Essential role for *puma* in development of postembryonic neural crest-derived cell lineages in zebrafish

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Abstract

Multipotent neural crest stem cells have been identified in late gestation amniote embryos. Yet, significant questions remain about the mechanisms by which these cells are generated, maintained, and recruited during postembryonic development. The zebrafish, *Danio rerio*, offers an opportunity to identify genes essential for these processes, by screening for mutants with defects in traits likely to depend on these cells during metamorphosis and adult life. One such trait is the pigment pattern formed by neural crest-derived pigment cells, or chromatophores, which include black melanophores, yellow xanthophores, and iridescent iridophores. Previous analyses have demonstrated that the adult zebrafish pigment pattern depends on the de novo differentiation of latent precursor cells during both early and late phases of pigment pattern metamorphosis. To better understand the development of these cells, in this study, we analyze the zebrafish *puma* mutant, which ablates most of the adult melanophores that differentiate during metamorphosis, but leaves intact early larval melanophores that differentiate during embryogenesis. We use epistasis analyses to show that *puma* promotes the development of both early-appearing metamorphic melanophores that depend on the kit receptor tyrosine kinase, as well as late-appearing metamorphic melanophores that depend on both the G-protein-coupled endothelin receptor B1 (ednrb1) and the kit-related fms receptor tyrosine kinase. We further demonstrate that, during pigment pattern metamorphosis, *puma* mutants have deficiencies in the numbers of cells expressing transcripts for kit, ednrb1, and fms, as well as the HMG domain transcription factor sox10. Because the *puma* mutant phenotype is temperature-sensitive, we use temperature-shift experiments to identify a critical period for *puma* activity during pigment pattern metamorphosis. Finally, we use cell transplantations to show that *puma* acts cell-autonomously to promote the expansion of pigment cell lineages during metamorphosis. These results suggest a model for the lineage diversification of neural crest stem cells during zebrafish postembryonic development.

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Introduction

Much of vertebrate diversity can be traced to differences in the patterning of neural crest-derived cells. In the early vertebrate embryo, neural crest cells arise along the dorsal neural tube and then disperse widely to generate an array of derivative cell types and tissues. These include pigment cells in the skin, glia and neurons of the peripheral nervous system, bone of the craniofacial skeleton, smooth muscle, endocardial cushion cells, endocrine cells, and others (Hall, 1999; Hörstadius, 1950; LeDouarin, 1982). Indeed, many of the shared derived characters of vertebrates have neural crest origins (Gans and Northcutt, 1983). In recent years, we have seen dramatic advances in our understanding of how embryonic neural crest cells become specified for particular fates (e.g., Dorsky et al., 1998; Henion and Weston, 1997; Jin et al., 2001), the morphogenetic consequences of such fate specification (Erickson and Goins, 1995; Reedy et al., 1998; Santiago and Erickson, 2002), and the factors that allow specified cells to complete their process of differentiation (Leimeroth et al., 2002; Paratore et al., 2002; Rawls and Johnson, 2001).

While it is clear that many embryonic neural crest cells terminally differentiate not long after leaving their origin at the dorsal neural tube (Le Douarin, 1984; Raible and Eisen,
1994), or even before dispersing from this location (Epperlein and Löfberg, 1990), there is increasing evidence that some neural crest-derived cells maintain their pluripotency even into postembryonic stages. In mammalian embryos, pluripotent neural crest-derived stem cells now have been isolated from various elements of the peripheral nervous system, and it appears that such cells may be widely distributed during fetal development and perhaps even in the adult animal (e.g., Bixby et al., 2002; Hagedorn et al., 1999; Kruger et al., 2002; Morrison et al., 1999; Stemple and Anderson, 1992). Stem cells also have been isolated that specifically contribute to postembryonic melanocytes (Nishimura et al., 2002). These findings have clear biomedical implications, as unregulated proliferation of such stem cells could contribute to tumorigenesis, whereas harnessing their developmental potential could allow new therapeutic strategies to treating human disease (Reya et al., 2001). In a phylogenetic context, such cells are especially intriguing as well, since many differences among species are manifested only late in development, and it is conceivable that some of this variation can be ascribed to evolutionary changes in how neural crest stem cells are deployed to particular pathways of differentiation even after embryogenesis. Thus, elucidating the mechanisms by which postembryonic neural crest stem cells are generated, maintained, and recruited to various fates is of paramount importance for both developmental and evolutionary research. Of particular interest will be the extent to which these mechanisms are shared with neural crest cells that differentiate during embryogenesis, and whether or not novel gene activities and cellular behaviors contribute to the development of neural crest stem cells during postembryonic stages.

For elucidating the genes and cell behaviors of vertebrate embryonic development, the zebrafish Danio rerio has proven to be a useful model organism, as forward genetic screens have identified a large number of mutants affecting a variety of traits. Despite its advantages for genetic and other analyses, this species remains underexploited as a model for postembryonic development. In the context of understanding neural crest stem cells, the zebrafish offers an opportunity to identify genes required for the development of these cells at postembryonic stages. Furthermore, if postembryonic neural crest stem cells depend on different gene activities than embryonic neural crest cells, then it should be possible to isolate mutants affecting one or another population but not both. Zebrafish also can be used to identify genes having essential roles during both embryogenesis and later development, as these fish develop over a relatively wide range of temperatures so that conditional, temperature-sensitive mutants can be isolated: individuals can be reared at a permissive temperature to bypass lethality of genes that have essential roles at embryonic stages, whereas shifting to a restrictive temperature during later development can reveal essential functions for the same genes during the larval-to-adult transition or beyond (Johnson and Weston, 1995).

One class of neural crest derivative for which it should be possible to dissect embryonic vs. postembryonic genetic requirements is the complement of pigment cells, or chromatophores, that generate the early larval and adult pigment pattern. During embryogenesis, a population of neural crest cells differentiates to form three major types of chromatophores: black melanophores, yellow xanthophores, and iridescent iridophores. These embryonic chromatophores generate a relatively simple early larval pigment pattern that consists of several well-defined stripes of melanophores and iridophores with widely dispersed xanthophores that cover much of the body (Kimmel et al., 1995). This embryonic/early larval pigment pattern persists through hatching and remains relatively unchanged during the first 2 weeks of development, though a few additional melanophores arise during this time (Kirschbaum, 1975; Milos and Dingle, 1978a,b; Milos et al., 1983). Subsequently, this pigment pattern is transformed into the considerably more complex pattern of the adult fish, which consists of alternating dark and light horizontal stripes (Fig. 1A). The adult dark stripes comprise principally melanophores and iridophores, whereas light stripes comprise principally xanthophores and iridophores.

The processes by which the zebrafish adult pigment pattern forms have been studied at the genetic and cellular level. Of importance for understanding the biology of neural crest stem cells, it is clear that some of the melanophores comprising the adult pigment pattern differentiate de novo from unmelanized precursors during the stages of pigment pattern metamorphosis (Johnson et al., 1995; Kirschbaum, 1995; Parichy et al., 1999; Parichy and Turner, 2003b). The onset of this metamorphosis is marked by the appearance of new melanophores over the flank in regions not previously occupied by these cells (ca. 2 weeks postfertilization). Some of these melanophores migrate into the positions of the future adult stripes, with the first two stripes (1D, 1V in Fig. 1A) forming just dorsal and ventral to the horizontal myoseptum. Melanophores arising during this early phase of pigment pattern metamorphosis depend on the kit receptor tyrosine kinase, as kit mutants lack this population of cells (Johnson et al., 1995; Parichy et al., 1999). Roles for Kit and its ligand have long been studied in amniote melanocyte development (e.g., Kunisada et al., 1998; Mayer and Green, 1968; Wehrle-Haller et al., 2001), though zebrafish differ from mammals in that even null alleles of zebrafish kit retain a population of kit-independent melanophores that differentiate already in the positions of adult stripes and generate a partial pattern of adult stripes (Fig. 2C).

