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Zebrafish *puma* mutant decouples pigment pattern and somatic metamorphosis

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Abstract

The genetic and developmental bases for trait expression and variation in adults are largely unknown. One system in which genes and cell behaviors underlying adult traits can be elucidated is the larval-to-adult transformation of zebrafish, *Danio rerio*. Metamorphosis in this and many other teleost fishes resembles amphibian metamorphosis, as a variety of larval traits (e.g., fins, skin, digestive tract, sensory systems) are remodeled in a coordinated manner to generate the adult form. Among these traits is the pigment pattern, which comprises several neural crest-derived pigment cell classes, including black melanophores, yellow xanthophores, and iridescent iridophores. *D. rerio* embryos and early larvae exhibit a relatively simple pattern of melanophore stripes, but this pattern is transformed during metamorphosis into the more complex pattern of the adult, consisting of alternating dark (melanophore, iridophore) and light (xanthophore, iridophore) horizontal stripes. While it is clear that some pigment cells differentiate de novo during pigment pattern metamorphosis, the extent to which larval and adult pigment patterns are developmentally independent has not been known. In this study, we show that a subset of embryonic/early larval melanophores persists into adult stages in wild-type fish; thus, larval and adult pigment patterns are not completely independent in this species. We also analyze *puma* mutant zebrafish, derived from a forward genetic screen to isolate mutations affecting postembryonic development. In *puma* mutants, a wild-type embryonic/early larval pigment pattern forms, but supernumerary early larval melanophores persist in ectopic locations through juvenile and adult stages. We then show that, although *puma* mutants undergo a somatic metamorphosis at the same time as wild-type fish, metamorphic melanophores that normally appear during these stages are absent. The *puma* mutation thus decouples metamorphosis of the pigment pattern from the metamorphosis of many other traits. Nevertheless, *puma* mutants ultimately recover large numbers of melanophores and exhibit extensive pattern regulation during juvenile development, when the wild-type pigment pattern already would be completed. Finally, we demonstrate that the *puma* mutant is both temperature-sensitive and growth-sensitive: extremely severe pigment pattern defects result at a high temperature, a high growth rate, or both; whereas a wild-type pigment pattern can be rescued at a low temperature and a low growth rate. Taken together, these results provide new insights into zebrafish pigment pattern metamorphosis and the capacity for pattern regulation when normal patterning mechanisms go awry.

Keywords: Melanophore; Zebrafish; Metamorphosis; Pattern regulation; Neural crest; Community effects

Introduction

The genetic and cellular bases for patterning adult form remain largely unknown. Yet, such information is vital for understanding the origins of many human disease syndromes, as well as evolutionary changes in adult phenotypes. A useful system for identifying mechanisms underlying the expression of adult traits is the metamorphosis of fishes and amphibians. Such metamorphoses entail changes in a variety of organ systems, including appendages and fins, the digestive tract, nervous system, sensory systems, the integument, and pigmentation (Brown, 1997; Gilbert et al., 1996; Shi, 2000; Tata, 1993; Webb, 1999). To maintain a properly functioning and integrated phenotype, these changes must be tightly coordinated with one another and with overall somatic growth. These different levels of coordination depend on both global hormonal influences and

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locally acting cellular processes. Studies of amphibian metamorphosis over many years have identified key roles for endocrine factors, such as thyroid hormones, as well as nuclear receptors that transduce these hormonal signals, and downstream genes that effect metamorphic changes (reviewed in Gilbert et al., 1996). Since many of these factors are likely to be important during postembryonic mammalian development as well, the metamorphosis of ectothermic vertebrates represents a useful system for identifying patterning mechanisms relevant to fetal and neonatal development (Tata, 1993, 1999).

To date, studies of metamorphosis have employed a variety of pharmacological, transgenic, and subtractive approaches to identify and test the functions of relevant genes and hormones (e.g., Schreiber et al., 2001; Tata, 1968; Wang and Brown, 1991). While these approaches have provided important information about hormonal interplay and cellular responses, they may not efficiently identify loci that act in as-yet-unidentified genetic pathways, or loci that act upstream of known genetic pathways. Forward genetic screens offer a complementary approach to identifying such genes. In recent years, the zebrafish *Danio rerio* has proven to be a tractable organism for developmental genetics and hundreds of mutants have been identified. Moreover, zebrafish undergo a metamorphosis much like amphibians, involving postembryonic changes in a variety of traits, including: resorption of the larval fin fold and development of adult unpaired fins; changes in the gut, peripheral nervous system, and sensory systems; alterations in physiology and behavior; development of scales; and formation of an adult pigment pattern (e.g., Kirschbaum, 1975; Ledent, 2002; Sire et al., 1997; also see Fuiman et al., 1998; Webb, 1999). Although most zebrafish mutants isolated thus far affect early patterning and are lethal at embryonic stages, mutant screens designed specifically to identify metamorphic and adult phenotypes have the potential to reveal novel genes and pathways required for postembryonic development.

A useful trait for understanding metamorphic remodeling is the pattern of pigment cells expressed during early larval and adult stages, because this pigment pattern undergoes extensive changes during metamorphosis, and because pigment cells are not essential for survival in the laboratory. Pigment patterns in zebrafish and other ectothermic vertebrates result from the arrangements of neural crest-derived pigment cells, including black melanophores, yellow xanthophores, and iridescent iridophores (Bagnara, 1998; Quigley and Parichy, 2002). In zebrafish and other danios, an embryonic/early larval (EL) pigment pattern arises during embryonic stages and persists largely unchanged for about 2 weeks (Fig. 1A). This pigment pattern consists of stripes of embryonic/early larval melanophores: dorsally, along the dorsal apex of the myotomes (ELD in Fig. 1A); ventrally, at the ventral apex of the myotomes (ELV); laterally, along the horizontal myoseptum (ELL); and covering the dorsal and ventral surfaces of the yolk (or later, the gut). Melanophores are not found elsewhere over the myotomes, though yellow xanthophores are widely dispersed over the flank at hatching (e.g., Kimmel et al., 1995).

During metamorphosis, a very different adult pigment pattern arises (Fig. 1B). The onset of pigment pattern metamorphosis can be defined by the first appearance of melanophores outside of the early larval stripes. Once this process begins (~2 weeks postfertilization), new melanophores continue to appear dispersed over the flank in regions not previously occupied by these cells. Then, beginning during the middle of pigment pattern metamorphosis (~3 weeks postfertilization), additional melanophores start to appear in the position of adult stripes, and simultaneously, some of the initially dispersed melanophores migrate into these stripes (Kirschbaum, 1975; Johnson et al., 1995; Parichy et al., 2000a). By terminal stages of pigment pattern metamorphosis (~4 weeks postfertilization), a juvenile/early adult pigment pattern has formed, consisting of an adult primary dorsal melanophore stripe (1D in Fig. 1B) and an adult primary ventral melanophore stripe (1V). These primary melanophore stripes also include iridophores and border a lighter interstripe region comprising xanthophores and iridophores. In addition, melanophores are found dorsally and in the developing dorsal scales (S). Pigment pattern metamorphosis can be considered complete when this juvenile/early adult pigment pattern has formed. During later juvenile and adult development, however, additional secondary melanophore stripes will form as the fish grows, with the first of these (2V) appearing at the ventral margin of the flank, just dorsal to the anal fin. The extensive metamorphic remodeling of the pigment pattern is evident by comparing the spatial relationships of the embryonic/early larval and adult melanophore stripes: the early larval lateral melanophore stripe occurs at the horizontal myoseptum, where the adult interstripe region will form; however, early larval melanophores are absent over the dorsolateral and ventrolateral surfaces of the myotomes, where the adult primary melanophore stripes will develop. Importantly, the fates of early larval melanophores during pigment pattern metamorphosis remain unknown.

