sensory neurons from zebrafish lateral neural plate

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SUMMARY

We examined the role of Delta signaling in specification of two derivatives in zebrafish neural plate: Rohon-Beard spinal sensory neurons and neural crest. *deltaA*-expressing Rohon-Beard neurons are intermingled with premigratory neural crest cells in the trunk lateral neural plate. Embryos homozygous for a point mutation in *deltaA*, or with experimentally reduced Delta signaling, have supernumerary Rohon-Beard neurons, reduced trunk-level expression of neural crest markers and lack trunk neural crest derivatives. Fin mesenchyme, a putative trunk neural crest derivative, is present in *deltaA* mutants, suggesting it segregates from other neural crest derivatives as early as the neural plate stage. Cranial neural crest derivatives are also present in *deltaA* mutants, revealing a genetic difference in regulation of trunk and cranial neural crest development.

Key words: Zebrafish, Neural crest, Delta, Notch, *deltaA*

INTRODUCTION

Patterning of the central nervous system (CNS) involves the progressive specification of individual neural cells to particular fates or subsets of fates (reviewed in Tanabe and Jessell, 1996). Recent models of dorsoventral CNS patterning have emphasized the importance of inductive signals emanating from adjacent non-neural tissues (reviewed in Baker and Bronner-Fraser, 1997a; LaBonne and Bronner-Fraser, 1998; Tanabe and Jessell, 1996). However, in some instances several cell types arise at nearly the same dorsoventral position in the neural plate or spinal cord, which implies that short-range signaling among CNS cells also plays a role in spinal cord patterning. Short-range signals within the CNS include diffusible factors like retinoic acid (Sockanathan and Jessell, 1998), and cell-surface signals like Delta (Artavanis-Tsakonas et al., 1995; Rooke and Xu, 1998).

Delta signaling is a mechanism for diversifying cell fates within an equivalence group, a domain of cells with apparently equivalent developmental potential (reviewed in Artavanis-Tsakonas et al., 1999; Rooke and Xu, 1998). It was first described in *Drosophila melanogaster* and is now known to function in vertebrate cell fate choice as well (reviewed in Lewis, 1996). In this signaling paradigm, a subset of cells within an equivalence group expresses high levels of the ligand, Delta, and adopts a preferred fate. Adjoining cells receiving this signal through the receptor, Notch, are inhibited from adopting the preferred fate and so adopt an alternative fate. In some cases, sequential rounds of Delta signaling mediate the specification of three or more cell types from a single equivalence group (Dorsky et al., 1997; Henrique et al., 1997). It is important to remember that an equivalence group refers to a group of cells that have the potential to adopt the same fates under specific conditions, rather than to a group of cells that are equivalent in all ways. Thus, the equivalence group concept does not preclude the possibility that a prepattern governs which cells in an equivalence group normally adopt the preferred fate.

Primary neurons are a class of neural cells apparently regulated by Delta signaling. These early-born neurons are found in anamniote vertebrates and mediate an early escape reflex (Kimmel et al., 1991). They include primary motoneurons (PMNs) found in the ventral spinal cord (Eisen et al., 1986; Myers et al., 1986) and spinal primary sensory neurons, called Rohon-Beard neurons (RBs), found in the dorsal spinal cord (Lamborghini, 1980; Metcalfe et al., 1990). PMNs and RBs are thought to derive from precursors found in the medial and lateral neural plates, respectively. PMN and RB precursors express Delta family members around the time they are born and, when Delta signaling is blocked, increased numbers of PMN and RB precursors form (Appel and Eisen, 1998; Chitnis et al., 1995; Haddon et al., 1998b). This suggests that PMNs and RBs are preferred fates of cells in two distinct equivalence groups, PMNs in the medial neural plate and RBs in the lateral neural plate. Alternative cell types that derive from the medial neural plate domain may include interneurons and a class of later-developing secondary motoneurons; both cell types are reduced in embryos with decreased Delta signaling (Appel and Eisen, 1998). Neural crest cells are specified in the lateral neural plate and later emerge from the CNS and migrate to various positions in the embryo where they differentiate into diverse derivatives. Because neural crest is thought to arise from the same part of the neural plate as RBs (DuShane, 1938), it is a candidate for an alternative cell type deriving from the lateral neural plate domain.

Here we show that RBs and trunk neural crest are intermingled in the lateral neural plate and that embryos carrying a mutation in *deltaA* not only have supernumerary RBs, but also have a concomitant decrease in trunk neural crest. These data support the model that Delta signaling segregates RBs and trunk neural crest precursors from a single equivalence group in the trunk lateral neural plate. The surprising presence of fin mesenchyme in *deltaA* mutant embryos suggests that this trunk neural crest derivative does not arise from the equivalence group that generates other neural crest derivatives and RBs. That similar molecular mechanisms underlie the specification of both RBs and neural crest is consistent with a recent proposal that neural crest originated from an RB-like cell in the vertebrate precursor (Fritzsch and Northcutt, 1993). We found no evidence that cranial neural crest is regulated by Delta signaling, revealing a previously unknown genetic difference in specification of trunk and cranial neural crest.

MATERIALS AND METHODS

Wild-type and mutant embryos

Embryos were reared in the University of Oregon Zebrafish Facility and staged by hours postfertilization at 28.5°C (h) (Kimmel et al., 1995).

To identify likely heterozygous carriers, sibling pairs from a *deltaA*^{dx2} outcross were mated and progeny scored for the upwardly curved tail phenotype (Appel et al., 1999). This allele is partially dominant (Appel et al., 1999; Riley et al., 1999); the upwardly curved tail phenotype is seen in an average of 1.7% of the progeny of a *deltaA*^{dx2} heterozygote and a wild-type fish (outcross) (Appel et al., 1999). Thus, pairs of fish were considered to be both heterozygotes only if they yielded clutches in which 15% or more of the embryos displayed the curved-tailed phenotype.

