The zebrafish *colourless* gene regulates development of non-ectomesenchymal neural crest derivatives

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SUMMARY

Neural crest forms four major categories of derivatives: pigment cells, peripheral neurons, peripheral glia, and ectomesenchymal cells. Some early neural crest cells generate progeny of several fates. How specific cell fates become specified is still poorly understood. Here we show that zebrafish embryos with mutations in the *colourless* gene have severe defects in most crest-derived cell types, including pigment cells, neurons and specific glia. In contrast, craniofacial skeleton and medial fin mesenchyme are normal. These observations suggest that *colourless* has a key role in development of non-ectomesenchymal neural crest fates, but not in development of ectomesenchymal fates. Thus, the *cls* mutant phenotype reveals a segregation of ectomesenchymal and non-ectomesenchymal fates during zebrafish neural crest development. The combination of pigmentation and enteric nervous system defects makes *colourless* mutations a model for two human neurocristopathies, Waardenburg-Shah syndrome and Hirschsprung's disease.

Key words: Zebrafish, Neural crest, Cell fate, Melanophore, Xanthophore, Iridophore, Neuron, Glia

INTRODUCTION

The process whereby precursor cells become specified to generate progeny with distinct fates is fundamental to development. The neural crest, a vertebrate tissue that generates cells of diverse fates (Le Douarin, 1982), has been widely used to address this issue. Crest-derived fates fall into 4 broad categories: pigment cells, neurons, glia and ectomesenchymal cells. Each category includes several distinct, terminally differentiated cell types; for example, in fish, ectomesenchymal cells include both craniofacial cartilage cells and median fin mesenchyme (Schilling and Kimmel, 1994; Smith et al., 1994).

Neural crest cells delaminate from the neural tube and migrate on well-characterised paths (Weston, 1970; Bronner-Fraser, 1986; Loring and Erickson, 1987; Krotoski et al., 1988; Serbedzija et al., 1989, 1990; Raible et al., 1992; Collazo et al., 1993). Premigratory neural crest is described operationally as a mixture of unrestricted and fate-restricted precursors. An unrestricted precursor generates progeny of two or more types, whereas a fate-restricted precursor generates progeny of only one type. However, these lineage-based definitions do not reveal cell potency, which must be tested independently from fate (see Eisen and Weston, 1993; Raible and Eisen, 1996). Clonal studies in vivo demonstrate that premigratory neural crest cells frequently generate clones containing more than one fate (chick, Bronner-Fraser and Fraser, 1991; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991; mouse, Serbedzija et al., 1994; frog, Collazo et al., 1993; zebrafish, Raible and Eisen, 1994). Cultured mammalian and avian neural crest cells also frequently generate mixed fate clones, as well as fate-restricted clones (Sieber-Blum and Cohen, 1980, Baroffio et al., 1988; Dupin et al., 1990; Baroffio et al., 1991; Stemple and Anderson, 1992; Rao and Anderson, 1997). At its most extreme, in vivo lineage studies suggest that zebrafish premigratory cranial crest consists of only fate-restricted cells (Schilling and Kimmel, 1994).

The mechanism and timing of specification of individual neural crest fates from unrestricted precursors has been a matter of debate and remains largely unknown. Baroffio and colleagues showed that cultured, migratory, avian cranial neural crest includes a very rare class of unrestricted cells, capable of generating progeny that differentiated into all classes of crest derivatives (Baroffio et al., 1988; 1991). However, most clones generated only a subset of fates. Such observations have lead to the suggestion that neural crest development involves a progressive series of restrictions in developmental fate. Thus, unrestricted precursors are postulated to generate progenitors restricted to a subset of fates, which then give rise to fate-restricted daughters (Dupin et al., 1990; Baroffio et al., 1991; Weston, 1991; Anderson, 1993; Le Douarin et al., 1994; Lo et al., 1994). Several progenitors generating specific subsets of fates have been well-characterised, including sympathoadrenal precursors (Anderson and Axel, 1986) and neuronoglial precursors (Dupin et al., 1990; Frank and Sanes, 1991; Pomeranz et al., 1993; Henion and Weston, 1997). The molecular genetic basis of these fate restrictions is poorly understood. Although in vitro some growth factors can direct the fate choice of individual neural crest stem cells, there is no evidence for progressive

restriction in this paradigm (Stemple and Anderson, 1992; Shah et al., 1994, 1996; Morrison et al., 1997; Shah and Anderson, 1997).

Models of neural crest specification by progressive fate restriction predict the existence of genes that regulate generation of combinations of neural crest fates. Mutants in such genes would essentially lack cells of those fates, with other derivatives being unaffected. Several mouse mutants have defects in specific subsets of neural crest fates, although the combinations of derivatives affected are perhaps surprising. Thus, mutations in 3 genes, *piebald (s)*, *lethal spotting (ls)* and *Dominant megacolon (Dom)* combine defects in the enteric nervous system and melanocytes. Homozygous mutant *s* and *ls* mice have a phenotype very similar to *Dom* heterozygotes, showing reduced melanocytes and posterior colon aganglionosis (Mayer and Maltby, 1964; Webster, 1973; Lane and Liu, 1984; Baynash et al., 1994; Hosoda et al., 1994; Pavan and Tilghman, 1994). Therefore, all three genes are implicated in development of both enteric neurons and melanocytes. In addition, *Dom* homozygotes have extensive peripheral nervous system (PNS) defects outside the enteric nervous system (Herbarth et al., 1998; Southard-Smith et al., 1998). Molecular characterisation shows that *s* and *ls* encode endothelin receptor B (Hosoda et al., 1994) and its ligand, endothelin-3 (Baynash et al., 1994) and *Dom* encodes the Sox10 transcription factor (Herbarth et al., 1998; Southard-Smith et al., 1998). The precise roles of these genes remains unclear; functions in proliferation, survival and differentiation of crest fates have been proposed (Lahav et al., 1996; Reid et al., 1996; Stone et al., 1997).

In zebrafish, in vivo clonal studies demonstrated that the majority of premigratory crest cells are fate-restricted (Raible and Eisen, 1994; Schilling and Kimmel, 1994). Clonal analysis of trunk crest cells is consistent with production of fate-restricted precursors as daughters of initially unrestricted cells (Raible and Eisen, 1994). Evidence for a completely unrestricted precursor, one forming all major classes of crest derivatives, has not yet been reported.