Previous studies of zebrafish have identified several genes essential for the development of different pigment cell lineages and for different components of the adult pigment pattern. Some loci completely ablate melanophores or xanthophores at both embryonic/early larval stages and adult stages (Lister et al., 1999; Parichy et al., 2000a). By contrast, a particularly interesting class of mutants ablates only subsets of pigment cells. For example, *kit* mutants develop embryonic/early larval melanophores, but these cells die beginning around the time of hatching and are completely lost by the onset of metamorphosis; only during middle and late stages of metamorphosis do new melanophores differentiate; *kit* mutants also lack dorsal and scale-associated melanophores. Conversely, *endothelin receptor b1 (ednrb1)* and *fms* mutants retain both embryonic/early

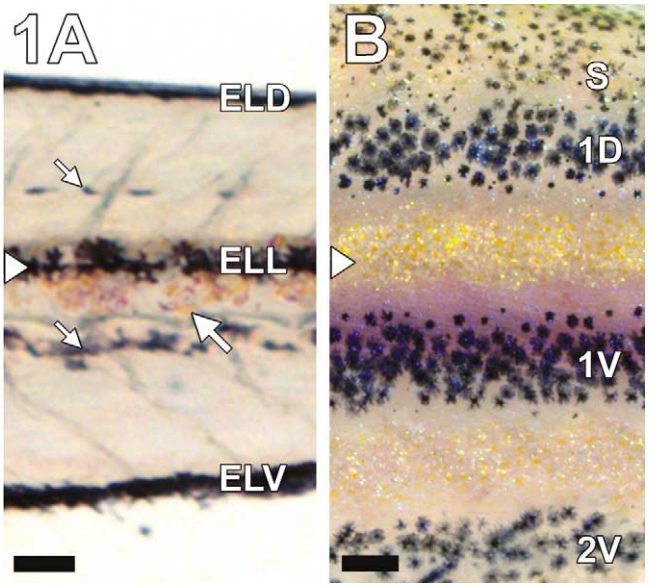


Fig. 1. Larval-to-adult transformation of the zebrafish pigment pattern. (A) Early larval pigment pattern. Shown is an individual at 14 dpf, at the onset of pigment pattern metamorphosis. Established during embryogenesis, the early larval pattern of melanophore stripes persists through the onset of metamorphosis. On the trunk, this pattern consists of an early larval dorsal melanophore stripe (ELD), an early larval lateral melanophore stripe (ELL) along the horizontal myoseptum, and an early larval ventral melanophore stripe (ELV). Below the plane of focus, melanophores also can be seen along the dorsal neural tube and the dorsal aorta (small arrows). At the onset of pigment pattern metamorphosis, a few xanthophores and iridophores (large arrow) are apparent immediately ventral to the early larval lateral melanophore stripe. (B) An early adult/juvenile pigment pattern develops by terminal stages of pigment pattern metamorphosis. Shown is an individual at 37 dpf. Two adult primary melanophore stripes have developed dorsal (1D) and ventral (1V) to a light interstripe region containing xanthophores and iridophores. The first adult secondary melanophore stripe (2V) has started to form near the base of the anal fin. Dorsally, melanophores and xanthophores are scattered over the myotomes and populate the dorsal scales (S). Arrowheads in (A) and (B) show location of the horizontal myoseptum; note that EL melanophores initially present at this location (A, ELL) are absent by terminal stages of pigment pattern metamorphosis (B). Images are not to the same scale: Scale bars, (A) 150 μm, (B) 500 μm.

larval melanophores and scattered early-appearing metamorphic melanophores, but lack stripe melanophores that normally arise during later metamorphosis (Johnson et al., 1995; Parichy et al., 1999, 2000a,b).

To identify additional loci that are required for pigment pattern and somatic metamorphosis, we are undertaking mutant screens specifically designed to isolate such phenotypes. In these screens, mutagenized fish are reared through embryonic and early larval stages at a low temperature (25°C), then just prior to metamorphosis, these individuals are shifted to a higher temperature (33°C) to complete development, during which time they are examined for mutant phenotypes. Since temperature-sensitive alleles are readily isolated in zebrafish (Johnson and Weston 1995; Rawls and Johnson, 2001), this approach should allow the identification of temperature-sensitive alleles of loci that might also have essential roles during embryonic development, thus bypassing early embryonic lethality.

In this study, we report a new zebrafish mutant, *puma*. We have chosen this mutant for analysis because early larvae show no apparent pigment pattern defects, whereas adults exhibit irregular pigment patterns with extensive variability among individuals (Fig. 2). *puma* mutants thus have the potential for providing insights into both pigment pattern metamorphosis and the mechanisms underlying individual variability in trait expression. Our analyses demonstrate that, at its most extreme, the *puma* mutant phenotype reflects a nearly complete failure of pigment pattern metamorphosis. In this respect, the *puma* mutation decouples pigment pattern development from many other events of somatic metamorphosis. The *puma* mutant thus identifies a novel class of gene, as compared with previously identified loci for which mutants ablate only subsets of metamorphic melanophores. We further demonstrate that, despite a severe reduction in melanophore numbers during metamorphosis, *puma* mutants recover substantial numbers of melanophores during later development. Finally, we demonstrate that the *puma* mutant phenotype is both temperature-dependent and growth-dependent, and we suggest a framework for understanding the variability of this phenotype within the context of normal interactions among pigment cells and their precursors.

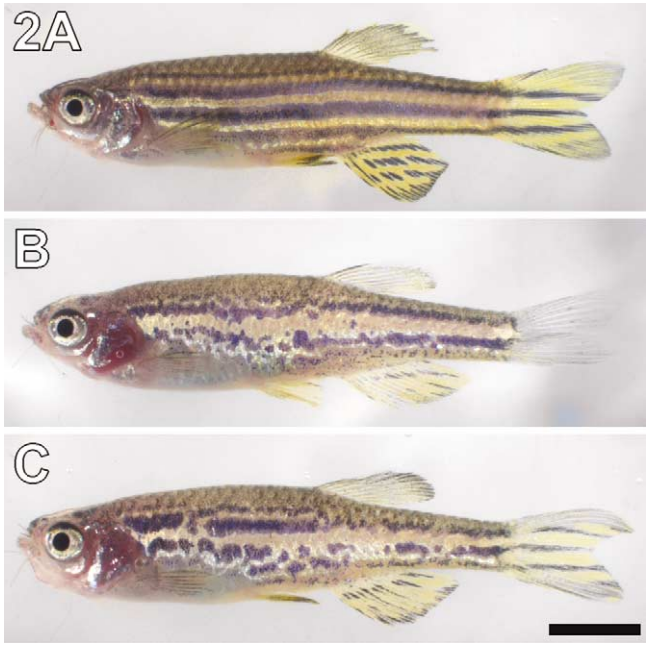
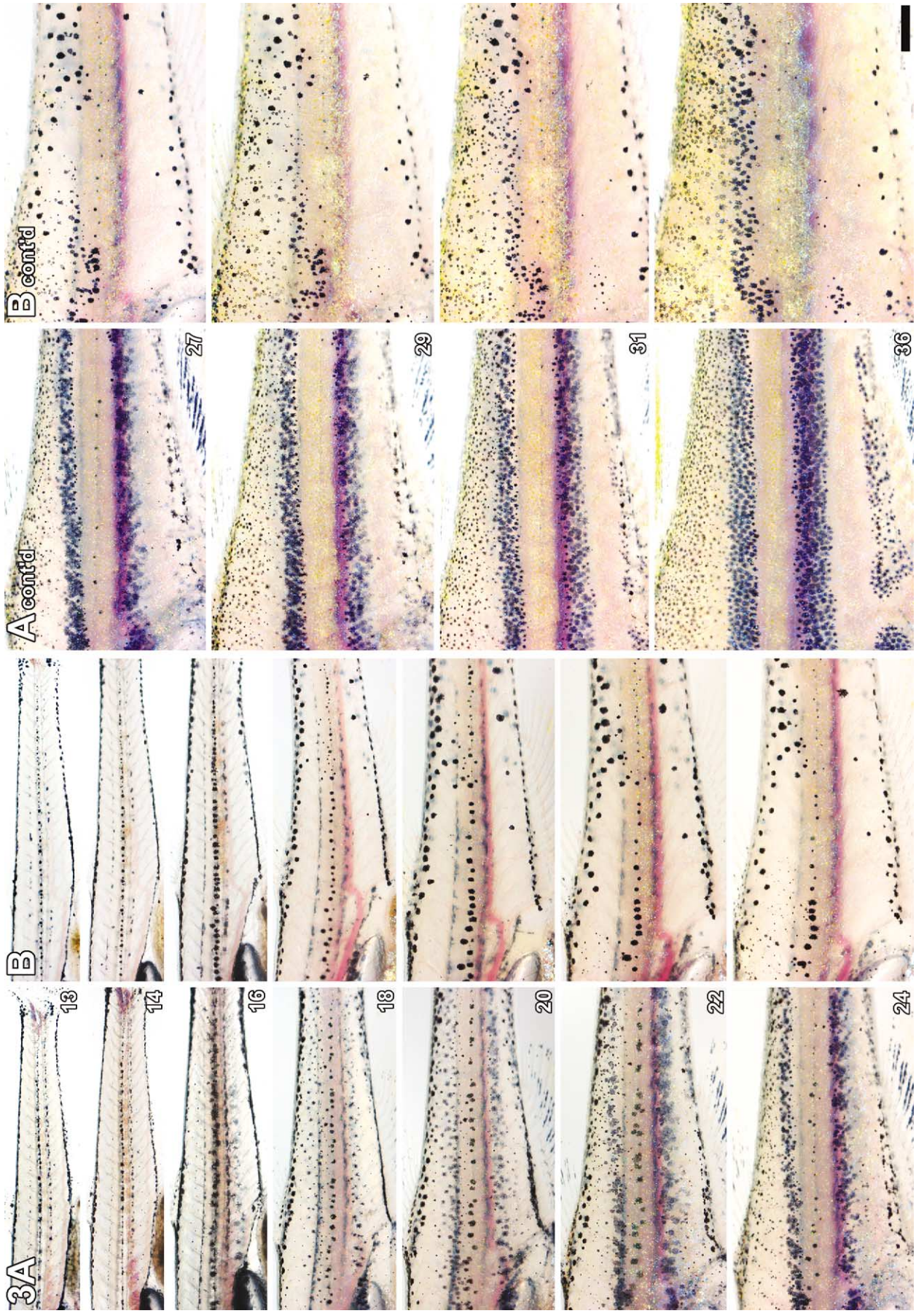