Immunohistochemistry, RNA in situ hybridization and β-galactosidase detection

For RNA in situ hybridization, antisense RNA probes were generated by digesting the following plasmids with the specified restriction enzyme and then transcribing RNA from them with the specified RNA polymerase: *fkd6* plasmid (gift of J. Odenthal and C. Nüsslein-Volhard) with *Bam*HI and T7 polymerase, *sna2* plasmid (Thisse et al., 1995) with *Xba*I and T7 polymerase, and an *msxB* plasmid (Akimenko et al., 1995) with *Sal*I and T7 polymerase. RNA in situ hybridization was performed as previously described (Appel and Eisen, 1998), except before prehybridization, all embryos were digested in 2 μg/ml Proteinase K (Boehringer-Mannheim) for 1 minute, refixed for 20 minutes in 4% paraformaldehyde in PBS and rinsed 4× 5 minutes in PBS with 0.1% Tween-20 (Sigma) (PBST). Double labeling with monoclonal antibody mAb 39.4D5, which recognizes Islet1 and Islet2 proteins (Tsuchida et al., 1994), and RNA in situ hybridization, were performed as described (Appel and Eisen, 1998). Monoclonal antibodies, zn12 (Trevarrow et al., 1990) and anti-acetylated tubulin (Sigma) were used at 1:4000 and 1:500, respectively, processed with the mouse peroxidase anti-peroxidase system (Sternberger Monoclonals, Inc), and developed in 0.5 mg/ml diaminobenzidine (DAB) and 0.003% H_2O_2. Sectioned embryos were processed for antibody labeling of tyrosine hydroxylase with 1:100 dilution of rabbit anti-tyrosine hydroxylase (Pel-Freez Biologicals), followed by the rabbit peroxidase anti-peroxidase system (Sternberger Monoclonals, Inc), and developed in DAB/H_2O_2. For detection of Hu, a neuron-specific RNA-binding protein (Marusich et al., 1994), fixed embryos with heads and tail tips removed were incubated in H_2O for 4-8 hours, blocked in PBS with 0.1% TritonX (Sigma) (PBSTx) with 2 mg/ml BSA, 4% normal goat serum, and 10% DMSO (blocking solution) for 1 hour, incubated overnight in 1:100 monoclonal antibody 16A11 (Marusich et al., 1994) in blocking solution at 4°C, rinsed in PBSTx for 6× 1 hour, and developed with mouse peroxidase anti-peroxidase (Sternberger Monoclonals, Inc) and DAB/H_2O_2, using overnight incubations of both secondary and tertiary antibodies. For detection of β-galactosidase activity, embryos were fixed in 2% paraformaldehyde in PBS with 0.02% NP-40 (Boehringer Mannheim) overnight at 4°C, rinsed 3×10 minutes in PBS with 0.02% NP-40 at RT, developed in 1 mg/ml X-gal in 10 mM sodium phosphate buffer (pH 7.3), 150 mM NaCl, 1 mM $MgCl_2$, 3.1 mM $K_4(Fe_3(CN)_6)$, and 3.1 mM $K_3(Fe_2(CN)_6)$ at 37°C for 10-30 minutes, and fixed again in 4% paraformaldehyde in PBS overnight at 4°C before processing for immunohistochemistry and RNA in situ hybridization. For analysis of jaw cartilage, fixed embryos were rinsed in PBST, bleached in 3% H_2O_2 and 1% KOH for 2 hours, rinsed again in PBST and then stained with Alcian green (Kimmel et al., 1998).

Cell counts

To count RBs, zn-12 monoclonal antibody-immunoreactive cells in the dorsal spinal cord (RBs, Metcalfe et al., 1990) were counted in 4-somite lengths in the trunk at 24 h.

To score for co-expression of Islet protein and *fkd6* mRNA within single cells, double-labeled 4-somite-stage embryos were deyolked and mounted under a coverslip. Presumptive RBs were scored over a distance of approximately three somite lengths of the neural plate immediately caudal to the most recently formed somite.

RNA synthesis and injection

X-dnDelta-1 (X-Delta-1^{STU}) and X-Su(H)^{DBM} plasmids were a gift from C. Kintner, and SP64T-nuc-lacZ plasmid a gift from M. Halpern. Capped mRNA was synthesized with the mMessage mMachine kit (Ambion), rinsed and concentrated with Microcon100 microconcentrators (Amicon). Approximately 2-5 nl of RNA at 0.05-0.2 mg/ml with 0.25% phenol red was injected with a pressure injection apparatus (ASI) into one or both cells of 2-cell-stage embryos still within their chorions.

Caged-fluorescein lineage labeling

Embryos were injected with 2-5 nl of 0.25% lysine-fixable caged fluorescein-labeled dextran (10,000 M_r; Molecular Probes, product no. D-7146) into both cells at the 2-cell stage; embryos were then raised in the dark. At the 4-somite stage (11 h), 4-6 neural plate cells were illuminated for approximately 5 seconds through a 40× water immersion objective using the DAPI filter set on a Zeiss UEM microscope; a few cells in the underlying mesoderm were also illuminated by this procedure. The light beam was narrowed with a pinhole aperture placed in the rear parfocal plane. Each embryo was examined briefly using a fluorescein filter set to verify that no more than 6 cells in the neural plate were labeled. At the 22-somite stage (20 h), embryos were examined for fluorescent cells. Neural crest cells were identified by their position and distinct morphology under DIC optics (see Raible et al., 1992). RBs were identified by their position and large soma size in live embryos and subsequently by expression of Islet. Mesoderm-derived muscle cells were also routinely seen. For simultaneous analysis of uncaged fluorescein and Islet expression, embryos were fixed in 4% paraformaldehyde in PBS overnight at 4°C. Endogenous phosphatases were inactivated by incubating embryos for 20 minutes at 70°C in PBST, then uncaged fluorescein was immunolocalized with anti-fluorescein-alkaline phosphatase at 1:15000 dilution. Alkaline phosphatase was developed for 5-30 minutes in 350 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 450 μg/ml nitro blue tetrazolium chloride in 0.1 M Tris pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween20. The reaction was stopped by rinsing 5×5 minutes in PBST and embryos were processed for immunolocalization of Islet.

Photography

Photographs were taken on a Zeiss Axioplan microscope and Kodak Elite chrome 160T film. In some images, dust and bubbles were removed from background with Adobe Photoshop.

RESULTS

Neural crest and RBs are intermingled at neural plate stages

To investigate mechanisms of cell fate specification in the lateral neural plate, we determined the relative positions of RBs and premigratory neural crest. RBs can be recognized in the dorsal spinal cord by morphology (Bernhardt et al., 1990), as well as by expression of *islet1* (*isl1*) and *islet2* (*isl2*) mRNAs (Inoue et al., 1994; Tokumoto et al., 1995), and of zn-12 antigens (Metcalfe et al., 1990). Presumptive RBs (pRBs) are defined as cells at the lateral edge of the neural plate that are recognized by an antibody to Islet1 and Islet2 proteins (Isl) (Appel and Eisen, 1998; Korzh et al., 1993; Tsuchida et al., 1994). Presumptive premigratory neural crest cells are also located at the lateral edge of the neural plate and express *forkhead6 (fkd6*) (Odenthal and Nüsslein-Volhard, 1998). We observed that *fkd6* was expressed in cells surrounding the pRBs but not within them (Fig. 1A). This expression pattern suggested that RBs and neural crest precursors were intermingled in a single domain in the lateral neural plate.

Because gene expression is not a reliable indicator of cell lineage (see Fraser and Harland, 2000), it is possible that cells expressing *fkd6* in the neural plate are different from later *fkd6*-expressing cells that become neural crest. To assess directly the fate of lateral neural plate cells adjacent to RBs, we photoactivated caged-fluorescein within clusters of 4-6 adjacent cells in the trunk lateral neural plate (Fig. 1B) and scored embryos at the 20-somite stage for the presence of labeled RBs and neural crest cells. We found that, in all 11 embryos that contained labeled RBs, neural crest cells were also labeled (Fig. 1C,D). Because trunk RBs are born by early somite stages (Haddon et al., 1998a; Stoesser, K., Appel, B., and J. S. E., unpublished), in our experiment, labeled RBs and labeled neural crest cells would most likely have arisen from adjacent neural plate cells rather than from a common precursor. Consistent with the intermingled, non-overlapping expression of *fkd6* and Isl, these data suggest that premigratory neural crest cells are adjacent to RBs in the neural plate.