Beginning during middle stages of pigment pattern metamorphosis in wild-type individuals (ca. 3 weeks postfertilization), additional melanophores appear within the developing adult primary melanophore stripes (Johnson et al., 1995). Melanophores arising during this phase of pigment pattern metamorphosis are likely to correspond to the same
population of cells that arises at this time and place in kit mutants. The development of these cells depends on the seven-pass G-protein-coupled receptor endothelin receptor b1 (ednrb1), which has long been studied for its role in melanocyte development in mammals (Mayer, 1965; Parichy et al., 2000b; Shin et al., 1999; Yoshida et al., 1996), as well as the kit-related receptor tyrosine kinase, fms, which has previously been known for roles in hematopoiesis and osteoclastogenesis (Dai et al., 2002; Marks and Lane, 1976; Parichy et al., 2000a; Tagoh et al., 2002). Thus, ednrb1 and fms mutant zebrafish each lack the late-appearing population of metamorphic melanophores. Nevertheless, each of these mutants retains a residual population of ednrb1- and fms-independent melanophores that generates an incomplete pattern of stripes and spots in the adult (Fig. 2E and G). Thus, the timing of melanophore appearance suggests that two populations of metamorphic melanophores contribute to the adult stripe pattern in zebrafish: an early-appearing population that depends on kit, and a later-appearing population that depends on ednrb1 and fms. Further evidence that early-appearing and late-appearing metamorphic melanophores are genetically separable populations comes from epistasis analyses, in which kit and either ednrb1 or fms mutations were shown to have additive effects on melanophore numbers: fish doubly mutant for either kit and ednrb1, or kit and fms lack virtually all body melanophores (e.g., Fig. 2I), whereas fish doubly mutant for ednrb1 and fms do not exhibit more severe melanophore deficiencies than either single mutant (Johnson et al., 1995; Parichy et al., 2000a). Taken together, these and other analyses (Parichy and Turner, 2003a; Rawls and Johnson, 2001) strongly suggest that undifferentiated precursors contribute to the zebrafish adult pigment pattern, and these precursors may be separable into distinct classes that generate early- vs. late-appearing populations of metamorphic melanophores. Nevertheless, it has not been known whether precursors to these different populations of metamorphic pigment cells share common genetic requirements, or the extent to which requirements by these precursors differ from precursors that differentiate as melanophores during embryonic stages.

In this study, we use the puma mutant to investigate the development of metamorphic melanophore precursors in zebrafish. Here, we use genetic analyses to show that puma promotes the development of both early-appearing (kit-dependent, ednrb1- and fms-independent) metamorphic melanophores as well as late-appearing (kit-independent, ednrb1- and fms-dependent) metamorphic melanophores. We also demonstrate that puma mutants have deficiencies in the numbers of cells expressing each of these three loci. Furthermore, we show that the puma locus acts autonomously to pigment cell lineages during the stages of pigment pattern metamorphosis to expand the population of these precursors, as well as cells expressing sox10, which has essential roles in generating pigment cell and glial lineages during embryogenesis (Dutton et al., 2001). Our analyses reveal an essential role for puma in the development of neural crest-derived lineages during metamorphosis and highlight the utility of the zebrafish for prospectively identifying genes that contribute to the generation, maintenance, and recruitment of postembryonic neural crest cell lineages.

Materials and methods

Fish stocks and rearing conditions

Wild-type stocks were of the largely inbred University of Texas isolate of AB, AB\textsuperscript{UT}. All pigment pattern mutants are maintained in this genetic background. puma was isolated by N-ethyl-N-nitrosourea mutagenesis (Parichy and Johnson, 2001; Solnica-Krezel et al., 1994). The pigment pattern
mutants kit<sup>b5</sup>, fms<sup>4el</sup>, and ednr<sub>B</sub><sup>b40</sup> have been described (Parichy et al., 1999, 2000a,b); all behave as null alleles relative to the pigment pattern. All fish were maintained at 28.5°C (14L:10D), except for temperature shift experiments that were conducted at 24 and 33°C.

**Temperature shift experiments**

To assess critical periods for *puma* activity, we reared fish in small groups at either 24 or 33°C to a common stage of development at which wild-type fish had developed a juvenile/early adult pigment pattern [equivalent to ∼36 days postfertilization (dpf) at standard temperature]. Individuals were shifted between temperatures either at an early larval stage (~1 day after the stage of first feeding) or during middle stages of pigment pattern metamorphosis (equivalent to 18–20 dpf at standard temperature). By the times of data collection, fish had reached a size of ∼16.6 mm standard length; nevertheless, final sizes differed somewhat across genotypes and treatments (genotype × treatment interaction, $F_{5,148} = 8.61, P < 0.0001$), with *puma* mutants maintained at 33°C or shifted to 33°C having slightly smaller sizes on average than *puma* mutants at 24°C (mean standard lengths ± SD at 33°C, 24°C = 14.7 ± 2.44 mm, 16.5 ± 2.78 mm; Tukey post hoc means comparisons, all $P < 0.05$). To assess pigment pattern development in these fish, melanophores were counted that overlapped each of two dorsal–ventral transects, at the anterior and posterior margins of the anal fin (Parichy and Johnson, 2001). Preliminary statistical analyses of these transects did not identify differences between locations, so subsequent analyses were performed by using the mean of each location as the estimate of linear melanophore density in each individual. This measure is highly correlated with other measurements of melanophore numbers. All comparisons of mean melanophore numbers, below, are based on analyses of covariance that controlled for variation in final size among individuals. Since the *puma* mutant phenotype depends in part on growth rate (Parichy and Turner, 2003b), this standardization controls for individual growth rate variability and provides for conservative tests for the effects of temperature treatment. Analyses of temperature effects that did not explicitly control for variation in size yielded equivalent results, though temperature effects on melanophore densities were greater (i.e., less conservatively estimated) than in analyses that controlled for size variation (data not shown).

**In situ hybridization**

Probes for pigment cell precursors included *kit*, *fms*, and *ednr<sub>B</sub>* (Parichy et al., 1999, 2000a,b). Probes for *sox10* and *foxd3* were generously provided by R.N. Kelsh and P. Henion, respectively. Larvae examined by in situ hybridization were reared at the standard temperature (28.5°C) as mortality could be considerably higher for all genotypes at the restrictive temperature used in temperature shift experiments. For mRNA in situ hybridization of larvae, standard protocols (2–3 day total times; e.g., Jowett and Yan, 1996) were modified to include longer incubations at each step (11–14 day total times). In brief, fish were fixed ~30 min in 4% paraformaldehyde, 1% dimethylsulfoxide (DMSO) in phosphate-buffered saline (PBS). After removing heads and tail tips to facilitate reagent penetration, larvae were fixed overnight at 4°C. Larvae were then transferred to 100% methanol overnight at ~20°C, then rehydrated through a series of methanol/PBS + 0.1% Tween 20 (PBST) washes. Larvae were then treated with 20 μg/ml proteinase-K, 1% DMSO in PBST for 30 min, postfixed for 20 min in 4% paraformaldehyde/PBST, washed in PBST, and prehybridized overnight at 68°C in a solution containing 50% formamide, 12.5 × SSC, 50 μg/ml RNA, 50 μg/ml heparin, 9.2 mM citric acid, and 0.1% Tween 20. Hybridizations were performed for ~48 h at 68°C by using digoxigenin-labeled riboprobes hydrolyzed to a predicted average length of 300 nucleotides and diluted 1:50–1:300 in hybridization solution. After initial washes, stringency washes were done in 0.2 × SSC, 0.01% Tween 20 at 68°C. Larvae were blocked overnight at 4°C in 5% heat-inactivated sheep serum, 2 mg/ml bovine serum in PBST, and were then incubated for ~48 h at 4°C in anti-digoxigenin alkaline phosphatase-conjugated Fab fragments (Roche) diluted 1:5000 in blocking solution. Larvae were washed with repeated changes of PBST for 48 h, transferred to alkaline phosphatase (AP) buffer (100 mM Tris, pH 9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl<sub>2</sub>, 0.1% Tween 20), and stained in NBT, BCIP in AP buffer with repeated changes for 12–96 h. Larvae were then fixed and washed in PBST. For whole-mount examination, larvae were blocked in 50% glycerol/50% PBST then 100% glycerol, and mounted on glass slides in 100% glycerol, 0.01% sodium azide. For examination in sections, larvae were incubated for several hours in 30% sucrose, embedded in OCT, and sectioned at 16 μm on a Microm 505E cryostat.