The advantages of zebrafish for in vivo analyses of cell behaviour are nicely complemented by extensive collections of mutations available for study. Many mutations affecting genes crucial for neural crest development have been isolated (Henion et al., 1995; Driever et al., 1996; Haffter et al., 1996; Kelsh et al., 1996; Neuhauss et al., 1996; Odenthal et al., 1996; Piotrowski et al., 1996; Schilling et al., 1996a,b). To date, characterisation of most of these mutant phenotypes are in early stages. *chinless*, the best-characterised zebrafish neural crest mutant, has profound defects in craniofacial cartilage consistent with involvement in cartilage specification (Schilling et al., 1996b). Mutants with defects in more than one neural crest category have been described (Henion et al., 1995; Kelsh et al., 1996; Neuhauss et al., 1996; Odenthal et al., 1996; Schilling et al., 1996a). However, it remains to be seen whether any of these mutations affect cell numbers of two or more derivatives, as would be expected for genes involved in progressive fate restriction. Thus, a zebrafish mutation affecting several, but not all, neural crest fates would be particularly interesting. We report here studies of mutations in a zebrafish gene, *colourless (cls)*, in which homozygous mutants show such a phenotype.

cls mutations were isolated independently in two large-scale mutagenesis screens (Kelsh et al., 1996; Malicki et al., 1996). Other than severe defects in pigmentation and ear morphogenesis (Malicki et al., 1996; Whitfield et al., 1996), these mutants show few overt defects prior to death at 10 days post-fertilisation (dpf). Initial phenotypic characterisation demonstrated a dramatic reduction in all three pigment cell types. Here we describe additional, pronounced neural crest defects in neural and pigment cell fates. However, ectomesenchymal fates are remarkably unaffected. Furthermore, we demonstrate that *cls* functions cell-autonomously within all three crest-derived pigment cell types. We propose that *cls* functions early in neural crest development and is important for generation of several fates. Such a phenotype is consistent with that of a gene functioning in development of a precursor restricted to non-ectomesenchymal crest fates.

MATERIALS AND METHODS

Fish husbandry

Embryos from the University of Oregon zebrafish colony were raised at 28.5°C and staged according to Kimmel et al. (1995). We examined embryos homozygous for 3 strong *cls⁻* alleles, *t3*, *tw2* and *tw11* on AB (*t3*) or mixed AB/Tübingen backgrounds (*tw2* and *tw11*); since no differences between alleles have been described we do not distinguish them here. Homozygous *cls⁻* embryos were obtained by mating known heterozygotes. We see no evidence of defects in heterozygotes and so refer to homozygous mutants as *cls⁻*. We have no evidence whether these alleles are nulls. *albino⁻ (alb⁻)* embryos were generated by mating *albᵇ⁴* homozygotes (Streisinger et al., 1986).

Neural crest cell counts

Neural crest cells on the lateral migration pathway (Raible et al., 1992) were counted by mounting anaesthetised embryos between No. 1 coverslips using No. 1 coverslips as spacers. All cells showing the characteristic fibroblastic morphology of migrating neural crest cells, and positioned between the epidermis and lateral surface of the muscles, were counted for somites 5-15 on one side of the embryo. These counts were performed on embryos between 26-somite and prim-24 stages (22-35 hpf). After counting, each embryo was released from between the coverslips and raised overnight in embryo medium so that mutants could be identified.

In situ hybridisation and immunocytochemistry

RNA in situ hybridisation was performed by the method of Thisse et al. (1993), except probes were not hydrolysed. Probes were labeled with digoxigenin or fluorescein using the Genius RNA Labeling Kit (Boehringer Mannheim) and detected with the appropriate antibody. A blue precipitate was formed by incubating with 5-bromo 4-chloro 3-indolyl phosphate/nitro blue tetrazolium. Probes used: *dopachrome tautomerase/tyrosinase-related protein 2 (dct)* (R. N. K., B. Schmid and J. S. E., unpublished); *forkhead-related gene 6 (fkd6;* Odenthal and Nuesslein-Volhard, 1998); *dlx2* (Akimenko et al., 1994); *sonic hedgehog (shh;* Krauss et al., 1993); *engrailed3 (en3;* Ekker et al., 1992); *krox20* (Oxtoby and Jowett, 1993); *c-ret* (Bisgrove et al., 1997).

Antibody staining was performed using peroxidase-antiperoxidase (Sternberger Monoclonals, Inc). Double immunolabelings of dissected zebrafish guts were examined with a Zeiss LSM 310 confocal microscope using fluorescent secondary antibodies (Jackson Immunoresearch Laboratories) and assembled using VoxelView (Vital Images). Primary antibodies used were: anti-Hu, mAb 16A11 (Marusich et al., 1994); anti-acetylated tubulin (Sigma); and anti-GFAP (Axell).

Embryos were processed as whole mounts. Immunolabeling of

Fig. 1. Melanoblasts are abnormal in *cls⁻* embryos. (A) Lateral view of wild-type embryo labeled for *dct* transcripts at 27 hpf reveals abundant melanoblasts along trunk and tail, with many along medial migration pathway (*). (B) *cls⁻* embryos have fewer melanoblasts, all dorsal to spinal cord (sc). (C) At higher magnification, wild-type melanophores show intense cytoplasmic *dct*-labeling (purple) and melanosomes (brown granules, arrowhead) in processes. (D) *cls⁻* melanophores show only faint *dct* labeling. In these and all subsequent lateral views, anterior is to the left and dorsal to the top. At both 24 and 27 hpf *cls⁻* embryos show only approximately one third the number of melanoblasts of wild types (data not shown). m, muscle fibres; n, notochord. Scale bar, 250 μm (A,B) and 50 μm (C,D).
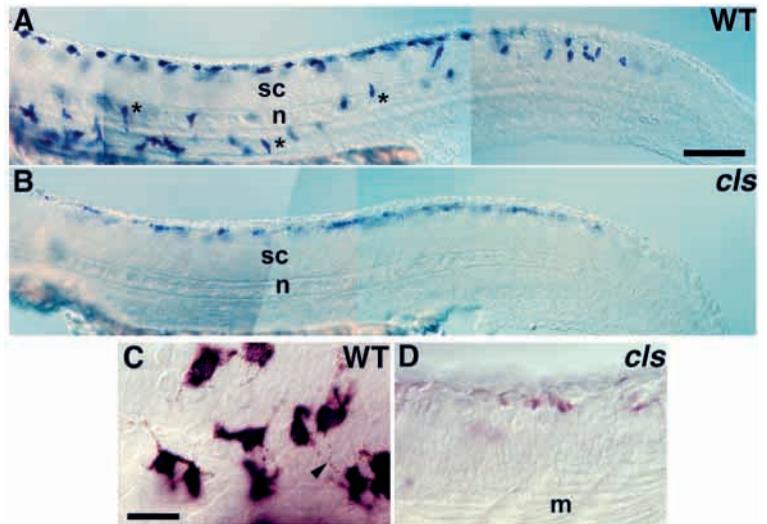
larval stages was performed on cryostat sections, as described by Raible et al. (1992). To view the enteric nervous system, larvae were fixed in 4% paraformaldehyde for 2 hours and intact guts (pharynx to anus) dissected out with fine needles and processed for whole-mount antibody staining.