To address whether RBs or neural crest cells are specified first, we performed double RNA in situ hybridization with probes to *isl1* and to *fkd6* (Fig. 1E). We observed that *isl1* is expressed nearly simultaneously in all trunk pRBs near the end of gastrulation (see Korzh et al., 1993). Expression of *fkd6* begins in presumptive cranial neural crest at this time and extends caudally over the next few hours (Odenthal and Nüsslein-Volhard, 1998). Thus, pRBs express *isl1* before surrounding cells express *fkd6* (Fig. 1E), consistent with the idea that RBs are specified before neural crest.

deltaA mutants have supernumerary RBs and decreased expression of neural crest markers

How could two cell fates be specified at such close proximity? pRBs transiently express *deltaA (dlA)* (Appel and Eisen, 1998; see also Haddon et al., 1998a) and are increased in number in

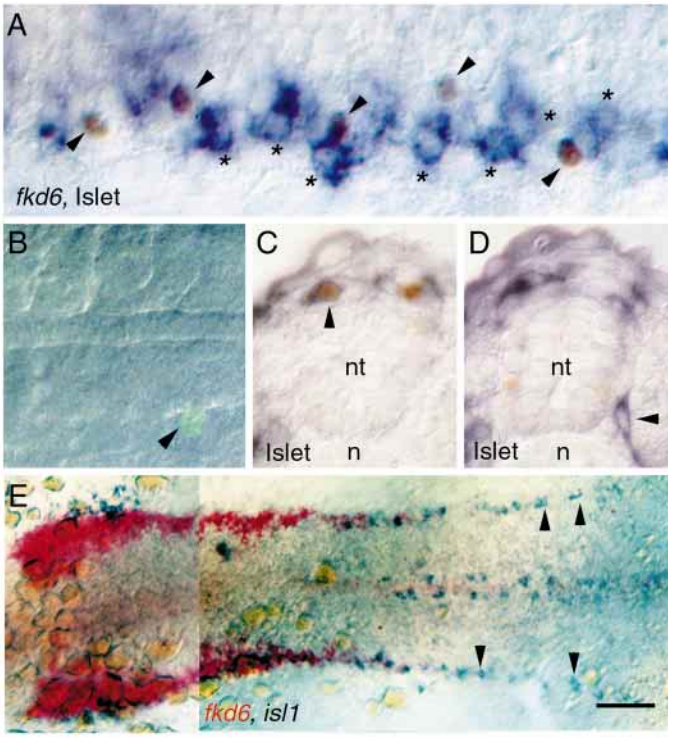

Fig. 1. Gene expression and labeling studies suggest pRBs are intermingled with premigratory neural crest cells in the lateral neural plate. (A) Dorsal view of lateral neural plate of 4-somite-stage (11.5 h) embryo, at the level of the last-formed somite, stained for Isl protein (brown, nuclear) and *fkd6* mRNA (blue, cytoplasmic). Anterior is to the left in this and all other figures unless otherwise indicated. Cells expressing *fkd6* (arrowheads) surround cells expressing Isl (asterisks); individual cells do not appear to express both genes (132 pRBs scored in 8 embryos). (B) Dorsal view of merged DIC and fluorescence images of a live 4-somite-stage embryo. Caged-fluorescein-dextran was photoactivated in a cluster of 4-6 adjacent cells in the lateral neural plate (arrowhead) just caudal to the last formed somite. (C) A 16 μm transverse section of a similarly labeled embryo, fixed at 22-somite stage (20 h) and processed to visualize Isl (brown, nuclear) and uncaged fluorescein (purple, cytoplasmic). Arrowhead indicates a doubly labeled RB on the left side of the neural tube. (D) The next section of the embryo shown in C. Arrowhead indicates a labeled migrating neural crest cell. In many experiments, labeled neural crest cells were identified by morphology in live embryos using DIC and fluorescence optics; labeled RBs were also identified by morphology. (E) Dorsal view of 3-somite-stage embryo (11.2 h). Double RNA in situ hybridization with *fkd6* (red) and *isl1* (blue) probes. *isl1* expression (arrowheads) is detectable further caudally than is *fkd6* expression. Since development proceeds from rostral to caudal, this expression is consistent with the theory that RBs are specified before neural crest. Scale bar: A, 15 μm; B, 50 μm; C, D, 20 μm; and E, 80 μm.

embryos carrying a *dlA* missense mutation (*dlA*^{dx2}) (Appel et al., 1999). Since neural crest cells are adjacent to RBs, we hypothesized that Delta signaling regulated formation of neural crest cells. To test this idea, we examined the expression of neural crest markers in *dlA*^{dx2} mutants.

The *dlA*^{dx2} allele is partially dominant and incompletely penetrant (Appel et al., 1999; Riley et al., 1999). Thus, it is possible that the protein encoded by the *dlA*^{dx2} allele interferes with normal function of other Delta family members or even