**Immunohistochemistry and histology**

For immunohistochemistry, larvae were euthanized, decapitated, fixed in Dent’s fix (80% methanol, 20% DMSO) overnight at 4°C, then bleached overnight in Dent’s bleach (70% methanol, 20% DMSO, 10% H<sub>2</sub>O<sub>2</sub>). Larvae were then rehydrated stepwise to PBST, equilibrated in 30% sucrose, 1% DMSO, frozen in OCT, and sectioned at 16 μm. Sections were collected on Superfrost Plus slides, air dried, rehydrated in PBS, postfixed with 4% paraformaldehyde, washed in PBST, then incubated in 100 mM glycine. Slides were blocked with an avidin/biotin blocking kit (Vector Laboratories) according to the manufacturer’s instructions. To stain for neurons and neurites, slides were then incubated with monoclonal anti-acetylated tubulin (Clone 6-11B-1; Sigma) diluted 1:400 for 2 h at room temperature,
washed, and incubated with biotinylated goat anti-mouse IgG antibody (Sigma) diluted 1:200 for 1 h at room temperature. Antibodies were then detected by using the Vectastain Elite ABC kit and DAB (Vector Laboratories) according to the manufacturer’s instructions. For double labeling with *sox10* riboprobe and acetylated tubulin, larvae were processed through the whole-mount in situ hybridization procedure (above), equilibrated in 30% sucrose, then processed for immunohistochemistry in sections as described.
Cell transplantation

Cell transplantations were performed between 3.3 and 3.8 h (Kimmel et al., 1995) on embryos by using a Narishige IM-9B micrometer-driven microinjection apparatus mounted on a Narishige micromanipulator. For operations, embryos were placed in wells formed in agar-lined dishes containing 10% Hanks solution (Westerfield, 1993) plus 1% penicillin/streptomycin. Typically 50–100 blastomeres were transplanted per embryo. To identify donor cells in host backgrounds, we used donors that were homozygous transgenic for GFP under the control of the β-actin promoter, which expresses ubiquitously across cell types (generously provided by K. Poss). We identified GFP+ donor cells under epifluorescent illumination using an EGFP filter set on a Zeiss Axioplan 2i microscope. To prevent melanin from quenching GFP fluorescence in melanophores, we treated fish with 2–3 drops of 10 mg/ml epinephrine prior to viewing, thereby causing melanin-containing melanosomes to be contracted toward cell centers; GFP fluorescence could then be clearly observed in cell peripheries. Individuals completely lacking GFP+ cells, or comprising greater than ~50% GFP+ cells, were discarded and are not included in the analyses below; typically, however, chimeras exhibited relatively low percentages of donor cells (<25%) that were often distributed widely in the adult fish with patches of donor cells comprising only one or a few cell lineages (see below).

Results

Essential role for puma in development of both early-appearing and late-appearing metamorphic melanophores

Epistasis analyses have suggested that genetically and temporally distinct populations of metamorphic melanophores contribute to the zebrafish adult pigment pattern: early-appearing melanophores dependent on kit, and late-appearing melanophores, dependent on ednrbl and fms (Johnson et al., 1995; Parichy et al. 1999, 2000a,b; reviewed in Quigley and Parichy, 2002). To test whether puma promotes the development of one or another of these populations, we asked whether double mutants for puma; kit, ednrbl, and puma; fms exhibit additional melanophore deficits as compared with single mutants for kit, ednrbl, and fms, respectively (each of which exhibits a null phenotype with respect to the pigment pattern; Parichy et al., 1999, 2000a,b). These analyses confirmed an essential role for puma in promoting the development of both early- and late-appearing melanophores.

Evidence that late-appearing metamorphic melanophores depend on puma comes from crosses between puma mutants and kit mutants, which lack early-appearing metamorphic melanophores and retain only late-appearing melanophores (Fig. 2C). Homozygous puma; kit double mutants lacked virtually all melanophores at early adult stages, resulting in a highly significant difference in melanophore density as compared with kit single mutants (Fig. 2C and D and 3). Thus, residual late-appearing metamorphic melanophores in kit mutants depend on puma for their development.

Evidence that early-appearing metamorphic melanophores require puma comes from crosses between puma mutants and ednrbl or fms mutants (Fig. 2E and G), which lack most late-appearing melanophores and instead exhibit residual early-appearing melanophores. Fish homozygous doubly mutant for either puma; ednrbl or puma; fms exhibited significant reductions in melanophore densities as compared with either ednrbl or fms single mutants alone (Figs. 2E–H and 3). These reductions also were accompanied by differences in melanophore patterning. ednrbl and fms single mutants each exhibit partial adult primary melanophore stripes, though this striped pattern can grade into spots, especially posteriorly and ventrally (Fig. 2E and G). By contrast, puma; ednrbl or puma; fms double mutants lacked even these residual adult primary melanophore stripes. Instead, only large early larval melanophores persisted in the middle of the flank (Fig. 2F and H), though numerous dorsal scale-associated melanophores were present as well, suggesting that this population is relatively less affected by the puma mutation. Together, these results suggest that puma promotes the development of late-appearing fms- and ednrbl-dependent metamorphic melanophores as well as the numbers and organization of early-appearing kit-dependent metamorphic melanophores.
Although *puma* mutants exhibit severe melanophore deficits through middle metamorphic stages, substantial numbers of melanophores are ultimately recovered during terminal stages of pigment pattern metamorphosis and during juvenile development (Parichy and Turner, 2003b). We reared *puma*; *kit* double mutants past the stages shown in Fig. 2D to determine whether these fish also could recover melanophores during later development, despite the near total absence of these cells at earlier stages. By 3 months postfertilization, *puma*; *kit* double mutants regained substantial numbers of melanophores (Fig. 2J), though melanophore numbers never approached those seen for either *puma* or *kit* single mutants. Given the near total absence of these cells in *puma*; *kit* double mutants at earlier stages, this result is consistent with the de novo differentiation of melanophores during later adult development. Together, these results indicate that, although *puma* promotes the development of both early- and late-appearing metamorphic melanophores, a larger population of melanophores develops at least partially independently of *puma* and *kit*.

**Requirement for *puma* during pigment pattern metamorphosis**

Epistasis analyses and imaging (Parichy and Turner, 2003b) indicate that *puma* is likely to be essential for expanding a population of melanophores or their precursors that will contribute to adult stripes. This possibility suggests two alternatives for how *puma* might promote the development of these cells: (1) *puma* could be required exclusively during pigment pattern metamorphosis to promote the expansion of melanophore populations; or (2) *puma* could be essential only during embryonic stages to expand a population of precursor cells that are later recruited to form the adult pigment pattern. To distinguish between these possibilities, we used temperature-shift experiments, as the *puma* mutant phenotype is temperature-sensitive at adult stages (Fig. 4; though not at embryonic/early larval stages; Parichy and Turner, 2003b).

We predicted that if *puma* is essential through metamorphosis for promoting normal melanophore numbers, then shifting to a restrictive temperature at the early larval stage prior to metamorphosis, or during metamorphosis, should result in significantly fewer melanophores than control fish reared at 24°C. Nevertheless, mean melanophore numbers did not differ detectably between *puma* mutants upshifted as either early larvae or during metamorphosis (*F*<sub>1,18</sub> = 0.001, *P* = 0.97). Thus, *puma* is essential during pigment pattern metamorphosis for promoting increased numbers of melanophores.

To further test whether a critical period for *puma* exists during metamorphosis, we reared fish at 33°C and then downshifted them at early larval or metamorphic stages to 24°C. We predicted that if *puma* is required throughout metamorphosis to expand a population of latent precursor cells that generates the adult pigment pattern, then individuals downshifted to the permissive temperature at any time during metamorphosis should recover additional melanophores, as compared with individuals reared at 33°C throughout development. If *puma* is required strictly during embryogenesis, then downshifting after this time should not rescue additional melanophores. Fig. 5B shows results from temperature downshift experiments. In wild-type individuals, melanophore densities did not differ significantly between treatments (*F*<sub>2,29</sub> = 0.90, *P* = 0.4). In *puma* mutants, however, melanophore densities were significantly increased by temperature downshift at early larval or metamorphic stages (*F*<sub>2,27</sub> = 7.55, *P* < 0.005). Melanophore densities did not differ depending on whether downshift occurred at early larval or metamorphic stages (*F*<sub>1,11</sub> = 0.14, *P* = 0.3). Melanophore densities observed after downshift also were indistinguishable from controls reared at 24°C (Fig. 5A; post hoc means comparisons, *P* > 0.05). These data support the idea that *puma* is required during pigment pattern metamorphosis to increase the numbers of melanophores comprising the adult pigment pattern.

Finally we asked whether *puma* might play a role in melanophore development in adult fish. To test this possibility, we reared fish though sexual maturity at the standard temperature of 28.5°C and divided them between 33 and 24°C treatments. After 3 weeks, we did not observe a significant difference in melanophore number between fish at 33 and 28.5°C (*F*<sub>1,13</sub> = 0.54, *P* = 0.5). In contrast, significantly more melanophores were present in fish shifted to 24°C (*F*<sub>1,15</sub> = 29.08, *P* < 0.0001; Fig. 5C). Together, these data suggest that *puma* is not required solely during embryogenesis, but also promotes melanophore development during and after pigment pattern metamorphosis.