Images were cropped, colour-balanced, contrast-enhanced and labeled using Photoshop (Adobe).

Craniofacial cartilage preparations

Cartilage was stained with Alcian green according to the method of Kimmel et al. (1998). Larvae were fixed in 4% paraformaldehyde overnight, washed in acid-alcohol (1.85% HCl, 70% ethanol) and incubated overnight in 0.1% Alcian green in acid-alcohol. Larvae were destained by extensive acid-alcohol washes, rehydrated, bleached using peroxide (1% KOH, 3% H_2O_2), cleared and stored in glycerol (0.25% KOH, 50% glycerol).

Mosaic analysis

Donor embryos were labeled with 10×10^3 M_r tetramethylrhodamine dextran (Molecular Probes) by intracellular injection into yolk cell cytoplasm during early cleavage stages. Genetic mosaics were generated by transplanting cells from late blastula donors into shield stage hosts. Known wild-type embryos were from AB stocks. To generate wild type→*cls⁻* chimaeras, we transplanted wild-type cells into host embryos from *cls* heterozygote crosses. Since at these stages wild-type embryos are indistinguishable from *cls⁻* siblings, these experiments generated many wild type→wild type control transplants. Donors and hosts were raised separately at 28.5°C until their phenotypes could be scored. To generate *cls⁻*→wild type chimaeras, donor and host embryos were reversed. In some experiments, wild-type embryos were replaced with *alb⁻* embryos, which completely lack melanin (Streisinger et al., 1986). The *alb⁻* mutation acts cell-autonomously in melanophores (R. N. Kelsh et al., unpublished).

Pigment cells were scored for pigment type (Bagnara and Hadley, 1973) and rhodamine label. Iridophores and xanthophores could be readily scored for fluorescent label between 3-6 dpf. In contrast, melanin strongly quenched the rhodamine fluorescence and only some melanophores could be reliably scored for rhodamine label at 2 dpf. Cells that could not be reliably scored were not considered.

RESULTS

cls⁻ embryos have fewer melanoblasts

Decreased melanophore number in *cls⁻* embryos (Kelsh et al., 1996) might result either from a decrease in melanoblast

precursors or in melanin synthesis. To distinguish these alternatives, we compared melanoblasts and melanophores in wild-type and *cls⁻* embryos using *dct* riboprobes (R. N. Kelsh et al., unpublished). In mice, *dct* is expressed in melanoblasts days before melanin becomes visible (Steel et al., 1992). In zebrafish, we detect melanoblasts several hours before melanisation. From 27 hpf, *cls⁻* embryos were readily distinguished from wild types by their lack of body melanin and distinct *dct* expression patterns (Fig. 1A,B). Wild-type embryos had many strongly labeled melanoblasts migrating throughout the body (Fig. 1A,C). In contrast, *cls⁻* embryos showed only a few, weakly stained melanoblasts, none anterior to the hindbrain, and none appeared to have migrated (Fig. 1B,D). Similar melanoblast defects were detected in 25% (presumably *cls⁻*) of embryos from a heterozygous *cls⁻* cross by 21 hpf, several hours before melanisation begins.

Since there are no published markers for iridoblasts and xanthoblasts, we examined *cls⁻* embryos for neural crest cells migrating on the lateral pathway. These cells are fated to become pigment cells (Raible and Eisen, 1994) and are strongly reduced in numbers in *cls⁻* embryos (Kelsh et al., 1996). Furthermore, they are severely depleted from very early stages (Fig. 2).

Most peripheral neurons are reduced in *cls⁻* embryos

The pigment phenotype in *cls⁻* embryos is reminiscent of *s*, *ls* and *Dom* mutant mouse phenotypes. These mutants also show enteric nervous system defects, so we asked whether the PNS was affected in *cls⁻* larvae. A pan-neuronal marker, anti-Hu antibody (Marusich et al., 1994), revealed large numbers of anti-Hu-positive enteric neurons around wild-type larval guts (Fig. 3A). *cls⁻* larvae have only about 13% as many enteric neurons as wild types along the entire length of the gut (Fig. 3B). Similar results were obtained by in situ hybridisation with another marker for enteric ganglion precursors, c-*ret* (Bisgrove et al., 1997; Marcos-Gutierrez et al., 1997; data not shown). Comparison of the rostrocaudal extent of enteric nervous system precursors using these 2 markers suggested that enteric neuron differentiation shows a rostrocaudal gradient. Thus, anti-Hu labeled neurons are relatively scarce in the caudal gut at 3 dpf, whereas c-*ret* labeling reveals many enteric neuron precursors in these caudal regions (data not shown).
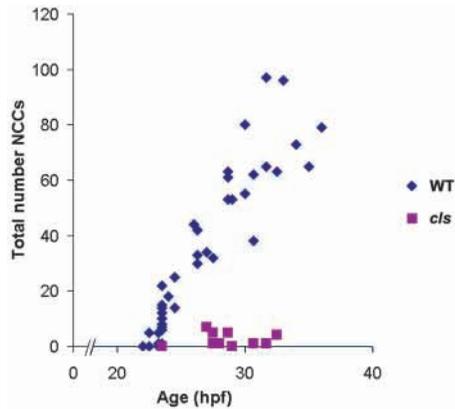
Fig. 2. Lateral migration path neural crest cells are severely reduced in $cls^{-/-}$ embryos from the earliest stages. Neural crest cell counts of each embryo are plotted as a single symbol (wild-type = blue, n=39; cls^- = pink, n=14) against the stage (expressed as age, hpf; Kimmel et al., 1995).
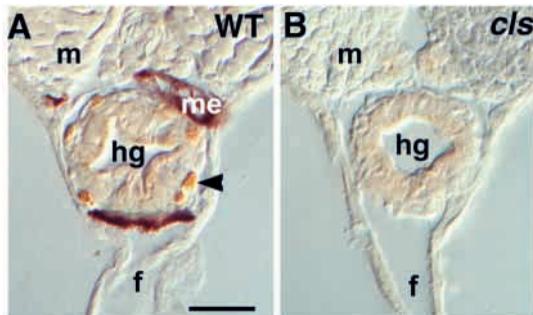