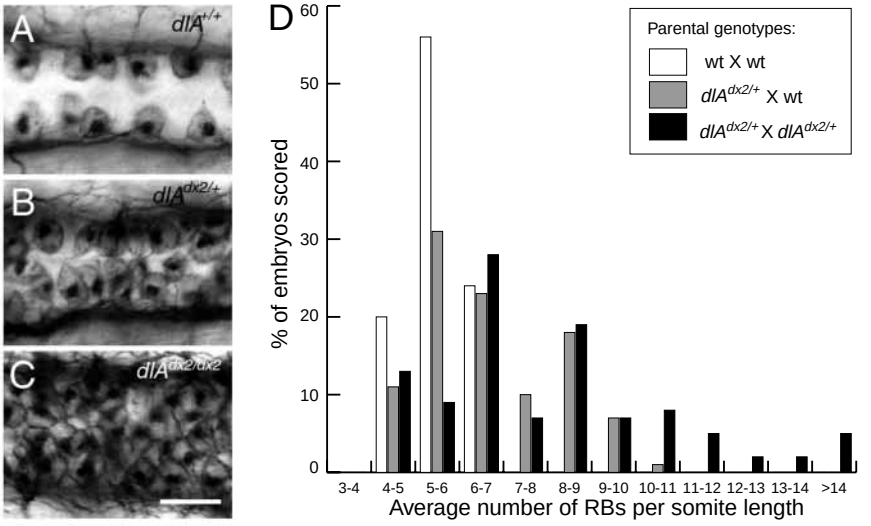

Fig. 2. *dlA*^{dx2} mutants have supernumerary RBs. Dorsal views of 24 h embryos processed for zn-12 immunoreactivity. Number of RBs in wild types (A) is considerably less that in presumed *dlA*^{dx2} heterozygotes (B). To examine the phenotype in heterozygotes, we crossed heterozygous mutant carriers to wild types. In the progeny of this cross, the mean number of RBs per somite length was 25% higher than in wild-type embryos. This significant difference (Student's *t*-test, $P<0.02$) clearly underestimates the dominant effect of the *dlA*^{dx2} allele, since the progeny of this cross should have been half heterozygotes and half wild types. (C) Presumed homozygous, strongly neurogenic *dlA*^{dx2}mutants have even more RBs. To examine the phenotype in homozygotes, we crossed two heterozygotes. In the progeny of this cross, the mean number of RBs per somite length was 55% higher than in wild types (Fig. 2C), showing that homozygous mutants have a significantly more severe phenotype that heterozygotes ($P<0.01$). (D) Average number of RBs per somite length in wild-type and *dlA* mutant embryos. RBs were counted over 4 somite lengths and averaged per somite length. White bars represent the progeny of wild-type fish (two clutches, $n=26$ embryos), gray bars represent the progeny of a wild-type and a heterozygous *dlA* mutant fish (three clutches, $n=130$ embryos) and black bars represent the progeny of two heterozygous *dlA* mutant fish (four clutches, $n=136$ embryos). Scale bar: 25 μm.

other Notch ligands. We found the phenotype of supernumerary RBs in *dlA*^{dx2} mutants to be quite variable, but to be generally more severe in homozygotes than in heterozygotes (Fig. 2). The progeny of two mutant carriers included a small class of embryos that were strongly neurogenic, defined as having a large excess of RBs. These embryos had 14 or more RBs per somite length, compared to wild types which have 5.5 RBs per somite length (Eisen and Pike, 1991; Grunwald et al., 1988). Because we never detected strongly neurogenic embryos in outcross progeny, we conclude that embryos with this phenotype were homozygotes. To assess the penetrance of this phenotype, we examined 385 embryos obtained from 9 pairwise matings of mutant carriers and found that 15 (4.2%) displayed the strongly neurogenic phenotype, ranging from 0% (0/37) to 11% (5/47) of individual clutches.

In homozygous, strongly neurogenic, *dlA*^{dx2} mutant embryos (hereafter referred to as *dlA* mutants) at the 4-somite stage, expression of *fkd6* was virtually eliminated from the trunk neural plate and spinal cord; in contrast, cranial *fkd6* expression appeared normal in these embryos (Fig. 3A,B). *sna2* is also expressed in zebrafish premigratory neural crest (Thisse et al., 1995), although slightly later than *fkd6* in the trunk (unpublished observations). Double RNA in situ hybridization analysis suggested that expression of *fkd6* and *sna2* defines a single population of cells in the CNS (Kelsh and Eisen, 2000a). In *dlA* mutants at the 10-somite stage (14 h), trunk-level expression of *sna2* was virtually eliminated, while cranial expression appeared normal (Fig. 3C,D). *msxB* is expressed in a longitudinal stripe about 5 cells wide in the lateral neural plate (Fig. 3E,F) and later in the dorsal neural tube and neural crest (Ekker et al., 1997). Although *msxB* is expressed in a broader band of cells in the lateral neural plate than is *fkd6* (compare Fig. 3E to Fig. 3A′), it resembles *fkd6* in that it is not expressed in pRBs (not shown). In *dlA* mutants at the 4-somite stage, trunk-level *msxB* expression was strongly reduced, but not eliminated, while cranial expression appeared unchanged (Fig. 3E,F). These data suggest that Delta function is involved in segregating RBs and trunk neural crest cells.

To determine the axial level where *fkd6* expression was lost in *dlA* mutants, we double-labeled 8-somite-stage (13 h) embryos with probes to *fkd6* and *myoD*, a marker of somites (Fig. 3G,H; Weinberg et al., 1996). *fkd6* expression was absent from the trunk of *dlA* mutants, but present rostral of somite 2. Surprisingly, at this stage *dlA* mutants had both *fkd6* expression (Fig. 3H) and supernumerary pRBs (Fig. 3J) adjacent to somites 1 and 2, the region giving rise to vagal neural crest. Together these data imply that, unlike trunk neural crest, specification of cranial neural crest does not depend on *deltaA* function, and that Delta-dependent and Delta-independent neural crest are mixed in the region of two or three most anterior somites at this stage.

Trunk neural crest derivatives are reduced in *dlA* mutants

To determine if altered gene expression in early somite-stage *dlA* mutants presaged a loss of neural crest derivatives, we examined mutants at 3-5 days. One major neural crest-derivative is pigment cells, of which there are three types in zebrafish: melanophores, iridophores and xanthophores (see Kelsh et al., 1996; LeDouarin, 1982). We examined pigment cells in 1207 progeny of 14 pairs of heterozygous *dlA* mutants. Of these larvae, 4.8% had severely reduced numbers of all three pigment cell types in the trunk and tail caudal to the level of the ear (Fig. 4A-D). Interestingly, pigments cells were typically absent from the region adjacent to the first two somites of *dlA* mutants. Within the progeny of a single pair, the number of embryos lacking pigment cells ranged between 0 and 23.5%, consistent with the variable penetrance described previously (Appel et al., 1999). Because the fraction of embryos lacking pigment cells resembles the fraction of highly neurogenic embryos, we suspect that highly neurogenic embryos become ones with the strong pigment phenotype.

Neurons of the dorsal root ganglia (DRGs) are also neural-

Table 1. Effect on *fkd6* expression of injected synthetic RNAs

RNA injected	Total embryos injected	Embryos with β-gal activity in both trunk and cranial lateral neural plate	Reduced trunk-level *fkd6* expression within area of β-gal activity	Reduced cranial-level *fkd6* expression within area of β-gal activity
X-dnDelta+lacZ	95	19	19	0
X-Su(H)^{DBM}+lacZ	80	22	22	0
lacZ	90	49	0	0

crest derived. DRGs, recognized by Hu immunoreactivity, are present lateral to the spinal cord in a segmentally reiterated pattern throughout the trunk and tail (Henion et al., 1996, Fig. 4E). DRGs were virtually absent from the trunk and tail of *dlA* mutants, with the exception of adjacent to the first two somites (*n*=11 embryos from four clutches), while sibling embryos with normal pigment also had normal DRG neurons (*n*=20 embryos from four clutches) (Fig. 4F). The reduction of pigment cells and DRG neurons concomitant with an increase of RBs in *dlA* mutants support the hypothesis that Delta signaling segregates RB and neural crest fates from an equivalence group in the lateral neural plate.

Fin mesenchyme appears normal in *dlA* mutants

Fin mesenchyme cells are thought to be derived from trunk neural crest in amphibians (references in LeDouarin, 1982) and zebrafish (Smith et al., 1994). These cells participate in morphogenesis of the median fin fold (Twitty and Bodenstein, 1941) and may eventually contribute to the fin rays of the dermal skeleton (Smith et al., 1994). Fin mesenchyme cells were readily visible in the median fin fold of 3-day wild-type embryos (Fig. 4A) and, unlike other trunk neural crest derivatives, they were present in *dlA* mutants (Fig. 4B). The presence in *dlA* mutants of fin mesenchyme cells suggests that specification of this cell type differs from specification of the trunk and tail neural crest that becomes pigment cells and DRG neurons.