**puma-dependent xanthophore development**

Given the persistent disorganization of melanophores in *puma* mutants and the requirement for interactions between melanophores and the xanthophore lineage to generate stripes (Parichy et al., 2000a; Parichy and Turner, 2003a,b), we asked whether *puma* mutants might be deficient for xanthophores in addition to melanophores. Figs. 6 and 7 show that, during metamorphosis, xanthophore numbers are significantly reduced in *puma* mutants compared with wild-
though this deficit is not as severe as for melanophores (averaged over 13–39 dpf, puma mutants exhibit ~73% as many xanthophores but only ~31% as many melanophores compared with wild-type). Although zebrafish also develop a third class of pigment cell, iridescent iridophores, the difficulty of reliably visualizing these cells precluded a rigorous examination of their distributions in puma mutants. Thus, the puma locus is required for promoting the development of at least one additional class of pigment cells, xanthophores.

**puma promotes expansion of postembryonic neural crest-derived lineages**

Epistasis analyses and temperature shift experiments point to a role for puma in expanding a population of
pigment cells or their precursors during metamorphosis. To test this possibility further, we examined the distributions of cells expressing molecular markers of pigment cell lineages. From epistasis analyses and analyses of xanthophore numbers, we predicted that puma mutants should exhibit fewer cells expressing kit, ednrb1, and fms during metamorphosis, as compared with wild-type larvae. Whereas kit is expressed by melanophores and their precursors, ednrb1 is expressed by all three major pigment cell lineages during embryogenesis: neural crest-derived pigment cell and glial precursors express sox10 and colourless.

Consistent with these predictions, we observed a severe reduction in the numbers of kit+ cells through metamorphosis (Fig. 8A–D). In wild-type larvae, these cells were widely distributed in the skin, but were found only rarely in puma mutants, particularly during early stages of pigment pattern metamorphosis. Nevertheless, the continued expression of kit in residual puma mutant melanophores (Fig. 8B) and other cell types (e.g., lateral line neuromasts; data not shown) suggests that, although kit+ cells are reduced in number, puma is not likely to be essential for expression at the kit locus. In contrast to metamorphic stages, gross differences in kit expression were not apparent between genotypes at embryonic stages (24–48 h; data not shown).

Similar to results for kit, we found reductions in the numbers of ednrb1+ and fms+ cells during early stages of pigment pattern metamorphosis in puma mutants as compared with wild-type. ednrb1+ and fms+ cells are widely distributed in the skin in wild-type fish at these stages (also see Parichy et al., 2000a,b), yet puma mutants exhibited variable reductions in these cell types (Fig. 8E–H). Since ednrb1 and fms are also expressed by xanthophores, which are not as severely affected in the puma mutant (and in the case of ednrb1, iridophores as well), our findings of only moderate reductions in the numbers of ednrb1+ and fms+ cells are not unexpected. Unfortunately, other reliable markers for these cell lineages at metamorphic stages are not yet available. Consistent with the eventual recovery of many xanthophores and some melanophores, deficits in ednrb1 and fms expression typically were not detectable by late stages of metamorphosis in puma mutants. Overall, these data support a model in which puma is essential for expanding a population of pigment cell precursors, but particularly the melanophore lineage, during pigment pattern metamorphosis.

Since puma acts on both melanophores and xanthophores during the larval-to-adult transition, we hypothesized that unspecified precursors to these cells might also be reduced in the puma mutant. Analyses of the zebrafish sox10 mutant, colourless, suggests that sox10 is upstream of kit, ednrb1, and fms in promoting the development of pigment cell lineages during embryogenesis: neural crest-derived pigment cell and glial precursors express sox10 and colourless.

![Fig. 6. puma promotes xanthophore development.](Image)

![Fig. 7. Quantitative analysis of xanthophore densities in wild-type and puma mutants during pigment pattern metamorphosis. Shown are mean xanthophore counts (bars, +95% confidence interval) from dorsal–ventral transects across the flank, averaged across individuals. Xanthophore numbers differed significantly between genotypes (F1,6 = 7.5, P < 0.05) after controlling for differences among individuals (F1,178 = 22.4, P < 0.0001) and days (F3,176 = 842.3, P < 0.0001). Xanthophore densities increased relatively linearly with time in both wild-type and puma mutants (genotype × day interaction, P = 0.4).](Image)
Fig. 8. *puma* promotes normal numbers of cells expressing markers of pigment cell lineages. All images are side views of whole-mount larvae during middle stages of pigment pattern metamorphosis with anterior to the left. (A, C, E, G) Wild-type larvae. (B, D, F, H) *puma* mutant larvae. (A–D) *puma* mutants exhibit fewer *kit*+ presumptive melanoblasts and melanophores as compared with wild-type. (A, C) In wild-type larvae, staining for *kit* transcripts is evident in cell peripheries, and unmelanized *kit*+ presumptive melanoblasts are scattered over the flank (C, arrow). (B, D) In *puma* mutant larvae, *kit* staining is detectable in melanophores (B, arrow), but unmelanized *kit*+ are dramatically reduced in number. (E, F) *puma* mutants exhibit fewer *ednrbl1*+ pigment cell precursors. (E) In wild-type larvae, *ednrbl1*+ cells are widely scattered over the flank, and these cells may represent precursors to melanoblasts, xanthophores, or iridophores; *ednrbl1* staining is typically patchy and cell boundaries are not easily detectable (arrow; Parichy et al., 2000b). (F) In *puma* mutants, fewer *ednrbl1*+ cells are detectable during early stages of pigment pattern metamorphosis (arrow), though these cells are recovered during later stages concomitant with the recovery of xanthophores. (G, H) *puma* mutants exhibit marginally fewer *fms*+ presumptive xanthophore precursors. (G) In wild-type larvae, numerous *fms*+ cells are present both in developing interstripe regions and, to a lesser extent, within developing melanophore stripes (also see Parichy and Turner, 2003b). (H) *puma* mutants exhibit a mild deficit of *fms*+ cells, consistent with the reduced xanthophore population, as compared with wild-type larvae. By later stages of pigment pattern metamorphosis, *puma* mutants do not exhibit detectably fewer *fms*+ cells compared with wild-type larvae (data not shown). Scale bar, 40 μm.

mutants lack these cells (Dutton et al., 2001; Kelsh and Eisen, 2000). Thus, we asked whether *sox10*+ cells are reduced in *puma* mutants. In situ hybridizations did not reveal differences in *sox10* expression between wild-type and *puma* mutants during embryonic stages (24–48 h; data not shown), but did show differences between genotypes during metamorphosis. In wild-type larvae, *sox10*+ cells are found medially surrounding dorsal root and sympathetic ganglia, within the myotomes, dorsal to the neural tube, and between the myotome and dermis (Fig. 9A, C, E, and G). These cells are especially prominent surrounding the main trunk lateral line nerve, which extends down the trunk along the horizontal myoseptum. In *puma* mutants, however, we observed a severe reduction in the numbers of *sox10*+ cells (Fig. 9B, D, F, and H). This deficit was especially evident within the myotomes and along the lateral line nerve (Fig. 9B and E), though *sox10*+ cells also could be fewer in number surrounding ganglia (Fig. 9H). As for cells expressing markers of pigment cell lineages, the defect in *sox10*+ cells was variable among individuals, and larvae at late stages of metamorphosis typically exhibited less severe defects, or even a complete recovery that was indistinguishable from wild-type (e.g., Fig. 9A) by terminal stages of metamorphosis.

The locations and morphologies of *sox10*+ cells suggested that many of these cells are likely to be peripheral glia, which are known to express *sox10* in zebrafish and other species (Britsch et al., 2001; Kuhlbrodt et al., 1998; Pusch et al., 1998; Sonnenberg-Reithmacher et al., 2001). To further assess this possibility, we examined the distributions of *sox10*+ cells relative to nerves and neurites stained with anti-acetylated tubulin. These comparisons confirm that many *sox10*+ cells are closely associated with nerves and neurites, consistent with these cells being Schwann cells (Fig. 10A and B). Since *sox10* mutants in both zebrafish and mouse exhibit defasciculation and other defects in peripheral nerves owing to the absence of glial cells (Britsch et al., 2001; Gilmour et al., 2002), we examined the morphology of peripheral nerve bundles in wild-type and *puma* mutants. A convenient and readily identifiable element of the peripheral nervous system is the posterior trunk lateral line nerve that runs along the horizontal myoseptum. In metamorphosing wild-type fish, this nerve is cohesive and well-organized (Fig. 10C and E). In metamorphosing *puma* mutants, however, the posterior lateral line nerve appears disorganized (Fig. 10D and F). Similar defects are observable affecting the cohesiveness and organization of other nerves in *puma* mutants as well. These observations support the interpreta-
tion that *puma* mutants exhibit a reduction in peripheral glia, in addition to deficits in pigment cells and their precursors.