Fig. 3. Enteric neurons are extremely reduced in cls^- larvae. Transverse sections of posterior trunk of (A) wild-type larva at 7 dpf stained for the anti-Hu epitope reveal numerous Hu-positive neurons (arrowhead) around the hindgut (hg). A sibling cls^- larva (B) lacks these cells. In whole-mount hindguts, dissected at 5 dpf, cls^- larvae have only about 13% as many enteric neurons (28.4±10.9; n=7), compared to wild types (212±20.9; n=3). f, ventral fin; m, myotome; me, melanophore. Scale bar, 35 μm.

Fig. 4. DRG sensory neurons are disrupted in cls^- larvae. (A-C) Transverse section of dorsal tail of (A) wild-type larva at 7 dpf stained with anti-Hu antibody reveals small clusters of Hu-positive neurons (arrowhead) in DRG. These are usually absent from the tail of cls^- homozygotes (B). cls^- larvae have an increased frequency of extramedullary cells dorsal to the neural tube (nt; arrow in C and G). (D-G) Lateral views of whole-mount larvae at 3 dpf stained with anti-Hu antibody reveal a segmental pattern of DRGs (arrowheads) throughout the trunk (D) and tail (F), but these cells are reduced and misplaced in the trunk (E) and absent from the tail (G) of cls^- larvae. N, notochord; sc, spinal cord. Scale bar, 25 μm (A-C) and 75 μm (D-G).

Bisgrove and colleagues (1997) suggest the identification of a cluster of c-ret-positive cells in 24 hpf embryos as enteric nervous system precursors, based primarily on their location and the later expression of c-ret in enteric neurons. However, it is perhaps surprising that these cells are apparently unaffected in homozygous *alyron* mutants, which have severe defects in neural crest formation (Cretekos and Grunwald, 1999), so this tentative identification of these cells needs to be confirmed by a lineage-tracing method. In situ hybridisation with c-ret probes of 24 hpf embryos from $cls^{+/-}$ crosses shows this cell cluster to be present in all embryos. Thus these putative enteric nervous system precursors appear to be properly specified, at least with respect to this marker, in the absence of cls function.

Dorsal root ganglion (DRG) sensory neurons are strongly affected in cls^- embryos. Anti-Hu immunostaining showed that wild types had a reproducible series of DRGs as early as 2 dpf, but this pattern was severely disrupted in cls^- larvae (Fig. 4). Posterior trunk and tail DRGs were almost absent (Fig. 4G), whilst in the anterior trunk the number was reduced, the pattern
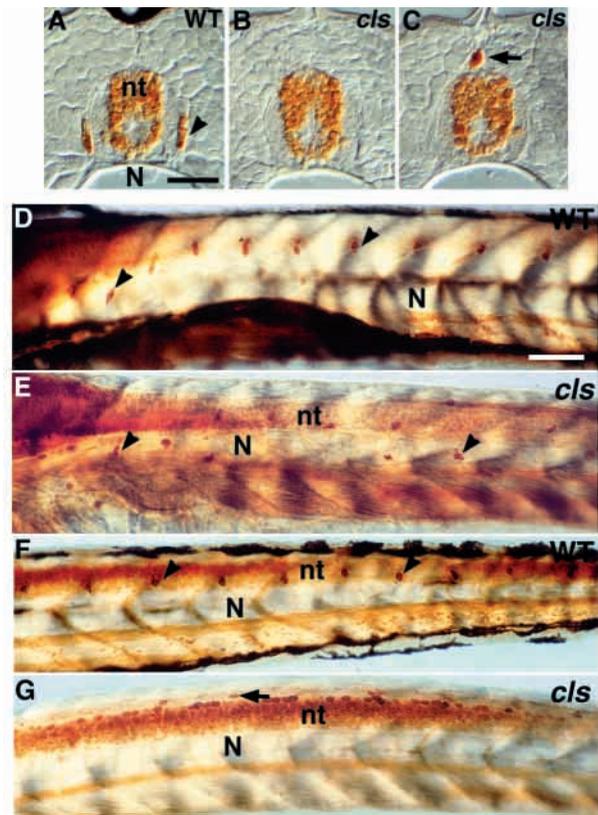
lost bilateral symmetry and positioning relative to the neural tube varied (Fig. 4E; Table 1). Although more posteriorly cls^- larvae had no DRGs, there were occasional anti-Hu-positive cells dorsal to the neural tube (Fig. 4C). These cells are reminiscent of extramedullary cells (EMCs) described in various amphibians and teleosts, including zebrafish (Hughes, 1957; Myers, 1985). EMCs were more abundant in cls^- larvae than in wild types.

Zebrafish sympathetic neurons are also detected by anti-Hu immunostaining and by an antibody to tyrosine hydroxylase (P.

Table 1. DRG numbers are reduced in cls^- homozygotes

	Number of DRG, mean±s.d. (n)*	
	48 hpf	72 hpf
Wild type	56.3±5.3 (8)	56.5±8.24 (7)
cls	24.7±6.5 (13)‡	21.8±4.44 (6)‡

*Counts of DRG neuronal clusters after anti-Hu antibody labeling of whole-mounts. Ganglia from both sides of trunk and tail are included.
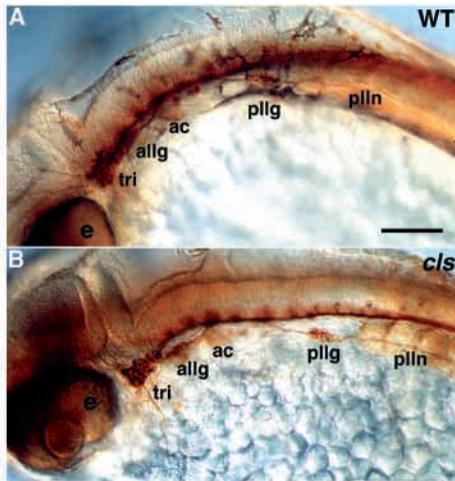‡P<0.01 (t-test).