Fig. 2. *puma* mutant adults exhibit disrupted stripes. (A) Wild-type zebrafish exhibit four to five regular dark horizontal stripes with lighter interstripe regions. (B, C) *puma* mutant adults exhibit fewer stripes with irregular borders. Two examples are shown. Scale bar, 5 mm.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Materials and methods

Fish stocks and rearing conditions

Wild-type fish were the partially inbred University of Texas isolate of AB, AB^{UT}. *puma*^{j115el} was isolated in an early pressure gynogenetic mutant screen for N-ethyl-N-nitrosourea-induced mutations (Solnica-Krezel et al., 1994). Mutagenized gynogenetic embryos were reared at 25°C until just prior to pigment pattern metamorphosis (~12 days postfertilization; dpf), and then were shifted to 33°C to complete development through juvenile or adult stages. Families were screened for mutant phenotypes and mutants of interest were isolated after repeated outcrosses. *puma* is maintained in the AB^{UT} genetic background and is recessive and homozygous viable. For image series, individual fish were reared in plastic containers in a flow-through water system and fed rotifers then dry flake food twice per day. All fish were maintained at the standard temperature of 28.5°C (14L:10D), except for temperature shift experiments that were conducted at 24 and 33°C.

Imaging

For recording melanophore behaviors during and after pigment pattern metamorphosis, AB^{UT} and *puma* mutant fish were repeatedly anesthetized with MS222 (Sigma) and imaged at 16× with a Zeiss Axiocam digital camera mounted on an Olympus SZX-12 stereozoom microscope. Images were recorded every 24 h from just prior to pigment pattern metamorphosis through early adult stages (13–39 dpf). Images were transferred to Adobe Photoshop for analysis.

Analyses of embryonic/early larval melanophore fates

To determine the fates of embryonic/early larval (EL) melanophores in wild-type and *puma* mutants, we followed in image series all visible EL melanophores within the early larval dorsal stripe and the early larval lateral stripe from 13–39 dpf. Time-lapse imaging of larvae for 24–48 h demonstrated that individual melanophores can be unambiguously reidentified from static image series during the stages examined (I. K. Quigley and D.M.P., unpublished data). Moreover, EL melanophores are easily distinguished by their position, initially larger size, greater pigment density, and blacker color, as compared with newly differentiating metamorphic melanophores. For each EL melanophore, we determined the distance traveled from its origin in the early larval stripes and whether it persisted or was lost by 39 dpf.

Analyses of metamorphic melanophore numbers and movements

To assess the requirement for *puma* by metamorphic melanophores, we defined 100-μm-wide dorsal–ventral transects at the approximate anterior and posterior margins of the anal fin, and we determined the numbers and positions of all melanophores within these transects between 13 and 39 dpf for each of six wild-type and six *puma* mutant fish. We excluded from these analyses EL melanophores as well as any melanophores that could not be confidently reidentified for more than 1 day. By accounting for virtually all melanophores within these transects on each day of development (sample sizes = 1,335 melanophores; 12,350 measurements), these analyses provide a relatively high resolution picture of melanophore numbers and fates over time.

Since considerable growth occurs during pigment pattern metamorphosis (see Appendix), we did not rely on absolute measures of melanophore positions, but instead determined the positions of melanophores relative to the dorsal and ventral margins of the flank. Thus, we assigned a value from 0 to 1 for each melanophore on each day of development, with 0 representing the dorsal-most edge of the flank; 1 representing the ventral-most edge of the flank; and fractions between 0 and 1 representing intermediate positions. We then determined the net changes in these relative dorsal–ventral positions by subtracting the starting position of each cell from its ending position; negative net changes thus represent dorsal movements, and positive net changes represent ventral movements. This approach controls for passive melanophore movements owing to isometric growth of the flank, but cannot correct for passive melanophore movements due to nonisometric growth; thus, some changes in melanophore positions may simply represent passive movements due to shape changes. Nevertheless, the wild-type and *puma* mutants we examined did not exhibit detectable differences in growth rate or other obvious differences in shape (see Appendix); thus, comparisons of net changes in melanophore position should reveal whether gross differences in melanophore movements are present between genotypes. Time-lapse imaging of larvae for up to 30 h revealed that most metamorphic melanophores are readily identifiable between static images. Furthermore, we limited our analyses by focusing strictly on melanophores that

Fig. 3. Pigment pattern metamorphosis in wild-type and *puma* mutant zebrafish. Numbers indicate days postfertilization. (A) Wild-type zebrafish exhibit a gradual increase in melanophore numbers and a gradual emergence of the adult pattern of dark primary and secondary melanophore stripes with light interstripe regions. (B) From embryonic to early larval stages (≤13 dpf), *puma* mutants exhibit pigment patterns indistinguishable from wild-type. Subsequently, however, *puma* mutants have fewer melanophores than wild-type fish during pigment pattern metamorphosis and juvenile development. Although some additional melanophores are present on the flank during middle stages of pigment pattern metamorphosis (e.g., 22 dpf), these are much less numerous than in wild-type larvae at corresponding stages. Finally, during terminal stages of pigment pattern metamorphosis and juvenile development (e.g., ≥27 dpf), more melanophores are present, and an irregular striped pattern forms that is nevertheless patchier than in wild-type fish. Scale bar, 500 μm.

could be reidentified unambiguously over ≥2 days in static image series. We did not attempt to estimate anterior and posterior movements. In some instances, actual distances traveled by melanophores could be greater than those reflected in the estimates of net dorsal–ventral changes in position (data not shown). Qualitatively similar results were found with alternative methods for analyzing cell movements by using absolute distances, other landmarks, or both. Melanophores were followed if they migrated out of the original transects, but cells migrating into transects were not included in analyses, thus avoiding inflated estimates of cells numbers per unit area. Although some melanophores could not be confidently reidentified and thus were lost during the course of the image series, only melanophores that unambiguously disappeared were counted as having been lost from the flank. The IPTK 4.0 package (Reindeer Graphics, Inc.) for Photoshop was used to determine the position of every identified melanophore relative to the dorsal and ventral margins of the flank as well as the horizontal myoseptum, and also to calculate nearest neighbor distances among melanophores. Since *puma* mutants lacked almost all ventral melanophores (see below), we restricted our analyses of melanophore movements to the dorsal half of the flank (analyses without these restrictions yielded identical results; data not shown).

Temperature and growth experiments

To assess environmental factors that may contribute to variability in the *puma* mutant phenotype, we reared fish under different conditions that promoted either high or low rates of growth. To achieve these different growth rates, fish were divided at the stage of first feeding between a low density treatment (2 fish per container, allowing a high growth rate) and a high density treatment (50 fish per container, yielding a low growth rate). Conditions experienced by fish in the low density/high growth rate and high density/low growth rate treatments were each more extreme than under standard rearing conditions. Fish were reared to an average size of ~12.6 mm standard length, and final sizes did not differ significantly between genotypes ($F_{1,67} = 0.30$, $P = 0.6$). Fish in low growth treatments took ~4 weeks longer to reach this size, as compared with fish in high growth treatments. Analyses of variance revealed a significant temperature × growth treatment interaction that affected size ($F_{1,68} = 9.30$, $P < 0.01$), and comparisons of means revealed that fish reared under high growth conditions at 33°C were slightly but significantly larger (average size of 13.6 mm standard length; Tukey post hoc means comparisons, all $P < 0.05$) as compared with fish reared in other treatments. Nevertheless, this small size difference did not bias melanophore densities upwards in either wild-type or *puma* mutants (see Results), and all comparisons below are based on analyses of covariance that controlled for small size differences among individuals (see Statistical methods). Replicate experiments that tested whether variability in the *puma* mutant phenotype depended on interactions between growth conditions and light levels revealed concordant effects of growth as are described below, but not significant effects of light intensity (data not shown).

Statistical methods

All analyses followed standard statistical methods (Sokal and Rohlf, 1981) and were performed by using the JMP Statistical Package v.4.0.4 (SAS Institute, Cary, NC). Factors associated with cell movements, total numbers, and births were tested by using partially hierarchical mixed-model nested analyses of covariance. Thus, comparisons between genotypes were made only after controlling for variation associated with individuals. Individuals and interactions with individuals were treated as random effects and nested within genotypes. This approach provides a more conservative test of effects associated with genotypic differences. For all parametric analyses, residuals were examined for normality and homogeneity of variances, and dependent variables were transformed as necessary to meet the assumptions of these linear models. To improve normality of the residuals, melanophore births were square root-transformed, whereas distances moved by early larval melanophores were log-transformed. To control for small differences in the sizes of individuals, comparisons of melanophore numbers and densities were made by using standard length (distance from the tip of the nose to the caudal peduncle) as a covariate; corrected least squares means are presented in figures below, though significance levels of analyses did not differ depending on the inclusion of standard length. Finally, analyses of melanophore deaths employed multiple logistic regression and maximum likelihood estimation, whereas contingency table analyses were used to assess frequencies of melanophore births and deaths in different defined regions of the flank. Details of analyses and complete statistical models are available from D.M.P. on request.