A second molecular marker of neural crest cells in embryos is *foxd3*, which is expressed in premigratory neural crest cells, but is subsequently downregulated as these cells disperse to the periphery (Gilmour et al., 2002; Odenthal and Nüsslein-Volhard, 1998). Recent evidence further suggests that *foxd3* promotes a neural crest fate, while repressing melanogenesis, and may be involved in maintaining the pluripotency of early embryonic stem cells (Dottori et al., 2001; Kos et al., 2001). Thus, *foxd3*+ cells may represent pluripotent cells in the embryonic neural crest, and perhaps also during later development. *foxd3* also is expressed by peripheral glia (Gilmour et al., 2002; Kelsh et al., 2000b). In wild-type metamorphosing larvae, *foxd3*+ cells were widely distributed in the myotomes (Fig. 9I and K). *foxd3*+ cells also were found along the posterior trunk lateral line nerve and other nerves, though these cells were fewer in number as compared with *sox10*+ cells. In *puma* mutants, we did not observe gross changes in the numbers of *foxd3*+ cells compared with wild-type from early through terminal stages of metamorphosis (Fig. 9J and L). Thus, *puma* is not essential for generating normal numbers of *foxd3*+ cells, despite its role in expanding populations of *sox10*+ cells as well as pigment cell precursors.

**Puma acts autonomously to promote the development of neural crest-derived and other cell lineages**

*Puma* could act either cell autonomously or cell non-autonomously to promote normal pattern formation during metamorphosis. For example, *puma* could act within pigment cell precursors to promote their proliferation or specification, or *puma* could affect the extracellular environment in which these cells reside. To distinguish between these possibilities, we transplanted cells between wild-type and *puma* mutant embryos, and reared these chimeras 8–10 weeks through development of an adult pigment pattern. With respect to pigment cell lineages, we predicted that if *puma* acts autonomously, then wild-type cells transplanted into *puma* mutant hosts should be capable of producing large numbers of pigment cells, whereas *puma* mutant cells transplanted into wild-type hosts should produce few if any pigment cells. Conversely, if *puma* acts non-autonomously, then wild-type cells should produce few if any pigment cells in *puma* mutant hosts, whereas *puma* mutant cells should be capable of producing many pigment cells in wild-type hosts. To distinguish donor from host cells, we used donors homozygous for GFP under control of the β-actin promoter, so that most or all cells express GFP.

Results of cell transplantation support the notion that *puma* acts cell autonomously during pigment pattern development, but these analyses also suggest that *puma* is likely to promote the normal metamorphosis of nonpigment cell lineages as well.

In wild-type → *puma* mutant chimeras, wild-type cells contributed to a diverse array of derivatives, including pigment cells (Fig. 11A–D; Table 1). Donor wild-type GFP+ melanophores and xanthophores frequently were distributed widely over the flank and could be interspersed with host *puma* mutant pigment cells, relatively independently of other donor-derived cell types. Such broad distributions are consistent with analyses of chimeras for other genotypes at adult stages, as well as the highly migratory, proliferative, and invasive nature of pigment cells and their precursors (Erickson, 1985; Parichy, 1996a,b; Parichy and Turner, 2003a; Tucker and Erickson, 1986). Although apparent sizes of donor wild-type melanophores (based on the distribution of melanin and GFP+ fluorescence) typically resembled the sizes of melanophores in wild-type adults, host *puma* mutant melanophores could be readily identified in chimeras by their larger size (Fig. 11A and B); these are likely to be residual embryonic/early larval melanophores that are apparent even during metamorphic stages in *puma* mutants (Parichy and Turner, 2003b). Whereas wild-type donor cells typically responded to epinephrine by rapidly contracting melanin granules toward cell centers, *puma* mutant host melanophores were refractory to epinephrine treatment and melanin granules often persisted in the cell peripheries (Fig. 11C and D).

In *puma* → wild-type chimeras, donor cells did not contribute detectably to pigment cell lineages; these chimeras developed normal striped patterns of wild-type cells. A contingency table analysis indicated that the different frequency of donor pigment cell derivatives at genotypes is unlikely to be due to chance alone (Table 1). The frequencies of other donor-derived cell types in *puma* → wild-type chimeras also differed from those observed in reciprocal transplants. In contrast to wild-type → *puma* mutant chimeras, no *puma* → wild-type chimeras exhibited *puma* mutant epidermis, and only one individual exhibited a single *puma* mutant neuron (Table 1; Fig. 11H). Moreover, for cell transplantsations performed at the same time, proportionally fewer *puma* → wild-type chimeras went on to exhibit any donor-derived cell types, as compared with wild-type → *puma* mutant chimeras (41 vs. 63%, *N* = 34, 63, respectively; *χ²* = 4.4, d.f. = 1, *P* < 0.05). *puma* mutant cells did, however, contribute to vertebrae and muscle in wild-type hosts (Fig. 11E–G). These data suggest an overall failure in the ability of *puma* mutant cells to develop properly in a wild-type background.

To further test the developmental potential of transplanted *puma* cells, we asked whether these cells might produce a broader array of derivatives if transplanted to *puma* mutant hosts. Consistent with this prediction, when we constructed *puma* (GFP+) mutant → *puma* mutant chimeras, we observed donor GFP+ cells contributing to an increased range of cell types as compared with *puma* → wild-type chimeras. When we tallied the frequencies of chimeras exhibiting from 0 to as many as 5 identifiable derivative cell types, we found a significant difference between *puma* host and wild-type host genotypes (*χ²* = 38.0,
Fig. 9. *puma* promotes development of *sox10*-expressing cells but is not essential for normal *foxd3* expression during metamorphosis. All images are side views of whole-mount larvae during middle stages of pigment pattern metamorphosis with anterior to the left. (A–H) mRNA in situ hybridization for *sox10*. (I–L) In situ hybridization for *foxd3*. (A, C, E, G, I, K) Wild-type larvae. (B, D, F, H, J, L) *puma* mutant larvae. (A) In wild-type larvae, *sox10*+ presumptive glia are abundant around the posterior trunk lateral line nerve (arrow). Melanophores can be seen in the skin, above the plane of focus. (B) In *puma* mutants, a severe reduction in *sox10*+ cells is evident particularly along the posterior trunk lateral line nerve. Only a few *sox10*+ cells are present at this location initially (arrow), though this number increases during later development. (C, E) Lower magnification views showing the flank of a wild-type larva, either superficially just beneath the skin (C), or deeper within the myotomes (E). Scattered *sox10*+ cells are evident in both locations, as well as lining the posterior trunk lateral line nerve (near the tops of the panels). (D, F) Corresponding views of an equivalent stage *puma* mutant larva showing the near complete absence of *sox10*+ cells both superficially and internally. A single *sox10*+ within the myotome is indicated (F, arrow). (G, H) Deeper views, just lateral to the larval midline. (G) In wild-type larvae, *sox10*+ presumptive glia are present in dorsal root ganglia (arrow), adjacent to the neural tube (nt). v, vertebrae. A few melanophores are present dorsal and ventral to the neural tube. (H) In *puma* mutants, *sox10*+ cells are present medially but appear relatively disorganized as compared with wild-type larvae. (I) In wild-type larvae, *foxd3*+ cells are widely distributed and are especially abundant within the myotomes (arrow). (J) In *puma* mutants, similar numbers of *foxd3*+ cells (arrow) are present compared with wild-type. (A, B) 60 μm; (C–F, I, J) 80 μm; (G, H) 80 μm; (K, L) 40 μm.
Since puma mutant cells contribute to a diverse array of derivatives in puma hosts but not wild-type hosts at adult stages, we asked whether this reflects a specific loss of puma mutant cells from wild-type hosts during metamorphosis, when temperature-shift experiments reveal a critical period for puma activity (above). Thus, we transplanted cells from puma mutant to wild-type embryos, and examined the distribution of donor puma mutant cells at an early stage, prior to metamorphosis. Donor cell types were examined in chimeras at 25–27 hpf, when melanophores and other pigment cells have differentiated anteriorly, but neural crest cell migration is continuing posteriorly. We chose this relatively early stage because the lighter melanization of melanophores would be less likely to interfere with GFP fluorescence in these cells, which do not acquire sensitivity to epinephrine for several days (preventing us from stimulating melanin transfer to cell centers to reveal GFP in cell peripheries, as in adults). Table 1 and Fig. 11K–R show that puma mutant cells contributed to a wider variety of cell types in wild-type embryos as compared with wild-type adults. Moreover, the numbers of derivatives produced by donor cells in puma mutant → wild-type chimera examined at embryonic stages did not differ from the numbers of derivatives produced in either wild-type → puma mutant chimeras (χ² = 2.6, d.f. = 4, P = 0.6) or puma mutant → puma mutant chimeras (χ² = 9.3, d.f. = 5, P = 0.1) examined at adult stages. puma mutant cells in wild-type host embryos were frequently found in epidermis, the peripheral nervous system, and other derivatives. puma mutant cells also were frequently identified in the neural crest and in neural crest migratory pathways, consistent with their acquisition of a neural crest fate. Nevertheless, ~50% of these cells were either small and poorly fluorescent, or exhibited a blebbled morphology typical of apoptotic bodies, which are not normally observed in cell transplants between other genotypes at these stages (e.g., Dutton et al., 2001; Parichy et al., 1999). These observations suggest that some donor puma mutant neural crest cells failed to complete their development in wild-type hosts even at these embryonic stages. Consistent with this interpretation, we were unable to identify donor GFP+ puma mutant cells that had differentiated as melanophores in these chimeras (despite the apparently wild-type embryonic/early larval pigment patterns exhibited by unmanipulated puma mutants; Parichy and Turner, 2003b).