Fig. 5. Cranial sensory neurons are unaffected in *cls⁻* embryos. Lateral views of whole-mount wild-type (A) and *cls⁻* (B) 24 hpf embryos stained with zn-12 and anti-acetylated tubulin antibodies. e, eye; tri, trigeminal ganglion; ac, acoustic ganglion; allg, anterior lateral line ganglion; pllg, posterior lateral line ganglion; plln, posterior lateral line nerve. Scale bar, 60 μm.

Henion, personal communication). We were unable to detect differentiated sympathetic neurons in sectioned 9 dpf *cls⁻* larvae, although they were readily seen in wild types (data not shown).

Cranial ganglion neurons appear normal in *cls⁻* embryos

To learn whether *cls* gene function is required for development of all peripheral sensory neurons, we examined cranial ganglia which are reported to contain neurons derived from both neural crest and ectodermal placodes (Le Douarin, 1982). The neural crest contribution to cranial ganglia has not been thoroughly investigated in zebrafish, although a contribution to the trigeminal ganglion has been shown by 24 hpf (Schilling and Kimmel, 1994). Both anti-Hu antibody and c-*ret* riboprobes label neurons in developing head ganglia at 24 hpf. We saw no obvious change in ganglia size, position or shape in *cls⁻* embryos compared with wild types using these (data not shown) and other markers, including zn-12 (Trevarrow et al., 1990) and zn-12 plus anti-acetylated tubulin (Chitnis and Kuwada, 1990) (Fig. 5A,B).

Peripheral ganglion glia appear reduced in *cls⁻* embryos

In avian and mammalian embryos, peripheral glia arise from neural crest (Le Douarin, 1982). The *fkd6* gene is expressed initially in premigratory neural crest (Odenthal and Nuesslein-Volhard, 1998) and later in what appear to be cranial ganglion satellite glia and cranial nerve-associated Schwann cells. At 24 hpf, *fkd6* was expressed in peripheral cells of cranial ganglia and cells extending along the posterior lateral line (PLL) nerve (Fig. 6A,C). Based on position and absence of anti-Hu labeling, we suggest these are glial cells (R. N. Kelsh et al., unpublished). *cls⁻* embryos had strongly reduced numbers of *fkd6*-expressing cells associated with cranial ganglia (Fig. 6B,D) and with the PLL and other cranial nerves (data not shown). Thus we propose that *cls* mutations affect glial cell numbers in the cranial PNS.
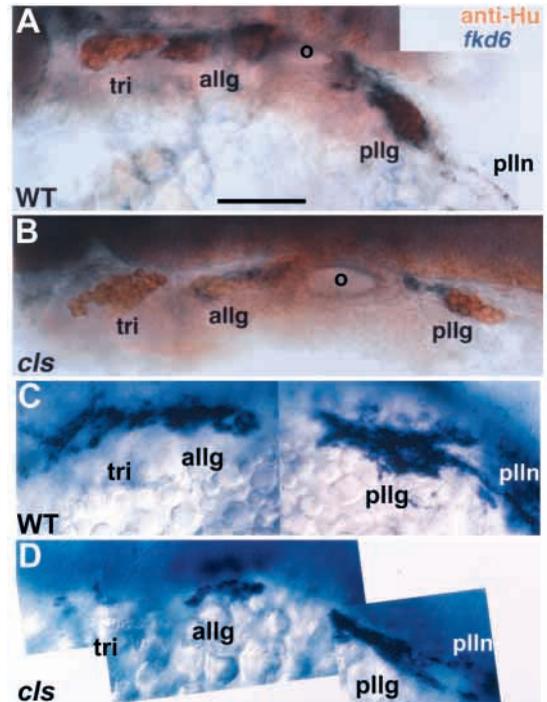

Fig. 6. Putative cranial glia are strongly decreased in *cls⁻* embryos. Lateral views of 24 hpf wild-type (A,C) and *cls⁻* (B,D) embryos labeled for *fkd6* expression (purple) and Hu epitope (orange, A,B only) show tight association of *fkd6* labeling with cranial ganglia. The number of *fkd6*-positive cells associated with cranial ganglia (A,B) and posterior lateral line nerve (C,D) is markedly reduced in *cls⁻* embryos. In A and B, *fkd6* labeling was stopped early to prevent masking of the anti-Hu staining. Abbreviations as Fig. 5. Scale bar, 40 μm.

Enteric glia are nearly absent from *cls⁻* embryos

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed in glia of the CNS (astrocytes and radial glia) and PNS (enteric glia; Jessen and Mirsky, 1980, 1983; Bjorklund et al., 1984; Jessen and Mirsky, 1985). In zebrafish larvae, an anti-bovine GFAP antibody cross-reacts with radial glia in the CNS, putative Schwann cells in the PNS (P. Henion, personal communication) and putative enteric glia in dissected zebrafish gut preparations. Wild-type embryos showed a network of GFAP-positive fibres extending over the gut (Fig. 7A), around and between Hu-positive enteric neurons.
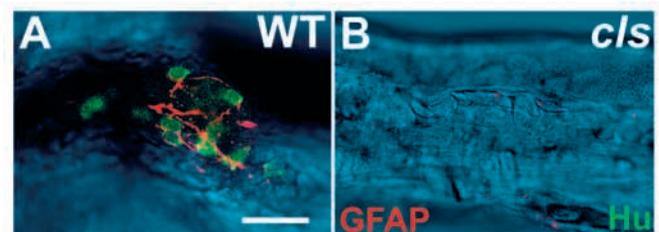

Fig. 7. GFAP-positive enteric glia are largely absent in *cls⁻* larvae. Dissected 5 dpf hindgut preparations stained with anti-Hu (green) and anti-GFAP (red) antibodies clearly reveal the enteric nervous system in the wild type (A) and its essential absence from *cls⁻* siblings (B). Confocal sections of the fluorescent labeling are superimposed on a DIC image of the gut. Scale bar, 20 μm.

Table 2. Wild-type neural crest cells form pigment cells in *cls⁻* host embryos

Experiment no.	No. chimaeras [wild type→*cls⁻*]	No. chimaeras with ectopic pigment cells	No. wild-type melanophores (at 2 dpf) present in chimaeras	Proportion of scored melanophores with label	Proportion of scored iridophores with label	Proportion of scored xanthophores with label
1	3	1	18	17/17	10/10	-
2	2	2	77	44/44	7/8	11/11
3	12	7	23	10/10	18/21	46/46
4	5	2	6	6/6	3/10	48/48
Total	**22**	**12**	**124**	**77/77**	**38/49**	**105/105**

In contrast, most guts from *cls⁻* larvae showed none of these fibres (Fig. 7B), although occasionally short isolated sections of gut were labeled (data not shown). Thus, *cls⁻* larvae have highly reduced or absent enteric glia.