Results

Disrupted pigment pattern metamorphosis and late pattern regulation in puma mutant zebrafish

The disrupted stripes of *puma* mutant larvae could result from a degenerative process in which stripes form normally, but fail to be maintained during adult stages. Alternatively, *puma* mutants could exhibit defects during pigment pattern metamorphosis that continue to be manifested by adults. To distinguish between these possibilities, we repeatedly imaged individual fish every 24 h between 13 and 39 dpf.

Fig. 3A shows a representative wild-type individual and reveals the overall sequence of pigment pattern metamorphosis. Metamorphosis begins (13–14 dpf) as a few melanophores appear over the middle of each ventral myotome,

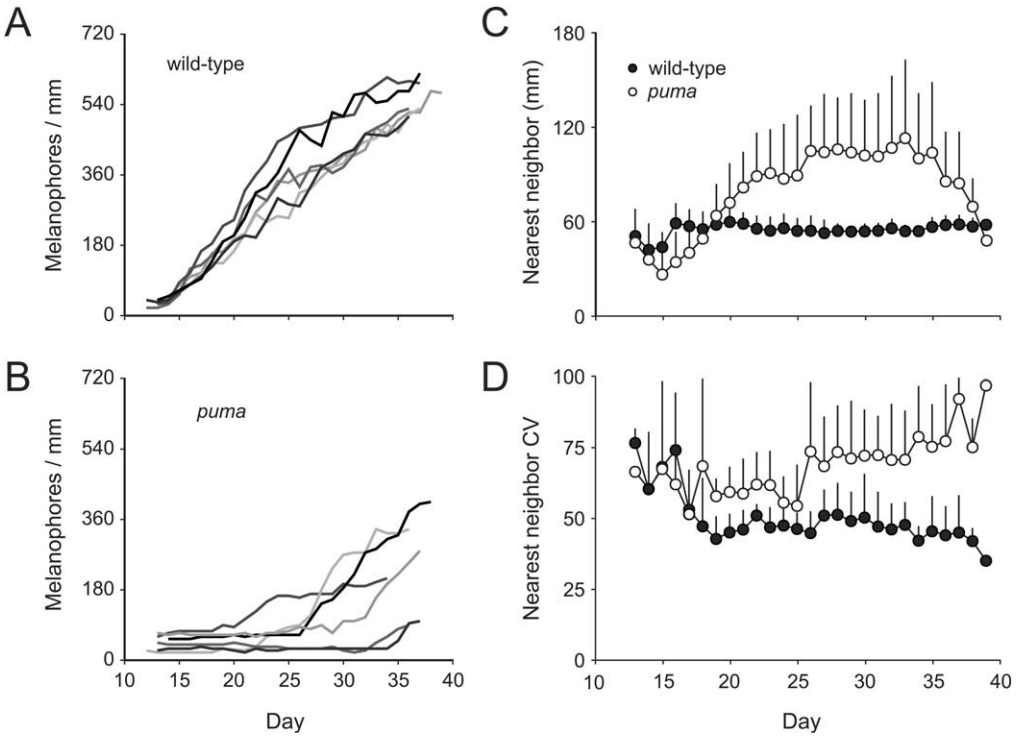
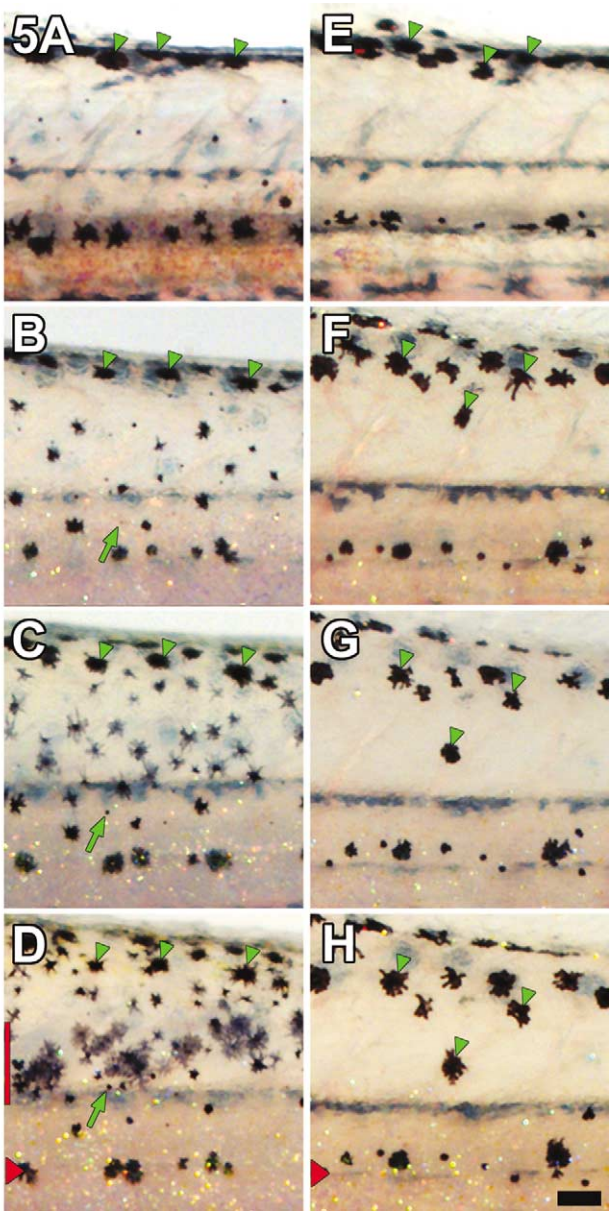


Fig. 4. Quantitative analyses reveal failure of normal pigment pattern metamorphosis but late pattern regulation in *puma* mutants. (A, B) Mean melanophore densities along two dorsal–ventral transects from each of six wild-type (A) and six *puma* mutants (B); each line represents a different individual. (A) In wild-type, melanophore densities increase steadily through pigment pattern metamorphosis. (B) *puma* mutants exhibit fewer melanophores, and a variable delay in the onset of melanophore number increases as compared with wild-type. (C, D) Quantitative analyses of melanophore distributions reveals partial pigment pattern regulation in *puma* mutants. Closed circles, wild-type fish. Open circles, *puma* mutants. (C) Mean nearest neighbor distances remain relatively constant in wild-type fish during pigment pattern metamorphosis and beyond. In *puma* mutants, however, mean nearest neighbor distances between melanophores increase during early and middle metamorphosis, reflecting the failure of normal stripe development; nearest neighbor distances then decline during later metamorphosis and juvenile development, reflecting the eventual recovery of many melanophores. (D) Coefficients of variation (CV) for nearest neighbor distances decline as wild-type fish develop, reflecting the increased regularity of melanophore arrangements in the adult pigment pattern. In *puma* mutants, the melanophore pattern remains significantly less organized as compared with wild-type fish. Error bars, +1 SD.

with melanophores beginning to appear over the dorsal myotomes shortly thereafter. Subsequently, melanophores continue to appear dispersed over the flank during early metamorphosis (14–20 dpf). Gradually, the primary adult dorsal and ventral melanophore stripes start to become visible, and the positions of these stripes are clearly discernable by the middle of metamorphosis (e.g., 22 dpf). During late metamorphosis (21–28 dpf), the primary melanophore stripes become more regular, a few melanophores typically present between these stripes are lost, and dorsal melanophores that will cover the scales increase in number. Finally, during terminal stages of metamorphosis (≥29 dpf), dorsal scales develop and are populated by melanophores, so that by juvenile stages (e.g., 36 dpf), the adult pigment pattern with two primary melanophore stripes (1D, 1V) is established and the first secondary melanophore stripe (2V) has started to form ventrally (compare with Fig. 1B). Quantitative analyses demonstrated that total melanophore numbers in wild-type fish increased nearly linearly with time along dorsal–ventral transects (Fig. 4A), correlated with the overall growth of the flank (see Appendix).