Together, these experiments suggest that puma acts cell autonomously to promote the expansion of pigment cell lineages; this activity occurs during metamorphosis but also is likely to be present at even earlier embryonic stages. Moreover, the differential distribution of epidermal and peripheral nervous system cell types across genetic backgrounds suggests that puma also may promote the development of these lineages during metamorphosis.

Discussion

The genetic and cellular bases for the development of neural crest stem cells, as well as the expression of adult traits in general, remain largely unknown. To better understand these mechanisms, we are using a mutational approach to dissect the processes of pigment cell specification and morphogenesis during the zebrafish larval-to-adult transition. Our analyses of the puma mutant provide new insights into the postembryonic development of neural crest-derived cell lineages. Specifically, our analyses suggest that the puma locus: (1) promotes the development of both early-appearing and late-appearing metamorphic melanophore populations; (2) contributes to the postembryonic development of nonmelanophore neural crest-derived lineages; (3) acts during metamorphosis; and (4) acts autonomously to neural crest-derived cells and possibly other lineages as well. These data suggest a model for how puma promotes the development of chromatophore and nonchromatophore lineages during the zebrafish larval-to-adult transition.

Essential role for puma in expanding metamorphic melanophore populations

Recent studies of amniotes have identified a population of melanocyte stem cells during postembryonic development (Nishimura et al., 2002; also see Grichnik et al., 1996; Kunisada et al., 1998). This study and other analyses of zebrafish (Johnson et al., 1995; Parichy et al., 2000a; Parichy and Turner, 2003a,b) suggest that analogous stem cells contribute to the adult pigment pattern of this species as well. Our results suggest a role for puma in expanding this population during metamorphosis, as the puma mutant largely ablates the population of melanophores that differentiates during metamorphosis, but leaves intact the population of early larval melanophores that differentiates during embryogenesis. In this respect, the puma mutant phenotype differs qualitatively from other described zebrafish pigment pattern mutants that either ablate melanophore lineages entirely (e.g., nacre mutants; Lister et al., 1999) or ablate only subsets of melanophores that differentiate at either embryonic or metamorphic stages (e.g., kit, ednrbb1, and fms mutants; see below).

Previous studies identified distinct populations of early-appearing and late-appearing metamorphic melanophores in zebrafish. Several lines of evidence in this and the accompanying report (Parichy and Turner, 2003b) suggest that puma promotes the development of both of these metamor-
phic melanophore populations. First, image series demonstrated that puma mutants exhibit a severe deficit in melanophore numbers during the stages when early- and late-appearing metamorphic melanophores normally would develop. Second, at a high temperature, puma mutants lack virtually all metamorphic melanophores. Third, epistasis analyses indicate that fish doubly mutant for puma and either kit, ednrb1, or fms have more severe melanophore deficits than single mutants alone. More specifically, kit single mutants lack early-appearing metamorphic melanophores but retain a population of early-appearing ednrb1- and fms-independent melanophores. When combined with the puma mutation, however, both ednrb1 and fms mutants exhibited more severe melanophore deficits, implicating puma in the development of early-appearing metamorphic melanophores as well.

Our analyses of molecular marker expression further support a model in which puma is required for expanding a population of undifferentiated stem cells that ultimately generate metamorphic melanophores. Specifically, we found a severe reduction in the numbers of kit+ presumptive melanoblasts during metamorphosis in puma mutants as compared with wild-type individuals. puma mutants also exhibit reductions in the numbers of ednrb1+ and fms+ cells, though these loci are expressed by nonmelanophore pigment cell lineages as well, which are less severely affected in the puma mutant (see below; Parichy et al.,

Fig. 10. Defects in peripheral nervous system organization in puma mutants. Staining for nerves and sox10 expression in wild-type (A, C, E) and puma mutant (B, D, F) larvae. All images are cross sections. (A) In wild-type larvae, sox10+ cells (blue arrow) can be observed along peripheral nerves and neurites stained for acetylated tubulin (red arrow), consistent with these cells being glia. (B) In puma mutants, residual sox10+ cells apparent in whole mounts (e.g., Fig. 9F) also can be observed in close association with nerves and neurites. (C) At late stages of pigment pattern metamorphosis in wild-type, the posterior lateral line nerve is cohesive and well organized (red arrow) and is surrounded by slow muscle fibers of the myotome. Blue staining for sox10 can be seen nearby as well. (D) In puma mutants at the same stage, the lateral line nerve appears disorganized and bundles of fibers can be seen both in the normal position (left red arrow) and medially (right red arrow). At this later stage, more sox10+ cells are observed as compared with early metamorphic stages (e.g., Fig. 9B). Arrowhead indicates a melanophore. (E) In a wild-type larva stained only for acetylated tubulin, the lateral line nerve is a solid rod of nerve fibers (arrow). e, epidermis. m, myotome. (F) In puma mutants, the nerve is disorganized and fewer nerve fibers are present (red arrows). Scale bars, (A, B) 10 μm; (C, D) 20 μm; (E, F) 20 μm.
Fig. 11. Cell transplantation reveals an essential role for puma in promoting multiple cell lineages during postembryonic development. All images are whole mounts. Red arrows, wild-type cells. Yellow arrows, puma mutant cells. (A–D) Wild-type cells transplanted into puma hosts contributed to a broad array of derivatives (Table 1), including pigment cells, when examined at adult stages. (A, B) Corresponding brightfield and fluorescence views. Many chimeras developed donor-derived GFP+/wild-type pigment cells. Large red arrow, example of a donor-derived wild-type melanophore. Although donor melanophores could be spread widely over the flank, they were typically interspersed with host GFP+ puma mutant melanophores, which retained their characteristic large size as compared with wild-type melanophores (yellow arrow). Donor wild-type cells also differentiated into placode-derived tissues and components of the peripheral nervous system: small red arrow, posterior trunk lateral line nerve. (C, D) Corresponding brightfield and fluorescence views. Higher magnification view of a different individual showing donor wild-type GFP+/puma mutant melanophores (red arrow) adjacent to host puma mutant melanophores (yellow arrow). Note that melanin (contained within melanosomes) remains partially dispersed in the peripheries of the puma mutant melanophores despite treatment with epinephrine, whereas melanin in wild-type melanophores is contracted toward the cell centers. (E–H) puma mutant cells transplanted into wild-type hosts contributed to only a limited array of derivatives (Table 1) when examined at adult stages. (E, F) Corresponding brightfield and fluorescence views showing donor GFP+/puma mutant contribution to vertebrae (v). nt, neural tube; a, aorta. A few wild-type host melanophores are visible in the background above the neural tube and below the aorta. (G) Donor puma mutant cells that differentiated as muscle fibers within the myotome (arrow). (H) In a single chimera, donor puma mutant cells differentiated as a single identifiable neuron (yellow arrow). Fluorescence from adjacent host GFP+ xanthophores (large red arrow) is color-shifted relative to GFP fluorescence. Note absence of GFP fluorescence in host melanophore as well (small red arrow). (I, J) puma mutant cells contribute to a broader spectrum of derivatives when transplanted to puma mutant hosts and examined in the adult. (I) Donor GFP+/puma mutant cells can be seen within the myotome (large arrow) and the vertebral column (small arrow). (J) puma mutant cells also contribute to pigment cell lineages in puma mutant hosts, including melanophores (arrow), in contrast to the behavior of these cells in wild-type hosts. (K–R) puma mutant cells are able to produce a wide variety of derivatives at embryonic stages, when transplanted to wild-type embryos. Shown are embryos at 25–27 hpf puma mutant cells contributing to the eye (small arrow), epidermis, and posterior lateral line nerve (large arrow, enlarged in L). (L) puma mutant donor cells contributing to the migrating posterior lateral line nerve (large arrow) as well as epidermis (small arrow). (M–R) puma mutant cells also are present within the premigratory neural crest and in neural crest migratory pathways, though these cells are often irregularly shaped. (M, N) Corresponding brightfield and fluorescence views of a donor GFP+/puma mutant cell within the epidermis dorsal to the neural crest (large arrow); morphology resembles that of typical zebrafish apoptotic bodies (Parichy et al., 1999; Parichy and Turner, 2003b). Small arrow, lightly melanized host wild-type melanophore. (O, P) GFP+/puma mutant cells within the dorsolateral neural crest migratory pathway is irregularly shaped and also resembles typical apoptotic body. (Q, R) Punctate GFP+/puma mutant cells within the neural crest. (A, B) 200 μm; (C, D) 100 μm; (E, F) 200 μm; (G) 100 μm; (H) 60 μm; (I, J) 100 μm; (K, L) 40 μm; (M, N) 40 μm; (O–R) 20 μm.
These results are consistent with epistasis analyses, and argue against a model in which *puma* is essential merely for the terminal differentiation of precursor cells into melanophores, as such a scenario would predict similar numbers of *kit*<sup>+</sup>, *ednrB1*<sup>+</sup>, and *fms*<sup>+</sup> precursors between wild-type and *puma* mutants (e.g., Kelsh et al., 2000a). Rather, we speculate that *puma* functions either to promote the proliferation and survival of stem cells or their specified progeny, or to promote the early events by which stem cells are recruited to melanophore and other lineages. Analyses are now underway to distinguish between these possibilities.