Cranial and some presumed vagal neural crest derivatives are present in *dlA* mutants

Since cranial expression of *fkd6*, *sna2* and *msxB* appeared normal in *dlA* mutants, we predicted that cranial neural crest derivatives would be present. We examined *dlA* mutants for the following cranial neural crest derivatives: head pigment cells (Fig. 5A,B), jaw cartilage cells (Fig. 5C,D) and cranial ganglia (Fig. 5E,F), composed of cells from both cranial neural crest and placodes (see references in LeDouarin, 1982). While we could not detect subtle changes in the numbers of these cells, it was clear that there was no gross under-representation of these cranial neural crest derivatives in *dlA* mutants (*n*=10 larvae).

Because some *fkd6* expression was maintained in vagal neural crest in *dlA* mutants, we expected also to find neural crest derivatives from this axial level. While, in zebrafish, the origin of enteric neurons has not been determined, in chick, most enteric neurons derive from vagal neural crest and migrate ventrally and posteriorly along the gut (Epstein et al., 1994). Enteric neurons were present in *dlA* mutants (Fig. 5G,H), suggesting that specification of at least some vagal neural crest does not depend on Delta signaling.

Sympathetic neurons and associated adrenal chromaffin cells are also derived from neural crest (LeDouarin, 1982). In 5-day zebrafish larvae, presumed sympathetic neurons can be recognized by tyrosine hydroxylase immunoreactivity (Guo et al., 1999). Lineage analysis in zebrafish has shown that early-developing sympathetic neurons are derived from vagal neural crest (Raible et al., 1992; D. W. Raible and J. S. E., unpublished). Early-developing sympathetic neurons and adrenal chromaffin cells were present in *dlA* mutants at 5 days (*n*=5 larvae; Fig. 5I,J).

Embryos with experimentally reduced Delta signaling resemble *dlA* mutants

The presence of cranial neural crest in *dlA* mutants suggests two models: either Delta signaling is not involved in specification of cranial neural crest or another Delta family member, unaffected by the *dlA* mutation, mediates signaling during formation of cranial neural crest. To test whether other Delta family members function in formation of cranial neural crest, we injected RNA encoding a dominant negative version of *Xenopus laevis* Delta, X-dnDelta-1 (Chitnis et al., 1995). This construct appears to block signaling by all known zebrafish Delta family members (Appel and Eisen, 1998; Haddon et al., 1998a). It is also possible that Notch ligands other than Delta, for example Serrate/Jagged (Fleming et al., 1990; Lindsell et al., 1995), mediate signaling in the head. Thus, we also injected RNA encoding a dominant negative version of *X. laevis* Suppressor of Hairless (X-Su(H)^{DBM}) (Wettstein et al., 1997). Suppressor of Hairless is an intracellular signaling molecule downstream of Notch and X-Su(H)^{DBM} appears to inhibit all Notch-mediated signaling involved in *X. laevis* neurogenesis (Wettstein et al., 1997).

To generate embryos with mosaically distributed RNAs, we injected these RNAs along with *lacZ* RNA into one cell at the 2-cell stage. At the 6-somite stage, we selected embryos in which β-galactosidase activity was present in the lateral neural plate at all axial levels and examined them for expression of *fkd6* and Isl. Like *dlA* mutants, embryos injected with *X-dnDelta-1* or *X-Su(H)^{DBM}* RNA, but not embryos injected with *lacZ* RNA alone, were missing trunk *fkd6* expression while cranial *fkd6* expression was normal (Fig. 6A,B; Table 1). These embryos also had excess pRBs [inset in Fig. 6B and not shown; see Appel and Eisen, 1998; Haddon et al., 1998b].

To examine the effect of blocking Delta signaling throughout the embryo, we injected both cells of a 2-cell-stage embryo with these RNAs. Similar to *dlA* mutants, a fraction of these embryos had severely diminished pigment cells (Fig. 6C,D; Table 2) and DRG neurons (not shown) over a significant portion of the trunk and tail. Cranial pigment cells, anterior to the ear, were present in all RNA-injected embryos (Fig. 6C,D). For each injected RNA, three embryos displaying strongly reduced trunk pigment cells were analyzed for the presence of