Fig. 3B shows a representative *puma* mutant fish. At early larval stages (≤13 dpf), no pigment pattern defects are present. Subsequently, however, *puma* mutants exhibit fewer melanophores and the arrangements of these cells differ from wild-type larvae. These defects were most severe during metamorphosis (14–28 dpf) and were somewhat less pronounced during later juvenile and adult development (≥29 dpf). Quantitative analyses confirmed the severe reduction in melanophore densities in *puma* mutants, although the magnitude of this defect varied among individuals: melanophore densities started to increase as early as 20 dpf in some fish but not until ~34 dpf in other fish, well after metamorphic melanophores normally appear (Fig. 4B). The pigment pattern defect in *puma* mutants is not attributable to an overall reduction in growth and development rate, as body size and shape did not differ between wild-type and *puma* mutants reared in isolation (see Appendix). Thus, disrupted stripes in *puma* mutants result from a failure of normal pigment pattern metamorphosis, rather than degeneration of the adult pigment pattern.

The zebrafish pigment pattern depends on both the numbers of melanophores and their arrangements. To further assess pigment pattern development in *puma* mutants, we



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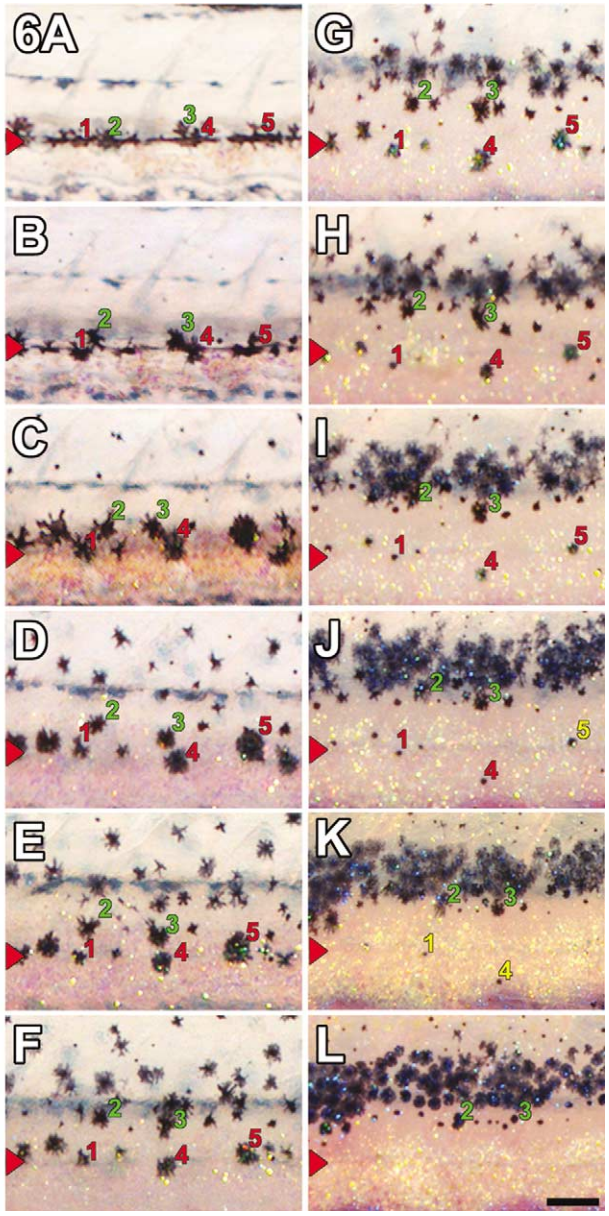

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Fig. 5. EL melanophore movements and appearance of metamorphic melanophores differs between wild-type and *puma* mutant larvae. (A–D) In wild-type larvae, most melanophores comprising the dorsal stripe of the embryonic/early larval pigment pattern remain near the dorsal aspect of the myotomes during early metamorphosis (arrowheads show representative cells). Additional newly differentiated melanophores appear as initially smaller, more lightly melanized cells scattered over the flank. Arrow in B–D, a newly differentiated melanophore moving dorsally to join the dorsal stripe. A–D, 15, 16, 19, 21 dpf, respectively. Red bar in (H), region of developing dorsal primary melanophore stripe. (E–H) In *puma* mutants, early larval dorsal stripe melanophores migrate further ventrally toward the middle of the flank than in wild-type (arrowheads). Newly differentiated melanophores are not evident. E–H, 15, 18, 19, 21 dpf, respectively. Images are scaled to maintain the same relative anterior–posterior positions of cells. Red arrowheads in (D) and (H), horizontal myosepta. Scale bar, 100 μm (H only).

Fig. 6. Movement or death of EL lateral stripe melanophores in wild-type larvae. Some EL melanophores initially comprising the lateral stripe move short distances dorsally to join the developing adult dorsal primary melanophore stripe (e.g., melanophores 2, 3). Early larval melanophores that fail to leave this region typically are lost (e.g., melanophores 1, 4, 5; last observed position in yellow). A–L, 14, 15, 16, 17, 18, 19, 20, 21, 23, 25, 26, 29 dpf, respectively. Images are scaled to maintain the same relative anterior–posterior positions of cells. Scale bar, 200 μm (L only).

thus examined two additional measures of melanophore distributions: mean nearest neighbor distances, and coefficients of variation (CV = 100 × SD/mean) for nearest neighbor distances. Whereas mean nearest neighbor distances are a good measure of melanophore packing within pattern elements such as stripes or spots, CVs for nearest neighbor distance are sensitive indicators of irregularities in this patterning (Parichy and Turner, 2003). In wild-type fish, mean nearest neighbor distances remained almost constant throughout metamorphosis and juvenile development (Fig. 4C). During this time, CVs for nearest neighbor distances dropped (Fig. 4D), reflecting the increased regularity of melanophore patterning. In *puma* mutants, mean nearest neighbor distances rose sharply during the normal stages of

metamorphosis, reflecting the failure of melanophore densities to keep pace with body growth, and subsequently fell to wild-type levels as new melanophores appeared during terminal metamorphic and juvenile stages (≥32 dpf; Fig. 4C). CVs for nearest neighbor distances continued to rise throughout development, however, reflecting persistent irregularity in melanophore patterning (Fig. 4D). These analyses confirm that *puma* mutants fail to develop normal pigment patterns during pigment pattern metamorphosis; rather, *puma* mutants achieve localized pattern regulation during terminal stages of metamorphosis and juvenile development, although melanophore organization remains deficient as compared with wild-type fish.

Embryonic/early larval melanophore fates and patterning during metamorphosis depend on puma

The defect in pigment pattern metamorphosis in *puma* mutants led us to ask whether melanophores that are present during these stages simply represent embryonic/early larval (EL) melanophores persisting from earlier stages. Since it has not been known what happens to EL melanophores even in wild-type fish, however, we first examined the fate of these cells during normal development. We find that, in wild-type larvae, some EL melanophores disappear, whereas others persist in the adult pattern. In *puma* mutants, fewer EL melanophores are lost, and the migratory behavior of these cells differs from wild-type.

As an initial test of EL melanophore fates in wild-type fish, we determined the dorsal–ventral distances moved by these cells during pigment pattern metamorphosis. EL melanophores present initially within the early larval dorsal stripe (ELD in Fig. 1A) shifted slightly ventrally, averaging ~60 μm from the dorsal margin of the flank by 21 dpf (Fig. 5A–D). By contrast, behaviors of EL melanophores within the early larval lateral stripe (ELL) were more complex (Fig. 6): a minority (19%) moved >100 μm dorsally and joined the ventral margin of the developing adult dorsal primary melanophore stripe (1D); a majority (81%), however, moved <100 μm from their initial position and thus remained in the middle of the flank. Because this region is fated to lack melanophores (Fig. 1), we asked whether EL melanophore survival correlated with emigration from this starting position: this analysis revealed that EL melanophores that migrated dorsally were more likely to survive than melanophores that remained at the horizontal myoseptum ($\chi^2 = 23.1$, d.f. = 1, $P < 0.0001$; Fig. 7). Thus, fates of EL melanophores were associated with their migratory behavior during metamorphosis.