Finally, our study suggests that, although *puma* promotes the development of *kit*<sup>+</sup>, *ednrB1*<sup>+</sup>, and *fms*<sup>+</sup> cells, *puma* may not be in the same genetic pathways as *kit, ednrB1*, or *fms*, as each of these genes continued to be expressed by residual cells in *puma* mutants. Thus, in comparison with pathways that have been long studied for their roles in pigment pattern development in both amniotes and anamniotes, the *puma* mutant may identify a novel genetic pathway essential for development of postembryonic melanophore lineages. It will be especially interesting to examine roles for *puma* in the development of pigment patterns in other species that undergo a metamorphosis, such as cichlid fishes in which melanophore populations and patterns are even more complex than in zebrafish (e.g., Couldridge and Alexander, 2002), as well as in amphibians in which larval and adult melanophore patterns are relatively independent, but distinct subpopulations of metamorphic melanophores have not been identified (Parichy, 1998, 2001).

*puma* promotes development of multiple neural crest-derived lineages during metamorphosis

Our analyses indicate that *puma* is essential for the development of multiple neural crest-derived cell lineages during zebrafish metamorphosis. In addition to defects in melanophore development discussed above, *puma* mutants also exhibited reductions in the numbers of xanthophores and *fms*-expressing presumptive xanthophore precursors. Nevertheless, this reduction was not as severe as for melanophores, indicating either that xanthophores exhibit a lesser dependence on *puma* activity, or that regulation and recovery of xanthophores is more rapid and complete than for melanophores.

Besides xanthophores and their precursors, our study demonstrates an essential role for *puma* in the development of *sox10*<sup>+</sup> cells during metamorphosis: *puma* mutants exhibit a severe reduction in the numbers of *sox10*-expressing cells along nerves, in the integument, and within the myotomes. In embryos, mRNA for the HMG-domain transcription factor *sox10* is expressed widely in neural crest-derived cells, and these transcripts continue to be detectable during the development of glial lineages (Dutton et al., 2001). In turn, the *sox10*<sup>+</sup> cells in this study may represent multiple lineages, including Schwann cells and satellite glia. The precise identities of these cells are somewhat uncertain as reliable molecular markers for these different lineages are only now being developed for these postmetamorphic stages (N.B.P. and D.M.P., unpublished data). Nevertheless, the location of *sox10*<sup>+</sup> cells along nerves and our finding that peripheral nerves are disorganized in *puma* mutants support the notion that at least some of the *sox10*<sup>+</sup> cells identified in this study are Schwann cells. Indeed, the disorganization of the peripheral nerves that we observe in *puma* mutants is reminiscent of *sox10* mutants in both mouse and zebrafish (Britsch et al., 2001; Gilmour et al., 2002). Since *sox10* was expressed robustly in residual cells in *puma* mutants, *puma* may not act in a genetic pathway immediately upstream of *sox10*, though it promotes the development of *sox10*<sup>+</sup> cells. In this respect, our results are similar to those for *ErbB3* mutant mice in which Neuregulin signaling is disrupted and Schwann cell numbers are severely reduced with accompanying defects in peripheral innervation (though other phenotypes of *ErbB3* mutant mice and *puma* mutants are dissimilar; Britsch et al., 1999; Garratt et al., 2000).

Besides glial cells, *sox10*<sup>+</sup> cells in this study also may represent precursors to pigment cell lineages as *sox10* mutants in both mammals and zebrafish embryos exhibit a nearly complete loss of pigment cells and their precursors (Britsch et al., 2001; Dutton et al., 2001; Kelsh and Eisen, 2000; Southard-Smith et al., 1999). Since *sox10* promotes melanocyte specification by directly interacting with the promoter of the key melanocyte regulatory gene *microphthalmia* (Goding, 2000; Potterf et al., 2000, 2001), and also upregulates the melanin synthesis gene *dopachrome tautomerase* (Britsch et al., 2001), it is possible that melanophore defects in *puma* mutants directly reflect the reduced number of *sox10*<sup>+</sup> cells during pigment pattern metamorphosis. In contrast to melanocytes and melanophores, essentially nothing is known about the molecular basis for specification of the xanthophore lineage (see Quigley and Parichy, 2002). Nevertheless, lineage analyses at embryonic stages in zebrafish indicate that single cells can generate either melanophores and glia, or unmelanized pigment cells and glia in wild-type embryos (Raible and Eisen, 1994), whereas in *sox10* mutant embryos, these fates either are not generated, or in the case of xanthophores, the cells die after differentiation (Dutton et al., 2001). A close relationship between pigment cell and glial lineages also is implied by clonal analyses and other studies of amniote embryos (e.g., Dupin et al., 2000; Henion and Weston, 1997; Sherman et al., 1993). Thus, our results are consistent with a model in which a defect in *puma* activity severely reduces the numbers of *sox10*<sup>+</sup> cells at metamorphosis, with concomitant reductions in the numbers of metamorphic melanophores and xanthophores as well as a subset of peripheral glia, possibly by affecting a common precursor of these cell lineages.

Our analyses of molecular markers in wild-type and
puma mutant larvae also suggest that puma does not act upstream of foxd3. The foxd3 transcription factor is implicated in the acquisition of a neural crest over neural fate, and in avian embryos suppresses a melanocyte fate (Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001). In zebrafish embryos, foxd3 is a marker for premigratory neural crest cells but is subsequently lost as these cells disperse, and is later reexpressed in cells believed to be Schwann cells (Kelsh et al., 2000b; Odenthal and Nüsslein-Volhard, 1998). We similarly observed foxd3+ cells in close association with nerves and within the myotomes. In contrast to sox10, however, neither the number of foxd3+ cells nor apparent levels of foxd3 expression differed between wild-type and puma mutants. A recent analysis suggests that foxd3 is essential for maintaining the pluripotency of stem cells during early mouse development (Hanna et al., 2002). By analogy, foxd3 may contribute to maintaining the pluripotency of neural crest-derived embryonic and metamorphic cell lineages in zebrafish. This model suggests that puma plays an early role in recruiting such pluripotent cells, and sox10 expression is an early manifestation of commitment to pigment cell and glial lineages. A second prediction of this model is that a failure of puma activity could result in the increased production of other cell lineages as pluripotent cells are freed to respond to signals specifying alternative fates. The puma mutant allele that we have analyzed exhibits a variably expressive and variably penetrant jaw hypertrophy; this and other aspects of the puma phenotype are currently being analyzed to gain a complete picture of how puma promotes the proper lineage segregation of neural crest-derived cells during and after metamorphosis.