Crest skeletal derivatives are unaffected in *cls⁻* embryos

In view of the widespread defects in all other major crest derivatives, we examined ectomesenchymal derivatives to learn whether they were also affected. *dlx-2,* a putative marker for early branchial arch crest (Akimenko et al., 1994), was expressed normally in *cls⁻* larvae (data not shown). Alcian green staining showed that all wild-type elements of the craniofacial skeleton were present in *cls⁻* embryos at 3-5 dpf (Fig. 8). However, later *cls⁻* embryos showed a slight retardation of arch development.

Neural crest also generates cells with another putatively ectomesenchymal fate in anamniote vertebrates, fin mesenchyme. These cells are prominent in median fins of zebrafish larvae, and at least some median fin mesenchyme cells have been reported to originate from neural crest (Smith et al., 1994; D. W. Raible, personal communication). Fin mesenchyme cells were found in similar positions and numbers in wild-type and *cls⁻* larvae (Fig. 8G,H). Thus both cranial and trunk ectomesenchymal crest derivatives appeared normal in *cls⁻* larvae.

Premigratory neural crest forms normally in *cls⁻* embryos

Since so many neural crest fates were strongly affected in *cls⁻* embryos, we investigated whether premigratory neural crest was reduced. Both *sna-2* (Thisse et al., 1995) and *fkd6* (Odenthal and Nuesslein-Volhard, 1998) have been reported to label premigratory neural crest cells. At both 14-24 hpf (*fkd6*) and 20-24 hpf (*sna-2*) the approximate number, position and intensity of labeling of premigratory crest cells was indistinguishable in mutants and wild type (data not shown).

cls mutations affect all three pigment cell types cell-autonomously

To learn whether *cls* mutations affect non-ectomesenchymal neural crest cells autonomously, we transplanted cells between wild-type and *cls⁻* embryos.

Cells transplanted from labeled wild-type donors into unlabeled *cls⁻* hosts were able to form neural crest and migrate on both medial and lateral pathways (Fig. 9A). Fluorescently labeled, wild-type pigment cells in *cls⁻* hosts developed wild-type pigment cell phenotypes (Figs

9C,D and 10; Table 2), even in close proximity to unlabelled *cls⁻* melanophores (data not shown). Only wild-type cells developed into normal chromatophores in *cls⁻* hosts and *cls⁻* pigment cells were not rescued by nearby wild-type cells. Therefore, the *cls* gene product appears to function cell-autonomously within crest-derived pigment cells.

We predicted that *cls⁻* cells transplanted into the neural crest of wild-type hosts would develop the characteristic *cls⁻* melanophore phenotype. However, this prediction remains untested as we cannot recognise these cells amongst the heavily pigmented wild-type melanophores. As an alternative approach, we transplanted *cls⁻* cells into *alb⁻* hosts (Table 3).
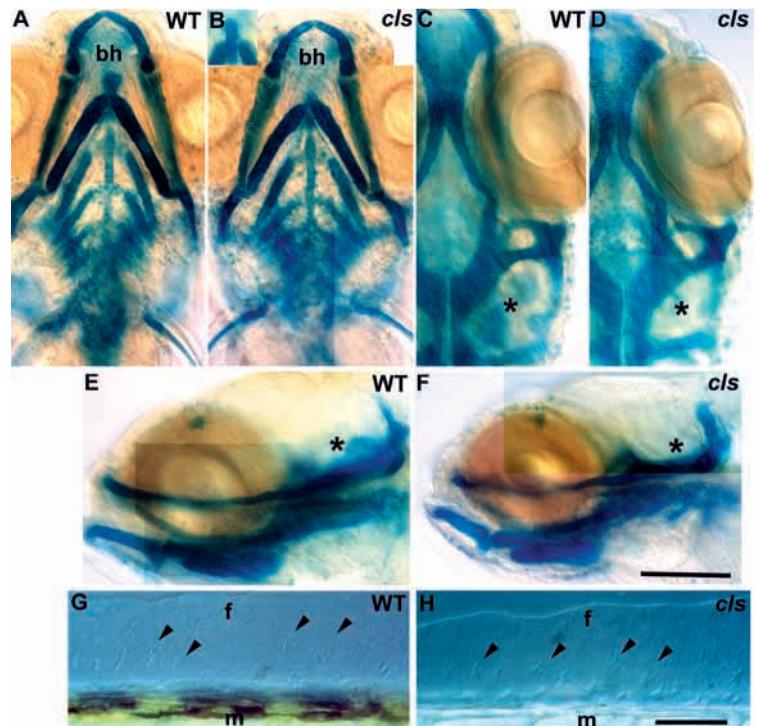

Fig. 8. Ectomesenchymal crest derivatives are essentially normal in *cls⁻* embryos. Alcian green stained craniofacial skeletons of 5 dpf wild-type (A,C,E) and *cls⁻* (B,D,F) larvae are shown in ventral (A,B), dorsal (C,D) and lateral (E,F) views. Mutant craniofacial skeletons are barely distinguishable from wild type, although development is slightly retarded (compare angle of main arch elements in A and B) and the basihyal cartilage is variable (bh in A and B, also inset in B). Ventral (C,D) and lateral (E,F) views show slight differences in the cartilages associated with the developing inner ear (asterisk) which remains small in *cls⁻*. Median fin mesenchyme (arrowheads) of the dorsal fin in lateral view with Nomarski optics in 5 dpf wild-type (G) and *cls⁻* (H) larvae; the number and position of cells is identical. f, medial fin; m, muscle blocks. Scale bar, 100 µm (A-F) and 50 µm (G,H).