Fates of *puma* mutant EL melanophores differed from wild-type. *puma* mutant EL melanophores became significantly more dispersed than wild-type melanophores (Levene's test, $F_{1,933} = 38.8$, $P < 0.0001$; Fig. 5E–H); these differences are particularly apparent in box plots (Fig. 8), which show both melanophore distribution means (diamonds) and ranges (limits of bars). *puma* mutant ELD

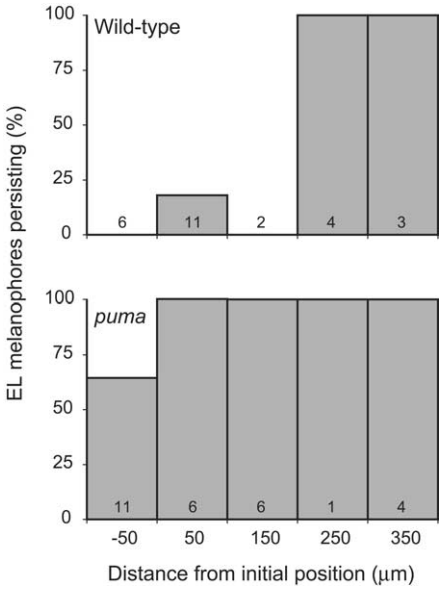

Fig. 7. Persistence of ELL stripe melanophores depends on movements during pigment pattern metamorphosis in wild-type but not *puma* mutants. Shown is the percentage of melanophores persisting through 39 dpf relative to their distance moved from an initial position along the horizontal myoseptum. Negative distances represent ventral displacements; positive distances represent dorsal displacements. Numbers of melanophores examined are indicated at the bases of vertical bars. Distances were measured at 21 dpf as most changes in position occurred by this time. (A) In wild-type larvae, ELL melanophores that failed to move from their origin at the horizontal myoseptum typically were lost, whereas melanophores that moved dorsally persisted through development of the juvenile/adult pigment pattern. (B) In *puma* mutants, most ELL melanophores persisted to adult stages, and there was no detectable relationship between distance moved and persistence.

melanophores moved further ventrally on average than wild-type melanophores, but the distributional limits of these cells differed even more dramatically: 16% of *puma* mutant ELD melanophores were found >100 μm from the dorsal margin of the flank, in comparison with only 1.5% of wild-type ELD melanophores. Behaviors of *puma* mutant ELL melanophores also differed from wild-type: most failed to leave their origin at the horizontal myoseptum, but a few moved further dorsally than wild-type ELL melanophores. Finally, the persistence of *puma* mutant ELL melanophores was greater than wild-type and was not correlated with distance migrated from the middle of the flank ($\chi^2 = 9.5$, d.f. = 1, $P = 0.1$; Fig. 7). Thus, *puma* is essential for the patterning and fate of EL melanophores during pigment pattern metamorphosis: in *puma* mutants, excess EL melanophores persist into adult stages, and some of these cells settle in ectopic locations.

puma-dependent expansion and patterning of metamorphic melanophore population

Quantitative image analyses showed that *puma* mutants exhibit fewer melanophores than wild-type larvae during

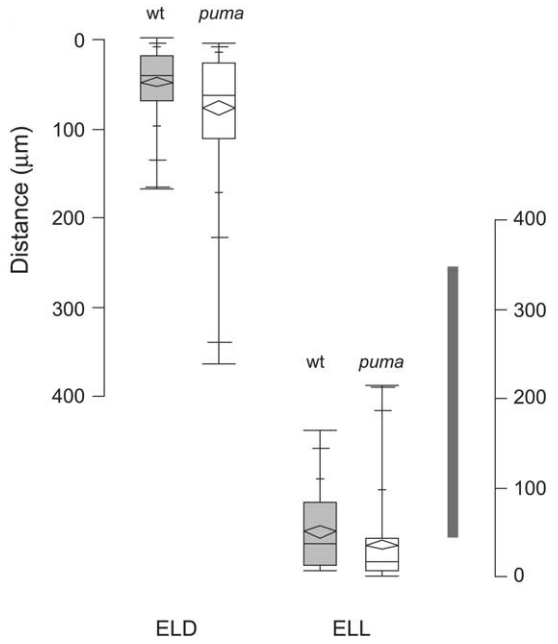

Fig. 8. Movement of EL melanophores during metamorphosis differs between wild-type and *puma* mutants. Shown are box plots indicating the distances traveled from starting positions either at the dorsal edge of the myotomes in the early larval dorsal stripe (ELD; left) or at the horizontal myoseptum in the early larval lateral stripe (ELL; right). Left and right axes are offset to reflect the average height of the dorsal flank at 21 dpf, when final measurements were taken, and orientations are reversed to reflect the relative orientation of ELD and ELL melanphore starting positions. Bar on the right indicates the average limits of the adult dorsal primary melanophore stripe (1D) at 21 dpf. In wild-type larvae, most ELD melanophores ($N = 256$) moved only short distances ventrally, whereas many ELL melanophores ($N = 156$) moved slightly dorsally to join adult stripe 1D. In *puma* mutants, however, distances traveled by EL melanophores were significantly more variable than in wild-type larvae. *puma* mutant ELD melanophores ($N = 239$) moved significantly further ventrally than in wild-type larvae ($F_{1,49} = 45.3$, $P < 0.0001$); mean distances moved by ELL melanophores did not differ significantly from wild-type ($F_{1,9} = 1.2$, $P = 0.3$) after controlling for variation among individuals ($F_{9,429} = 3.1$, $P < 0.005$). Diamonds, mean distances traveled. Boxes denote interquartile ranges of data (i.e., 75% of data lie within regions indicated by box limits, with median of data indicated by horizontal line within each box); limits of bars indicate range of data, with hatchmarks indicating ranges containing successive 5% intervals of data points.

pigment pattern metamorphosis (above). Since the total number of melanophores represents a balance between their appearance and loss (Parichy et al., 2000a), we tested whether wild-type and *puma* mutants differ in their frequencies of metamorphic melanophore births (defined as new cells appearing by differentiation or proliferation) or metamorphic melanophore losses (defined as cells disappearing by death or dedifferentiation). Fig. 9A shows the temporal and spatial patterns of melanophore births (also see Fig. 5). In wild-type fish, melanophore births reached maximal rates during middle stages of pigment pattern metamorphosis (~21 dpf) and declined thereafter (also see Appendix). Melanophore births occurred in both dorsal and ventral regions of the flank. In *puma* mutants, there were fewer melanophore births than in wild-type ($F_{1,308} = 62.3$, $P < 0.0001$), though the magnitude of this deficit varied among days ($F_{1,308} = 12.5$, $P < 0.001$): there were very few melanophore births during early and middle pigment pattern metamorphosis (14–27 dpf), but a gradual increase during terminal pigment pattern metamorphosis and juvenile development (reaching a peak at ~34 dpf). Proportionally fewer melanophores were born in ventral regions of the flank in *puma* mutants as compared with wild-type ($\chi^2 = 25.9$, d.f. = 4, $P < 0.0001$).

In contrast to melanophore births, melanophore losses did not differ dramatically between genotypes and were slightly more frequent in wild-type than *puma* mutants (9.9%, 4.9%, respectively; $\chi^2 = 4.7$, $P < 0.05$). Thus, *puma* promotes normal numbers of metamorphic melanophores principally by affecting the proliferation or differentiation of these cells, rather than the survival of melanophores that already have differentiated.

Analyses above showed that *puma* has a role in patterning EL melanophores during metamorphosis. Given this observation, we asked whether *puma* might also contribute to patterning newly arising metamorphic melanophores during these stages. Thus, we tested whether movements of metamorphic melanophores differed between genotypes. In wild-type fish, metamorphic melanophores often moved short distances dorsally or ventrally to join developing adult stripes (Fig. 9B). In *puma* mutants, however, these movements were significantly reduced. This effect was principally associated with diminished movements by the few *puma* mutant melanophores appearing during middle stages of pigment pattern metamorphosis (21–26 dpf; Table 1). During terminal metamorphosis and juvenile development (27–39 dpf), directional movements could not be detected for either wild-type or *puma* mutant melanophores. Since the majority of *puma* mutant melanophores appeared at these later stages, this result argues that pigment pattern regulation in *puma* mutants occurs principally through the appearance of melanophores in situ, as opposed to directed movements of these cells to sites of stripe formation. Taken together, these analyses show that *puma* is essential for expanding the population of melanophores that normally arises during metamorphosis, and that pattern regulation in *puma* mutants does not depend on the same dorsal–ventral melanophore movements as during normal stripe formation.

Temperature-dependent and growth-dependent patterning in puma mutants

The *puma* mutant phenotype is highly variable among individuals reared under standard conditions, with some individuals exhibiting much more severe pigment pattern defects than other individuals, even within the same family. To identify the sources of this variability, we tested environmental factors that might contribute to pigment pattern development. The *puma* mutant was isolated in a screen for temperature-sensitive mutations affecting pigment pattern