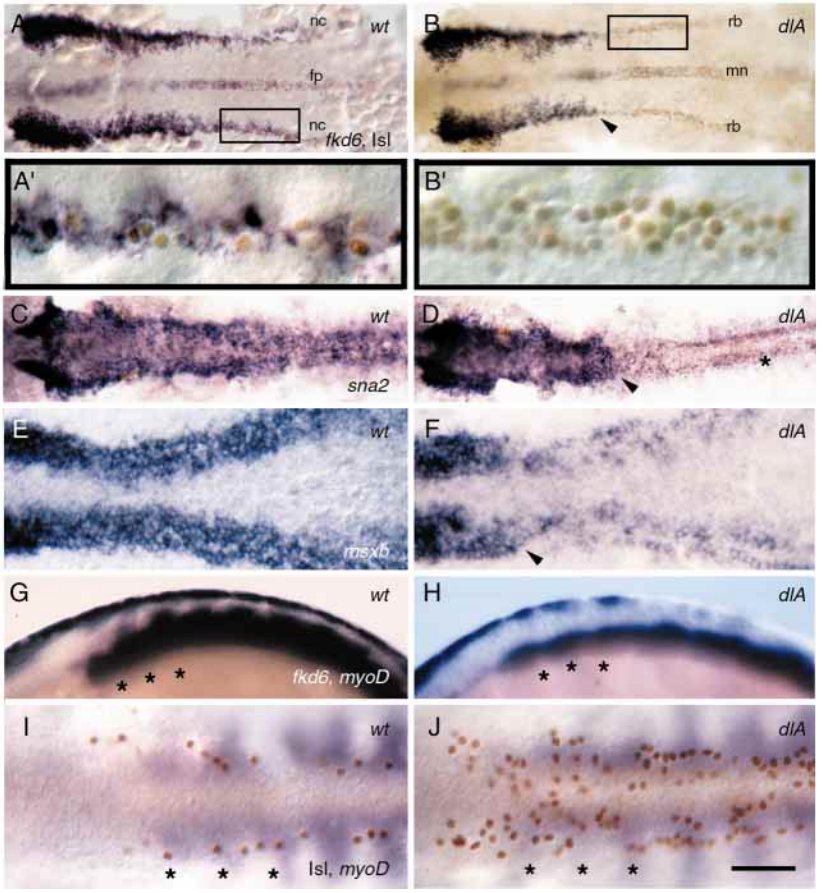

Fig. 3. Trunk expression of neural crest markers is reduced in *dlA* mutants. **(**A) Dorsal views of 4-somite-stage wild-type and (B) *dlA* mutant embryos labeled for pRBs (Isl, brown, nuclear) and neural crest precursors (*fkd6* mRNA, blue, cytoplasmic). In wild-type embryos, pRBs and *fkd6*-expressing cells are intermingled, as seen at higher magnification in A′ (corresponds to box in A). In strongly neurogenic *dlA* mutants, concomitant with a large increase in pRBs, there is a strong reduction of trunk-level expression of *fkd6* as shown at higher magnification in B′ (corresponds to box in B). Cranial expression of *fkd6* (left of arrowhead) appears normal in mutants. Floor plate expression of *fkd6* (fp) is apparent in both wild types and mutants. Supernumerary motoneurons (mn), which also express Isl, are apparent in *dlA* mutants (see Appel and Eisen, 1998). (C) Dorsal views of 10-somite-stage wild type and (D) *dlA* mutant labeled for pRBs (Isl, brown, nuclear) and *sna2* mRNA (blue, cytoplasmic). In mutants, cranial neural crest expression (left of arrowhead) of *sna2* looks normal and trunk-level neural crest expression is strongly reduced. Somite expression of *sna2* is visible in D (asterisk). **(**E) Dorsal views of 4-somite-stage wild-type and (F) *dlA* mutant embryos labeled for *msxB* expression. In the mutant, trunk-level expression is strongly reduced, while cranial expression appears normal (left of arrowhead). (G) Lateral views of 8-somite-stage wild-type and (H) *dlA* mutant embryos labeled for *fkd6* and *myoD* expression, to reveal premigratory neural crest and somites, respectively. In mutants, expression of *fkd6* appears normal at cranial levels, reduced adjacent to somites 1-3 (asterisks), and absent further caudally. **(**I) Dorsal views of 8-somite-stage wild-type and (J) *dlA* mutant embryos labeled for *myoD* (blue) and Isl (brown) expression. As in the trunk, supernumerary pRBs are present adjacent to and just rostral to somites 1-3 (asterisks) in *dlA* mutants. nc, neural crest, fp, floor plate, rb, presumptive Rohon-Beard neurons, mn, presumptive motoneurons. Scale bar: A-D, 140μm; E,F, 80 μm; G,H, 75μm; I,J, 50 μm.

Table 2. Effect on pigment cells of injected synthetic RNAs

RNA injected	Injected embryos surviving at 3 days (no. of experiments)*	Embryos lacking melanophores over 5 or more continuous trunk or tail somites (percent)‡
X-dnDelta-1	326 (7)	53 (16%)
X-Su(H)^{DBM}	185 (3)	28 (14%)
lacZ	60 (1)	0 (0%)

*Mortality of approximately 5% occurred with each injected RNA

‡At the concentrations of RNA used (0.05-0.2 mg/ml), the two constructs were similarly efficient at blocking formation of pigment cells over several segment lengths in the trunk. However, *X-Su(H)^{DBM}* mRNA was considerably more effective than *X-dnDelta-1* mRNA at eliminating pigment throughout the trunk and tail (as in Fig. 5**).** Because the tail forms later than the trunk, the different efficiency of the two constructs at blocking formation of tail pigment likely results from differential stability of the injected RNAs or proteins they encode.

jaw cartilage, cranial neurons, enteric neurons and early sympathetic neurons. In each case, these cranial and vagal neural crest derivatives were present (not shown). These observations support the model that Delta-Notch signaling is not involved in specification of vagal and cranial neural crest.

There was one striking difference between *dlA* mutant embryos and the RNA-injected embryos. The median fin folds of 3-day *X-dnDelta*-injected and *X-Su(H)^{DBM}*-injected embryos displaying a severe reduction in trunk pigment cells also had crumpled, unextended median fin folds (Fig. 6E,F). Because of this aberrant morphology, it was impossible to tell whether fin mesenchyme cells were present. These data suggest that Delta signaling, but not specifically by *deltaA*, regulates some aspect of fin morphogenesis.

DISCUSSION

Delta signaling segregates RBs from trunk neural crest

We propose that RBs are the preferred fate of an equivalence group in the zebrafish trunk and tail lateral neural plate, and that cells prevented from taking the preferred fate by Delta signaling become neural crest instead. Several lines of evidence support this model. First, pRBs express high levels of Delta homologues soon after they are born (Appel and Eisen, 1998; Haddon et al., 1998b). Second, our analysis of cell clusters reveals that cells surrounding RBs in the neural plate normally become neural crest. Finally, when Delta signaling is decreased through mutation of *deltaA*, or through injection of RNA constructs encoding proteins that disrupt Delta-Notch signaling, more cells become RBs and fewer cells become

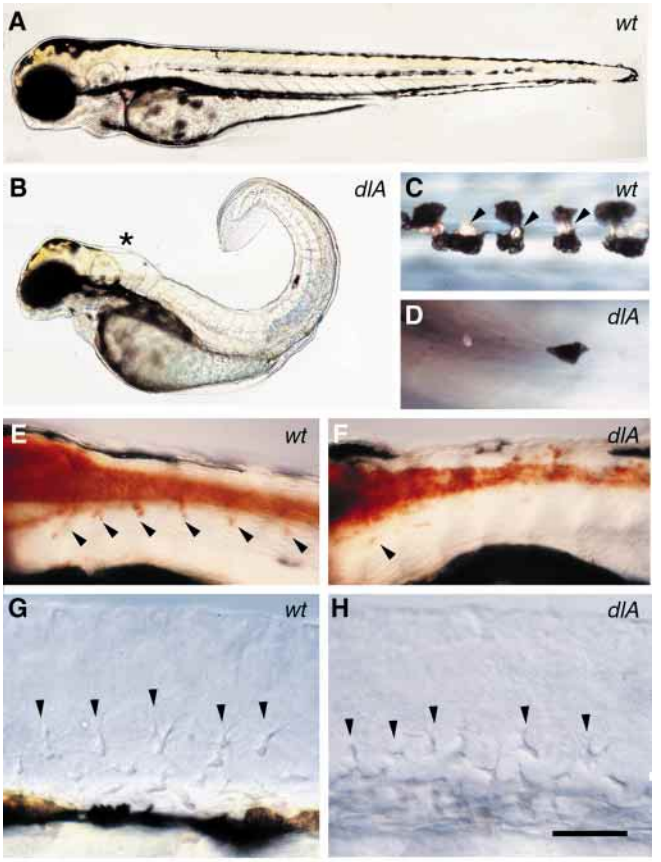

Fig. 4. Trunk neural crest derivatives are diminished in *dlA* mutants. (A) Lateral views of 3 day wild-type and (B) *dlA* mutant embryos. Melanophores (black pigment cells) and xanthophores, which account for the yellowish tinge of the trunk and tail in wild-type embryos, are strongly reduced in the trunk and tail of *dlA* mutants. Pigment cells are also reduced in the vagal region (asterisk). (C) Dorsal views of 3 day wild-type and (D) *dlA* mutant embryos. Iridophores (arrowheads) are strongly reduced in the mutant. (E) Lateral views of 3 day wild-type and (F) *dlA* mutant embryos labeled with anti-Hu antibody to reveal neurons. DRG neurons (arrowheads) are visible lateral and ventral to the neural tube in wild types but are absent from mutants except adjacent to somites 1 and 2. **(**G) Dorsal median fin fold in trunk of 2 day wild-type and (H) *dlA* mutant embryos. Fin mesenchyme cells (arrowheads) are visible in both. Scale bars: A,B, 290 μm; C,D, 95 μm; E,F, 90 μm; G,H, 40 μm.