Table 3. Wild-type, but not *cls⁻*, neural crest cells form melanophores in *alb⁻* host embryos

Experiment no. (total no. transplants)	No. chimaeras [wild type→*alb⁻*]	No. chimaeras from wild type donor with normally pigmented melanophores (%)	No. wild type melanophores present	Proportion of scored melanophores with label	No. chimaeras [*cls⁻*→*alb⁻*]	No. chimaeras from *cls* donor with normally pigmented melanophores (%)
1 (28)	18	13 (72)	151	15/15	10	0 (0)
2 (12)	12	9 (75)	n.d.	n.d.	0	0 (0)
3 (28)	27	21 (78)	377	25/25	1	0 (0)
Total	**57**	**43 (75)**	**528**	**40/40**	**11**	**0 (0)**

n.d., not determined

Fig. 9. Wild-type→*cls⁻* chimaeric embryos show enhanced numbers of pigment cells. Wild-type donor cells labeled with rhodamine dextran (red) and transplanted into an unlabeled *cls⁻* host embryo at shield stage frequently contributed to neural crest. Three cells are seen here migrating past the notochord (no) at 24 hpf (A). Some chimaeric *cls⁻* embryos show many (C) or few (D) melanophores by 2 dpf; all such embryos are clearly distinguishable from wild types (B). (D) All these melanophores have a wild-type appearance [compare wild-type melanophore (arrowhead) and mutant melanophore (asterisk)]. A-C, lateral views; D, dorsal view of posterior head. nt, neural tube. Scale bars, 50 μm (A) and 250 μm (B-D).
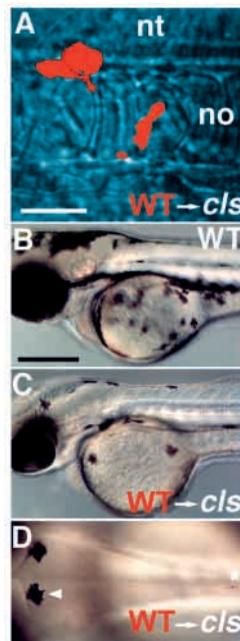

Fig. 10. *cls* acts cell-autonomously in all three pigment cell fates. Chimaeric embryos generated by transplanting rhodamine-dextran labeled wild-type cells into *cls⁻* hosts show ectopic iridophores (A), melanophores (C) and xanthophores (E). These chromatophores are entirely derived from the labeled donor (red; B,D,F). A and C are views with Nomarski optics, B and D are superimposed Nomarski and fluorescence views. A labeled xanthophore shows its characteristic autofluorescence (E) and rhodamine fluorescence (F). vf, ventral fin; nt, neural tube; no, notochord. Scale bars, 50 μm (A-D) and 25 μm (E,F).
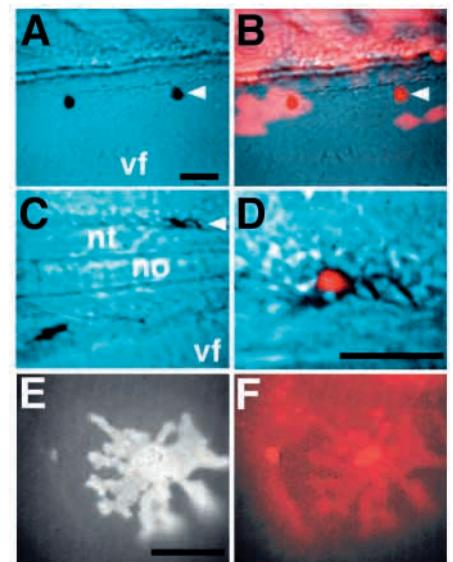
alb⁻ embryos have a full complement of melanophores, based on *dct* in situ hybridisation (R. N. Kelsh et al., unpublished), but do not become pigmented (Chakrabarti et al., 1983; Streisinger et al., 1986). Wild-type cells transplanted into *alb⁻* hosts often formed normal melanophores, but did not rescue the nonpigmented phenotype of *alb⁻* mutant melanophores (data not shown). Since nearly 75% of wild type→*alb⁻* chimaeras had at least one melanophore, we clearly targeted wild-type cells to the neural crest of *alb⁻* hosts at high frequency. In contrast, we never saw any melanophores with wild-type pigmentation when *cls⁻* cells were transplanted into *alb⁻* hosts, consistent with the conclusion that *cls* mutations act cell-autonomously. However, somewhat surprisingly, we did not see melanophores with the distinctive *cls⁻* phenotype in these *alb⁻* hosts.

DISCUSSION

cls affects non-ectomesenchymal, but not ectomesenchymal, crest derivatives

Here we demonstrated an unexpectedly broad, but still selective, requirement for the *cls* gene during neural crest development. We confirmed previous findings (Kelsh et al.,

Fig. 11. Model of *cls* gene function. The *cls⁻* defects are consistent with an early segregation of precursors for ectomesenchymal and non-ectomesenchymal derivatives. We indicate multipotent neural crest stem cells (NCSC) generating progeny restricted to either ectomesenchymal (E) or non-ectomesenchymal (NE) fates. These then produce progeny restricted to individual fates within each class: craniofacial (C) or fin mesenchyme (F); pigment cells (P), neurons (N) or glia (G). *cls* gene function is necessary for some aspect of normal development of non-ectomesenchymal, but not ectomesenchymal, progenitors. The broad arrows indicate that we do not know whether a series of intermediate progenitors may exist.
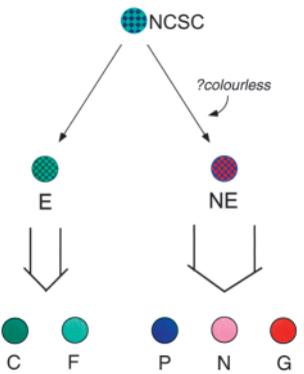
1996) that *cls* mutations affect pigment cell fates prior to migration, and showed that they severely reduce sensory, sympathetic and enteric neurons and enteric and putative cranial glia. In contrast, craniofacial skeletal derivatives and

median fin mesenchyme are initially unaffected in *cls*⁻ embryos. In view of the significant contribution of neural crest to the cranial ganglia in mammalian and avian embryos (Le Douarin, 1982), we expected significant reduction in the neuronal populations of at least some cranial ganglia in *cls*⁻ embryos. We suggest that the lack of defects at 24 hpf reflects a predominantly ectodermal placode derivation for at least these early neurons, although we cannot rule out later contributions from undifferentiated neural crest cells. However, gross abnormalities of the posterior lateral line ganglion are not visible even in 5 dpf *cls*⁻ mutant larvae. Previous studies have shown that some cranial ganglion neurons are crest-derived (Schilling and Kimmel, 1994; Dutton and R. N. K., unpublished), but the relative contributions of ectodermal placode and neural crest to these ganglia is still unknown. Clearly, much work is required to clarify the origins of zebrafish cranial neurons, before this surprising result can be fully explained.