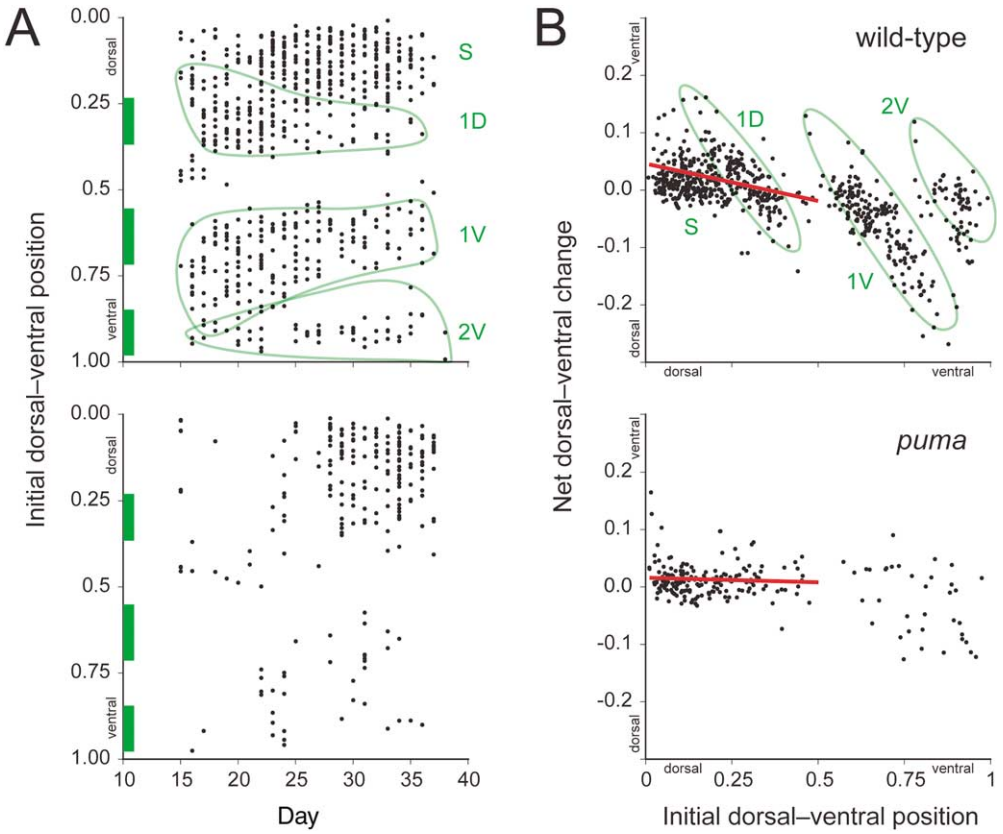

Fig. 9. Melanophore births and movements in wild-type (*upper plots*) and *puma* mutants (*lower plots*) through metamorphosis. (A) Patterns of melanophore births in wild-type and *puma* mutants. Shown are the initial dorsal–ventral positions of melanophores first appearing on each day of imaging. Dorsal–ventral positions are determined relative to the total height of the flank such that 0 is the dorsal-most point on the flank and 1 is the ventral-most point on the flank. Because of overlap, points occasionally represent more than one melanophore. (*Upper plot*) In wild-type larvae, melanophores appear widely distributed on each day of imaging. Green ellipses are drawn around cells that ultimately contributed to dorsal and scale melanophores (S), the adult primary dorsal melanophore stripe (1D), the adult primary ventral melanophore stripe (1V), and the first adult secondary melanophore stripe at the ventral margin of the flank (2V). Compare with Fig. 1B. Green bars indicate the dorsal–ventral limits of melanophore stripes at completion of the image series. Thus, some initially dispersed melanophores become more tightly localized into stripes. (*Lower plot*) In *puma* mutants, melanophores appear late in development and are more numerous in dorsal regions of the flank that normally contribute to dorsal and scale-associated melanophores (compare with *upper plot*). (B). Net changes in dorsal–ventral positions of melanophores (*y* axes) are plotted against the initial dorsal–ventral positions at which these cells were first identified (*x* axes). Net changes in dorsal–ventral positions over time reflect dorsal–ventral melanophore movements during pigment pattern metamorphosis. Since net changes are calculated by subtracting the starting position from the final position for each melanophore, net negative changes represent dorsal movements, whereas net positive changes represent ventral movements. Slopes (red lines) are drawn for melanophores that appeared between 21 and 26 dpf (Table 1). (*Upper plot*) In wild-type larvae, cells initially in more ventral positions tend to move dorsally, whereas cells initially in more dorsal positions tend to move ventrally. (*Lower plot*) In *puma* mutants, far fewer cells are present and most of these develop during late phases of metamorphosis when melanophore movements are reduced. Slope drawn through dorsal cells is considerably reduced as compared with wild-type, reflecting the reduced net movements of late-developing *puma* mutant melanophores as compared with wild-type melanophores.

and postembryonic development. Thus, we asked first whether the *puma* mutant phenotype is temperature-sensitive, by rearing *puma* mutants at either 33 or 24°C. These initial tests showed that *puma* mutants that developed at either 33 or 24°C exhibited embryonic/early larval pigment patterns that were indistinguishable from wild-type. During and after pigment pattern metamorphosis, however, *puma* mutants that developed at 33°C exhibited more severe melanophore deficiencies than *puma* mutants that developed at 24°C (see below). Thus, the *puma* mutant adult phenotype is temperature-sensitive.

Given the variability of the *puma* mutant phenotype even at the standard temperature of 28.5°C, we further speculated that differences in individual growth rates might contribute to differences in their pigment patterns. To test this possibility, we reared *puma* mutants at different densities and food levels to achieve different rates of somatic growth, and then we quantified melanophore densities at a size and stage when an adult pigment pattern had formed in wild-type controls. Because of the temperature-sensitivity of the *puma* mutant phenotype, we further compared the effects of different growth rates between permissive (24°C) and restrictive (33°C) temperatures.

In wild-type larvae, melanophore densities differed somewhat across growth rate and temperature treatments (growth × temperature interaction: $F_{1,28} = 5.63$, $P < 0.05$), but this difference was relatively small in magnitude (Figs. 10 and 11). In *puma* mutants, however, melanophore densities varied dramatically across treatments ($F_{1,34} = 10.43$,

Table 1
Movements of metamorphic melanophores in wild-type and *puma* mutant fish

dpf[a]	wild-type		*puma*		wild-type vs. *puma*[d]	
	coeff.[b]	*n*[c]	coeff.	*n*		
14–20	$-0.32 \pm 0.037^{***}$	113	$-0.25 \pm 0.052^{**}$	19	$F_{1,127} = 1.3$	$P = 0.2$
21–26	$-0.10 \pm 0.023^{***}$	199	0.02 ± 0.034^{NS}	50	$F_{1,245} = 7.7$	$P < 0.01$
27–39	-0.03 ± 0.017^{NS}	135	-0.01 ± 0.012^{NS}	132	$F_{1,255} = 0.9$	$P = 0.3$

[a] Days post fertilization, corresponding to early metamorphosis (14–20 dpf) when early-appearing *kit*-dependent metamorphic melanophores first appear; middle to late metamorphosis (21–26 dpf) when late-appearing *ednrb1*- and *fms*-dependent metamorphic melanophores first appear; and terminal metamorphosis and juvenile development (27–39 dpf), when scales develop and are populated by dorsal melanophores.

[b] Partial regression coefficient describing the relationship between net dorsal–ventral change in melanophore position and initial dorsal–ventral position; see text for details. ***, $P < 0.0001$; **, $P < 0.001$; NS, slope not significantly different from 0.

[c] *n*, number of melanophores examined included.

[d] Comparison of partial regression coefficients to test for differences in pattern of melanophore movements between wild-type and *puma* mutants.

$P < 0.005$). As expected from preliminary tests, *puma* mutants reared at 33°C had significantly fewer metamorphic melanophores than *puma* mutants reared at 24°C. Effects of growth rate were equally marked, however. At a low growth rate at 24°C, melanophore densities and patterns were indistinguishable from wild-type (post hoc means comparisons, all $P > 0.05$). In contrast, at a high growth rate at 24°C, effects on melanophore densities and patterns were much more severe and were indistinguishable from *puma* mutants reared at 33°C (post hoc comparisons of means, all $P > 0.05$). Thus, the pigment pattern phenotype of *puma* mutants depends on temperature, but also somatic growth rate. This result supports a model in which *puma* is essential for expanding a population of precursor cells that contribute to the adult pigment pattern; in the *puma* mutant, expansion of this cell population does not keep pace with body growth, resulting in an uncoupling of pigment pattern development and somatic metamorphosis.

Discussion

Our study indicates that *puma* mutant zebrafish exhibit a gross defect in postembryonic pigment pattern develop-