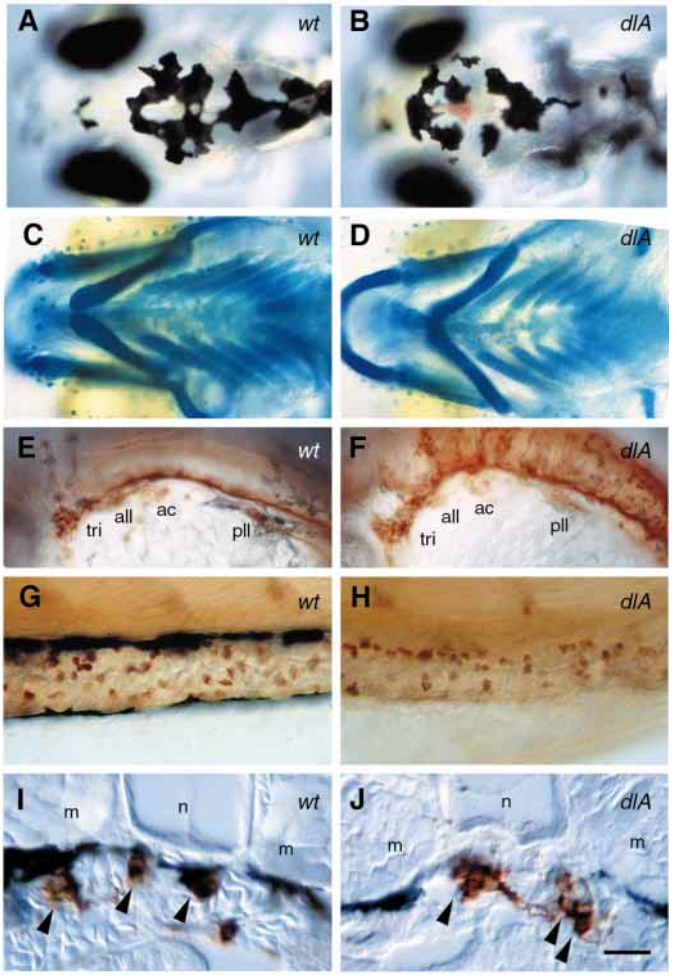

Fig. 5. Cranial neural crest derivatives are present in *dlA* mutants. (A) Dorsal views of 3 day wild-type and (B) *dlA* mutant embryos. Melanophores (black) and xanthophores (yellow) are present in both rostral of the otic vesicle (just to right of these photographs). (C) Ventral views of 5 day wild-type and (D) *dlA* mutant embryos labeled for jaw cartilage, which appears essentially normal in the mutant. (E) Lateral views of 30 h wild-type and (F) *dlA* mutant embryos, labeled with zn-12 and anti-acetylated tubulin monoclonal antibodies to reveal cranial ganglia, all of which were present in the mutant**. (**G) Lateral views of gut region of 3 day wild-type and (H) *dlA* mutant embryos labeled with anti-Hu antibody to reveal enteric neurons, which are readily visible in mutants. The black cells in G are melanophores, which are absent from this region in the mutant (H). (I) Transverse sections just caudal to the ear of 5 day wild-type and (J) *dlA* mutant larvae. Sympathetic neurons and chromaffin cells (arrowheads) are immunolabeled with anti-tyrosine hydroxylase. Sympathetic neurons at this level were present in the *dlA* mutant in similar numbers to wild types. n, notochord, m, muscle**.** tri, trigeminal, all, anterior lateral line ganglion, ac, acoustic ganglia, pll, posterior lateral line ganglion. Scale bar: A-D, 50 μm; E,F, 79 μm; G,H, 40 μm; I,J, 20 μm.

trunk neural crest. Because trunk-level expression of markers of premigratory neural crest is also drastically reduced in these embryos, it appears that the initial specification of trunk neural crest is blocked. These data support the hypothesis that RBs and trunk neural crest are two derivatives of an equivalence group in the lateral neural plate.

Is all trunk neural crest derived from the same neural plate domain? Trunk neural crest derivatives include pigment cells, DRG neurons, peripheral glia and fin mesenchyme cells (Eisen and Weston, 1993). Pigment cells and DRG neurons are essentially absent from the trunk and tail of *dlA* mutants; for lack of a suitable molecular marker, we were unable to score glia. As *dlA* mutants lose expression of two early markers for neural crest precursors, *fkd6* and *sna2*, we predict that *fkd6*$^{+}$, *sna2*$^{+}$ cells generate all trunk pigment cells, DRG neurons and, probably, glia. In contrast, fin mesenchyme cells are present in *dlA* mutants, suggesting they do not derive from *fkd6*$^{+}$, *sna2*$^{+}$ cells. We plan to explore this issue by fate-mapping studies in the neural plate.

Our data support the model that ectomesenchymal neural crest cells, defined as those destined to populate cartilage,

dermis or connective tissue, segregate from non-ectomesenchymal neural crest cells very early in development (Weston, 1991). Initial evidence for this model came from transplant studies in avian embryos, in which only cranial neural crest has ectomesenchymal potential. When neural tube plus neural fold was transplanted from cranial levels to trunk levels, a subset of transplanted neural crest cells differentiated into connective tissue and cartilage. These data were interpreted to mean that ectomesenchymal neural crest cells were determined at a time when other neural crest cells were still susceptible to inductive signals (LeDouarin and Teillet, 1974). More recently, Dorsky et al. (1998) found that altering Wnt signaling levels in single zebrafish cranial neural crest cells altered the fates of pigment and neural precursors, but not of cartilage precursors, demonstrating distinct genetic regulation of the ectomesenchymal population. This model is also supported by the recent finding that fish homozygous for the *colourless* mutation lack essentially all neural crest derivatives except fin mesenchyme and jaw cartilage (Kelsh and Eisen, 2000b). The differential effect of the *dlA*^{dx2} mutation on fin mesenchyme and other trunk neural crest cells is further genetic evidence for distinct regulation of ectomesenchymal and non-ectomesenchymal neural crest cells.

The aberrant median fin folds of embryos injected with RNA encoding proteins that block Delta signaling is a surprising difference from *deltaA* mutants. Interestingly, embryos homozygous for the *mindbomb* mutation, which have a phenotype reminiscent of disrupted Delta-Notch signaling (Haddon et al., 1998a; Jiang et al., 1996; Schier et al., 1996), also have similarly affected median fin folds (our unpublished observations). Since fin mesenchyme cells are thought to participate in morphogenesis of median fin folds, one possibility is that these cells are reduced in our RNA-injected embryos and in *mindbomb* mutants. This would imply that specification of fin mesenchyme cells depends on signaling mediated by a Notch ligand other than DeltaA. However, without a molecular marker for fin mesenchyme it is impossible to know whether it is present in embryos with such distorted fin folds. Thus, it is also possible that a step in fin-fold morphogenesis other than specification of fin mesenchyme depends on signaling mediated by a Notch ligand. Isolation of a molecular marker for fin mesenchyme precursors will resolve this issue.