We propose that the *cls*⁻ phenotype reflects an early segregation between ectomesenchymal and non-ectomesenchymal crest precursors in which *cls* gene function is required only for correct development of non-ectomesenchymal precursors (Fig. 11). Non-ectomesenchymal precursors generate neuronal, glial and/or pigment cells, but not ectomesenchymal cells. The *cls*⁻ phenotype provides genetic evidence consistent with the suggestion that ectomesenchymal fates segregate early from other crest fates (Weston, 1991; Le Douarin et al., 1994). One prediction of our hypothesis is that trunk crest-derived ectomesenchymal fates segregate early from non-ectomesenchymal fates, probably before neural crest migration. Zebrafish crest lineage studies have not yet examined segregation of the ectomesenchymal lineage (Schilling and Kimmel, 1994; Smith et al., 1994). Similarly, we are not aware of any mutant with defects in both crest-derived ectomesenchymal fates. *chinless*, the best-studied craniofacial mutation, is not reported to affect median fin mesenchyme (Schilling et al., 1996b). However our model is supported by recent studies showing that altering activity of *Wnt* signalling in zebrafish non-ectomesenchymal cranial crest cells changes their fate, but altering *Wnt* signalling in cartilage precursors does not affect their fate (Dorsky et al., 1998).

Although we propose early segregation of ectomesenchymal and non-ectomesenchymal precursors, we cannot rule out two other possibilities. First, that single fate precursors are directly specified from unrestricted neural crest cells. In this model, the *cls* gene product would be required in all precursors that generate neuronal, glial or pigment cell derivatives. Second, that there is no segregation of ectomesenchymal and non-ectomesenchymal precursors. Instead, crest cells might be specified with a wide variety of partially restricted but overlapping potentials, as proposed by Le Douarin and colleagues in avian crest (Le Douarin, 1986; Baroffio et al., 1988, 1990; Le Douarin, 1990; Baroffio et al., 1991; Le Douarin et al., 1991). In this model, the *cls* gene product would be necessary for generation of *any* non-ectomesenchymal fate from all these various progenitors.

cls mutations as a model for human Waardenburg-Shah syndrome

The *cls* mutant phenotype is reminiscent of mouse pigmentation mutants with defects in both melanocytes and enteric nervous system (Lane and Liu, 1984; Baynash et al., 1994; Hosoda et al., 1994; Southard-Smith et al., 1998). The milder defects in *s* and *ls* mutants suggest that these genes function only in melanoblasts and enteric nervous system precursors. In contrast, the more extreme phenotype of *Dom* homozygotes implies an earlier function in a wider range of derivatives (Southard-Smith et al., 1998). *cls*⁻ embryos show wide-ranging neural crest defects reminiscent of *Dom* homozygous mutants and so *cls* gene function is also likely to act early in crest development. Whilst *cls*⁻ mutants lack enteric neurons along the entire extent of the gut, *s* and *ls* homozygous mutants show aganglionosis only in the posterior gut. This aspect of the phenotype is most reminiscent of *c*-ret mutants, which show aganglionosis throughout the gut, but which have no pigment defects (Schuchardt et al., 1994); and *Dom* homozygous mutants. However, a major difference between *cls* and *Dom* homozygous mutant phenotypes is the severe otic vesicle defect in *cls*⁻ embryos; inner ear defects have not been described for *Dom* homozygotes. It will be interesting to know whether *cls* encodes a zebrafish homologue of any of these mice loci or whether it defines a new gene involved in neural crest development. We are currently testing these ideas. Mice with mutations in *s*, *ls* and *Dom* are all models for human Hirschsprung's disease, characterised by aganglionosis of the colon, and Waardenburg-Shah syndrome, in which colonic aganglionosis is associated with pigmentary defects (Passarge, 1973; Badner and Chakravarti, 1990; Dow et al., 1994; Attié et al., 1995; Edery et al., 1996; Hofstra et al., 1996; Southard-Smith et al., 1998). We propose that *cls* mutations are a zebrafish model for these diseases. Given the ease of in vivo lineage analysis (Raible and Eisen, 1994), we believe that this new model will contribute significantly to our understanding of Hirschsprung's disease and Waardenburg-Shah syndrome.

What is the function of the *cls* gene?

Importantly, *cls* gene function is not required for formation of neural crest per se, since premigratory crest markers are expressed normally in *cls*⁻ embryos. A priori, *cls* might function in specification, proliferation, survival, or differentiation of non-ectomesenchymal neural crest precursors. The observations described here are consistent with possible roles in specification, proliferation and differentiation, and we are accumulating evidence for a possible role in survival (K. Dutton and R. N. K., unpublished observation). Further studies should clarify which of these processes is primarily affected in *cls*⁻ mutants.

The earliest defects that we see in *cls*⁻ embryos are present at 21 hpf or shortly thereafter. Thus, *dct*-expressing melanoblasts are reduced in number as early as 21 hpf and lateral path neural crest cell migration is defective at 24 hpf. The eventual fate of these pigment cell precursors is unclear. Some differentiate into the residual population of pigment cells present in *cls*⁻ mutants, but others presumably die or change fate. Future studies of crest cells, including clonal analysis and investigations of cell death, should distinguish these possibilities.

cls⁻ embryos show a migration defect for crest-derived pigment cell precursors and possibly DRG neurons (Kelsh et al., 1996; this work). However, a primary defect in migration cannot fully explain the *cls*⁻ phenotype since such a role would predict that crest derivatives differentiating near their origin,

for instance melanophores in the dorsal pigment stripe, would develop normally. However, this is the location of the few melanophores seen in *cls⁻* mutants, yet even here none differentiate normally. Conversely, our c-*ret* studies provide evidence that migration of putative enteric nervous system precursors may be normal until at least 24 hpf, although a contribution of migratory difficulties to the later enteric neuron phenotype cannot be ruled out. Thus, we suggest that migratory defects are a secondary effect of the *cls* mutation.

Finally, whatever its role, we have demonstrated that the *cls* gene product acts cell-autonomously, at least in pigment cell precursors. The cell-autonomy of *cls* gene action in other crest fates remains to be tested. Although we expect *cls* to function cell-autonomously in all non-ectomesenchymal crest derivatives, autonomy in pigment cells and non-autonomy in enteric neurons is proposed for the *s* gene in mice (Mayer, 1965; Kapur et al., 1995) and may be the case here as well.

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