### Postembryonic requirement for puma activity

Temperature shift experiments indicate that puma is required during metamorphosis for adult pigment pattern development. The allele of puma that we have analyzed was derived from a screen for mutants affecting metamorphosis and postembryonic development. In this on-going screen, mutagenized embryos are reared through embryonic and early larval stages at a low (permissive) temperature, shifted to a higher (restrictive) temperature just prior to the onset of metamorphosis, and then screened for mutant phenotypes during later metamorphic and juvenile stages. This screening strategy should identify several classes of mutants, including: (1) null or hypomorphic alleles of genes that are not essential during embryogenesis but are essential during postembryonic development; (2) temperature-sensitive alleles of genes that have essential functions during both embryogenesis and postembryonic development; (3) temperature-sensitive alleles of genes that do not have essential functions during embryogenesis, but are required during metamorphosis or juvenile development. In addition to potentially identifying genes that function during both embryonic and postembryonic stages, temperature-sensitive alleles offer an opportunity to identify critical periods for gene activity through temperature shift experiments. In zebrafish for example, temperature-sensitive alleles and reciprocal temperature shift experiments have shown that Kit is essential for late stages of melanophore development in the fin (Rawls and Johnson, 2001).

Our analyses of the puma115e1 mutant reveal a require-

### Table 1

Percentages of chimeras exhibiting donor-derived cell types

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Chimera genotype</th>
<th>puma (n = 40)b</th>
<th>puma (n = 14)</th>
<th>puma (n = 11)</th>
<th>puma (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatophores</td>
<td></td>
<td>33</td>
<td>0</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Trunk neural crest</td>
<td></td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Peripheral nervous system</td>
<td></td>
<td>43</td>
<td>7i</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Central nervous system</td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Epidermis</td>
<td></td>
<td>28</td>
<td>0</td>
<td>27</td>
<td>64</td>
</tr>
<tr>
<td>Craniofacial skeleton/cartilage</td>
<td></td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
<td>30</td>
<td>21</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>Notochord, vertebrae, other endoskeleton</td>
<td></td>
<td>25</td>
<td>13</td>
<td>91</td>
<td>16</td>
</tr>
</tbody>
</table>

a Additional derivatives observed at low frequency in only one or another donor–host combination included: gut, vasculature, gills (wild-type→puma); fin skeleton (puma→puma); Rohon–Beard neurons (puma→wild-type embryo).
b Numbers of chimeras exhibiting at least some GFP+ donor cells.
c P < 0.005, significantly different percentages across genotypes after controlling for multiple comparisons.
d Not applicable.
e Lateral line, eye.
ment for *puma* during the larval-to-adult transition: fish upshifted to a restrictive temperature just before or during metamorphosis exhibited more severe melanophore deficiencies than fish reared at a permissive temperature throughout development; conversely, fish downshifted to a permissive temperature just before or during metamorphosis recovered substantial numbers of melanophores compared with fish reared only at the restrictive temperature. Downshifting adult fish also resulted in partial recovery of melanophore numbers. These results are most simply interpreted to mean that *puma* is essential during and after pigment pattern metamorphosis for recruiting normal numbers of cells to the melanophore lineage. Analyses of chimeras between *puma* mutant and wild-type fish also support the interpretation that *puma* acts during metamorphosis. When *puma* mutant cells were transplanted to wild-type embryos, these cells generated a broad array of derivatives, including presumptive neural crest cells at embryonic stages. By contrast, *puma* mutant cells did not contribute to neural crest-derived cell types in wild-type hosts when chimeras were examined as adults. This difference suggests that *puma* mutant cells comprising neural crest-derived lineages were lost by adult stages, consistent with an essential role for *puma* in maintaining (and expanding) these cell types during metamorphosis.

Despite the apparently normal development of *puma* mutant embryos and larvae, our results are consistent with a model in which *puma* functions not only during metamorphosis but during embryogenesis as well. In chimera analyses in which *puma* mutant cells were transplanted to wild-type hosts, these cells were identified in neural crest migratory pathways but were often punctate or blebbed, resembling apoptotic bodies (Dutton et al., 2001; Parichy et al., 1999). These observations suggest that some *puma* mutant cells were specified as neural crest cells, but were unable to complete their development in wild-type hosts. Indeed, we did not observe *puma* mutant donor cells contributing to the population of differentiated melanophores at these early stages. This interpretation might seem contradictory to temperature shift experiments, in which *puma* mutants reared at 33°C during embryogenesis recovered substantial numbers of melanophores when subsequently downshifted to 24°C, since curtailing *puma* activity during embryogenesis could be expected to ablate precursor cells so that melanophores would not later be recoverable. Nevertheless, it is presently not known whether the *puma*\(^{115e1}\) phenotype at the restrictive temperature represents a complete inactivation of *puma*, or only a partial loss-of-function. Thus, persistence of melanophores in *puma*\(^{115e1}\) may simply reflect the escape of a limited number of cells that would otherwise be lost in a null allele. Molecular cloning of the *puma* locus will allow a definitive analysis of whether or not *puma* is required at embryonic stages, in addition to its requirement during metamorphosis. Likewise, analyses of additional *puma* alleles will indicate whether defects in *puma*\(^{115e1}\) mutants, and functions inferred from these defects, are typical of the *puma* locus more generally.

### Cell autonomy of *puma* function and roles for *puma* in development of neural crest-derived and non-neural crest cell lineages

Lineage diversification of neural crest cells in embryos depends on a variety of cell intrinsic and extrinsic influences (Dorsky et al., 2000). Our cell transplantation studies indicate that *puma* acts cell autonomously to promote the expansion of neural crest-derived cell lineages during metamorphosis, as *puma* mutant cells failed to generate pigment cells in wild-type hosts, whereas wild-type cells produced large numbers of pigment cells in *puma* mutant hosts. Nevertheless, our finding that other cell lineages are differentially represented in chimeras between wild-type and *puma* mutants raises the possibility that the *puma* locus acts more widely to promote the metamorphosis of other cell lineages as well; a requirement that is only revealed when *puma* mutant and wild-type cells are juxtaposed in the same embryo. Thus, it is conceivable that an overall deficit in proliferative potential or another cellular process results in the loss of *puma* mutant cells in wild-type hosts, whereas gross defects in unmanipulated *puma* mutants are only manifested in cell populations that undergo extensive remodeling at metamorphosis, such as pigment cells.

Our analyses suggest a model for *puma* activity and *puma*-dependent cell lineages during the metamorphosis of neural crest-derived lineages (Fig. 12). During embryonic stages, neural crest cells (nc, in the figure) differentiate to form a diverse array of cell types that contribute to the early larval body; these include early larval melanophores (ELM), early larval xanthophores (ELX), and early larval peripheral glia (ELG). Specification of these fates during embryogenesis requires *sox10* (Dutton et al., 2001; Kelsh and Eisen, 2000), and *puma* may or may not contribute to the development of these cells. In addition, some neural crest-derived cells are set aside as metamorphic stem cells (m) that will generate chromatophores and other cell types both during and after metamorphosis. As metamorphosis occurs, some early larval melanophores (ELM) are lost, whereas others persist and contribute to the pigment pattern of adults. *puma* is essential for the normal patterning of these cells, probably by indirectly influencing their morphogenesis via effects on other *puma*-dependent cell lineages (Parichy and Turner, 2003b). Simultaneously, metamorphic stem cells are recruited in a *puma*-dependent manner. *sox10* specifies nonectomesenchymal fates, though it is uncertain precisely when cells become committed to particular cell lineages. In turn, specification and recruitment of terminal cell types depends on a battery of signaling molecules and transcription factors (e.g., for melanophores: *microphthalmia, ednrbl, kit*) of which only a few are likely to have been identified so far (e.g., Johnson et al., 1995; Lister et al., 1999; Parichy et al., 1999, 2000a,b). Finally, terminal dif-
differentiation of metamorphic melanophores (MM; including both early-appearing and late-appearing metamorphic populations), metamorphic xanthophores (MX), and metamorphic glia (MG) depends on loci encoding proteins essential for function of the differentiated cells (e.g., for melanophores: melanin synthesis enzymes; Kelsh et al., 2000a; Ziegler et al., 2000) as well as the activities of genes that promote their survival and morphogenetic behavior during these stages (such as kit and fms, not shown; Rawls and Johnson, 2001; Parichy and Turner, 2003a). Identification of the puma locus and other genes essential for postembryonic neural crest patterning will provide an additional test of this model and will reveal the extent to which embryonic and metamorphic pathways of lineage diversification are conserved.

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References