Dependence on Delta function distinguishes trunk and cranial neural crest

There is evidence for differential regulation of cranial and trunk neural crest development (LeDouarin, 1982). For example, cranial neural crest has a far greater potential to regenerate after ablation than trunk neural crest (Suzuki and Kirby, 1997). In addition, targeted deletion of mouse *Ap2*, a gene expressed in neural crest at all axial levels, severely disrupts cranial neural crest while at least one trunk neural crest derivative, DRGs, appears normal (Zhang et al., 1996). The differential dependence on Delta function demonstrated here represents an additional distinction between trunk (Delta-dependent) and cranial (Delta-independent) neural crest.

Interestingly, both premigratory neural crest cells and supernumerary RBs are present in the vagal region of *dlA* mutants. This is surprising since our model predicts that, in the absence of Delta signaling, all cells within the equivalence domain should become RBs. Instead, the vagal region appears to contain a mixture of Delta-dependent and Delta-independent neural crest populations. One possibility is that these populations are initially spatially segregated, but become mixed by the stage we examined (8-somite stage). Alternatively, they may be mixed before Delta signaling occurs. Fate mapping of this region will resolve this issue.

Neural crest may have evolved from an RB-like spinal cord neuron

Neural crest is usually considered a defining feature of vertebrates, distinguishing them from chordate sister groups, cephalochordates and urochordates. The evolutionary appearance of neural crest is associated with the transition to predatory behavior and increased success of vertebrates relative to other chordates (Gans and Northcutt, 1983). Shared features between vertebrates and cephalochordates are assumed to have existed in the chordate precursor from which both of these modern groups evolved. While amphioxus, a commonly studied cephalochordate, lacks neural crest derivatives, it has dorsal spinal sensory neurons that may be homologous to RBs (Bone, 1960), suggesting this cell type predates neural crest. It has been proposed that, during neural crest evolution, a subset of RBs migrated from the neural tube and became extramedullary sensory neurons similar to modern DRG neurons (Fritzsch and Northcutt, 1993). Thus the earliest neural crest derivative may have been a sensory neuron, which then diversified to form the many modern neural crest derivatives (see Baker and Bronner-Fraser, 1997b; Hall and Horstatdius, 1988). The phenotypes of the *dlA* mutant, supernumerary RBs at the expense of neural crest, and the *narrowminded* mutant, lacking both RBs and neural crest (Artinger et al., 1998), together demonstrate that the regulation of RB and neural crest fates are tightly linked and thus potentially evolutionarily related. A population of cells in the amphioxus neural plate expresses *snail*, a gene related to zebrafish *sna2*, and might be homolgous to vertebrate neural crest (Langland et al., 1998). It will be interesting to learn whether dorsal spinal sensory neurons arise from this population.

With respect to this model, why should Delta signaling regulate specification of trunk, but not cranial neural crest? Perhaps cranial neural crest originated from a class of cranial sensory neurons that has been lost. It has been suggested that the vertebrate precursor had dorsal sensory neurons throughout the neuraxis but that these cells were lost from the hindbrain of jawed vertebrates (Fritzsch and Northcutt, 1993). Indeed, dorsal sensory neurons are present in the head of amphioxus, but it is unclear what relationship these cells have to RBs (Bone, 1960). Interestingly, jawed vertebrates, including zebrafish, have dorsal sensory neurons in a hindbrain nucleus, mesencephalic V (Kimmel et al., 1985); these may be homologous to RBs. It will be important to learn whether Delta signaling regulates these neurons and whether they are derived from an equivalence group that includes neural crest.

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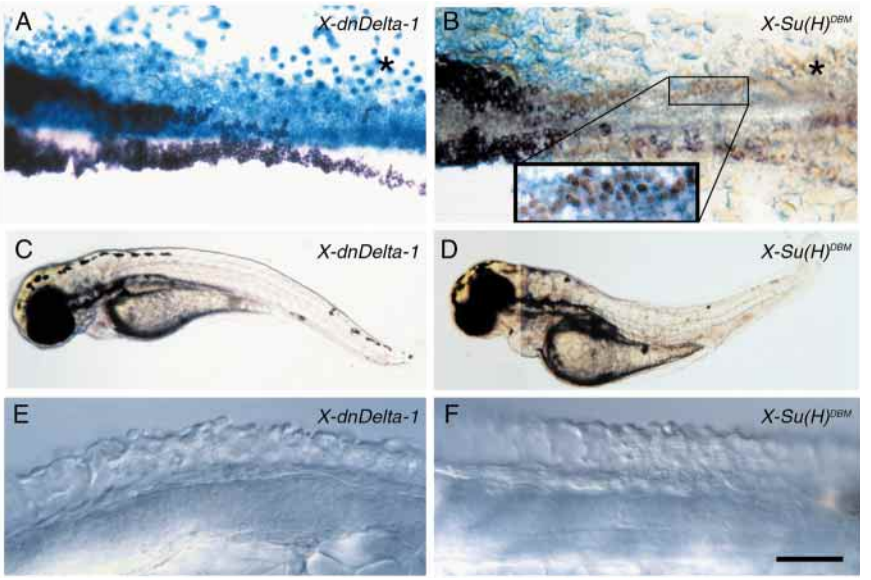

Fig. 6. Embryos with experimentally reduced Delta-Notch signaling lack derivatives of trunk neural crest but retain those of cranial neural crest. (A,B) Dorsal views of flat-mounted embryos at 6-somite stage. (A) *fkd6* expression (black) and *β-galactosidase* activity (blue) in embryos co-injected with RNAs encoding X-dnDelta-1 or (B) X-Su(H)^{DBM} and β-gal. Asterisks indicate injected side. In both cases, despite presence of injected RNA in both cranial and trunk neural crest domains, *fkd6* expression is highly reduced at trunk but not cranial levels. Isl expression is concomitantly upregulated in the trunks of *X-dnDelta-1*-injected embryos (not shown; see Appel and Eisen, 1998) as well as in *X-Su(H)^{DBM}*-injected embryos (inset in B). Lateral views of 3 day (C) *X-dnDelta-1* and (D) *X-Su(H)^{DBM}*-injected embryos. Pigment cells are highly reduced in the trunk and tail, but still present in the head. Pigment cells are present at vagal levels in the *X-dnDelta-1*-injected embryo shown (D), but absent from many other embryos (not shown). Dorsal median fin fold in trunk of 2 day (E) *X-dnDelta-1* and (F) *X-Su(H)^{DBM}*-injected embryos. The dorsal median fin fold is crumpled relative to wild types (compare to Fig. 4G) and *dlA* mutants (Fig. 4H) and fin mesenchyme cells are not apparent. Scale bar: A,C, 90 μm; B,D, 380 μm; E,F, 50 μm.

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