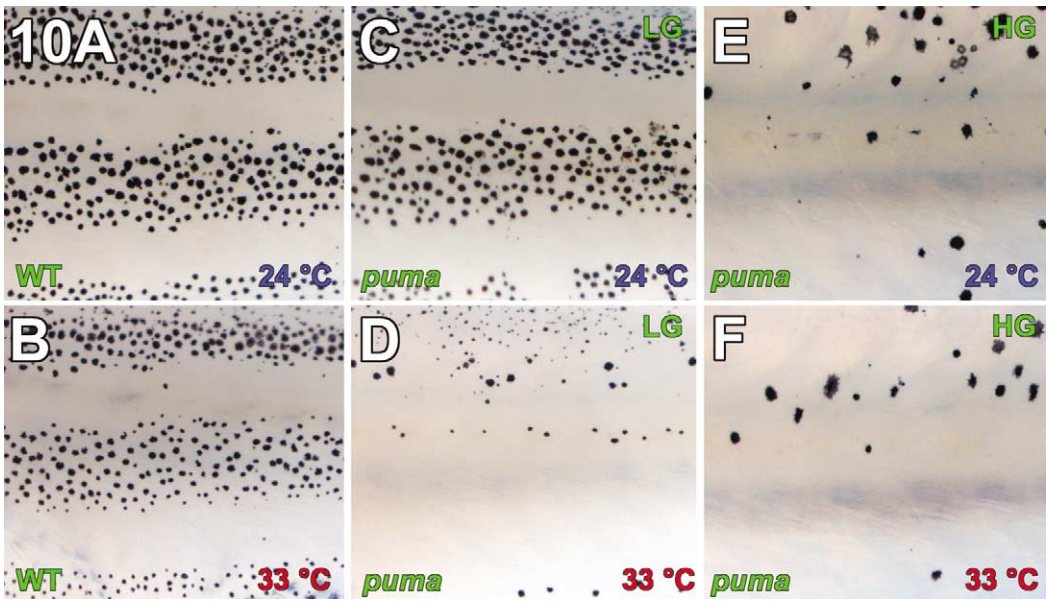

Fig. 10. Severity of the *puma* mutant phenotype depends on temperature and growth rate. (A) Wild-type individuals reared at 24°C develop typical wild-type melanophore stripes. (B) Wild-type individuals reared at 33°C also develop typical wild-type stripes. (C, D) The *puma* mutant phenotype is temperature-dependent. (C) At 24°C, melanophore densities and patterns approach that seen in wild-type individuals. (D) At 33°C, however, a severe melanophore deficiency and pattern defect is observed. (C–F) The severity of the *puma* mutant phenotype depends on individual growth rate as well as temperature. LG, low growth. HG, high growth. (C, D) *puma* mutants reared under conditions favoring a low growth rate (LG) exhibit markedly different phenotypes depending on the temperature they experience during development. (E, F) *puma* mutants reared under conditions favoring a high growth rate (HG) exhibit similar phenotypes across temperatures, since even individuals reared at 24°C exhibit a severe melanophore deficiency. In contrast to *puma* mutants, phenotypes of wild-type fish did not differ dramatically given different growth rates (e.g., A, high growth; B, low growth). In all images, the region shown is immediately dorsal to the anal fin.

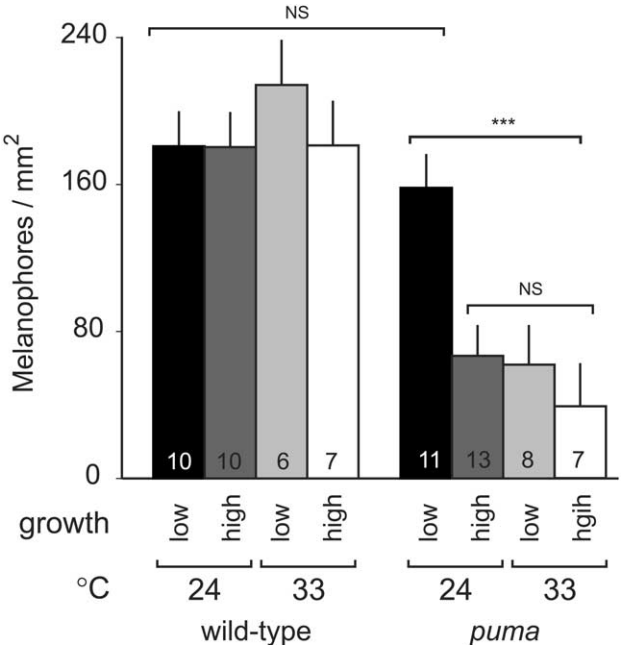

Fig. 11. Quantitative analyses of melanophore densities in wild-type and *puma* mutants reared under different growth and temperature regimes. Temperature and growth rate was not associated with marked variation in melanophore densities among wild-type fish. In contrast, higher growth rates resulted in significantly reduced melanophore densities in *puma* mutants, and this effect was particularly evident among fish reared at 24°C. Shown are means ± 95% confidence intervals. Numbers at bases of bars represent numbers of individuals examined.

ment: at its most extreme, the *puma* mutant phenotype represents a complete failure of pigment pattern metamorphosis and the retention of an embryonic/early larval (EL) population of melanophores into adult stages. Thus, the *puma* mutant defect is qualitatively different from previously analyzed adult pigment pattern mutants in zebrafish that either ablate melanophores completely, or distinguish between different classes of metamorphic melanophores. The *puma* mutation thus decouples pigment pattern metamorphosis from many other aspects of somatic metamorphosis (also see Parichy et al., 2003). Our analyses of this defect in comparison to wild-type development provide new insights into the fates of EL melanophores, the cellular bases for pigment pattern metamorphosis, and the potential for pattern regulation when normal metamorphic events are disrupted.

Fates of embryonic/early larval melanophores during pigment pattern metamorphosis

The extent to which phenotypes are remodeled at metamorphosis remains largely unknown. One way in which metamorphic remodeling can occur is through the loss of premetamorphic cells and their replacement by new cell populations that are recruited to differentiate during and after metamorphosis (Alberch and Gale, 1986; Alley, 1989). In zebrafish, image series and genetic analyses demonstrate that new melanophores differentiate during metamorphosis (this study; Johnson et al., 1995; Parichy et al., 2000a). The fate of melanophores comprising the early larval pigment pattern has remained unclear, however. Such cells might persist or might be lost during the larval-to-adult transition; these possibilities have different implications both for pigment pattern development and for pigment pattern evolution. For example, if selection favors qualitatively different pigment patterns in larvae and adults, then a more complete cellular and genetic uncoupling of larval and adult patterns should permit faster evolutionary changes at one or both stages; if larval and adult patterns share common cellular bases and genetic determinants, then selection response at one stage may be biased by selection at the other stage (Ebenman, 1992; Haldane, 1932). Mutational analyses have identified genetically separable populations of melanophores that arise during pigment pattern metamorphosis. Yet, these analyses have not been able to partition contributions of EL melanophores and metamorphic melanophores because previously identified mutants that ablate EL melanophores also ablate some or all metamorphic melanophores (e.g., Lister et al., 1999; Parichy et al., 1999). Our analyses show that EL and metamorphic melanophores are genetically separable, as *puma* mutants retain EL melanophores into adult stages, but fail to develop a normal population of metamorphic melanophores.

The finding that EL melanophores persist in *puma* mutants led us to examine the fates of EL melanophores in wild-type fish. We find that some wild-type EL melanophores persist and contribute to the adult pigment pattern, whereas others disappear during pigment pattern metamorphosis. Thus, early larval and adult pigment patterns are not completely independent at the cellular level: temporally distinct melanophore populations arise in embryos/early larvae and during metamorphosis, but the final striped pattern of melanophores in adults depends on contributions from each of these populations. Although our analyses suggest that EL melanophores constitute a relatively small proportion of the total number of adult stripe melanophores (being found principally along the ventral edge of the adult dorsal primary melanophore stripe), their presence suggests caution when interpreting mutant phenotypes in adults. For example, stripes and spots of melanophores that persist in *ednrb1* and *fms* mutants (Johnson et al., 1995; Parichy et al., 2000a,b) could represent early-appearing metamorphic melanophores, EL melanophores (or their progeny) persisting to adult stages, or both.

We further demonstrate that *puma* is essential for the normal patterning of EL melanophores during metamorphosis. In *puma* mutants, movements of EL melanophores can be greater than in wild-type larvae, though many melanophores moved little if at all. *puma* mutant EL melanophores also persisted ectopically in the middle of the flank. Formally, these differences from wild-type could reflect cell-autonomous roles for *puma* in promoting the normal migra-

tion or death of these cells. A more compelling explanation, however, is that *puma* activity is indirect, and EL melanophore fates depend on other events of pigment pattern metamorphosis that are directly dependent on *puma*. For example, enhanced variability in EL melanophore movements could reflect the severe deficit in the numbers of metamorphic pigment cells (see below): an absence of "population pressure" and contact-stimulated migration would keep some EL melanophores from leaving their initial positions, whereas EL melanophores stimulated to move could migrate further owing to persistence of directionality and reduced contact inhibition (Thomas and Yamada, 1992; Tucker and Erickson, 1986). Likewise, the persistence of EL melanophores in the middle of the flank may result from the failure of *puma* mutants to produce normal numbers of xanthophores (Parichy et al., 2003), which normally occupy this region and are associated with the death of isolated melanophores (Goodrich et al., 1954; Goodrich and Greene, 1959; Parichy et al., 2000a).

puma-dependence of pigment pattern metamorphosis

Our analyses demonstrate that *puma* promotes the development of normal numbers of melanophores that appear during pigment pattern metamorphosis: in comparison with wild-type larvae, metamorphosing *puma* mutants exhibit a dramatic reduction in the numbers of melanophore births, reflecting a failure of metamorphic melanophore differentiation or proliferation. This deficit persists from early through late stages of pigment pattern metamorphosis (and is especially apparent at high temperature; see below). Thus, only when pigment pattern metamorphosis is largely completed in wild-type larvae do *puma* mutants recover substantial numbers of melanophores.

An intriguing aspect of the *puma* mutant phenotype is the patchiness and irregularity of the pattern that does develop. This patchiness is not likely to reflect directional migration of initially dispersed melanophores into irregular clusters, as our analyses revealed comparatively little movement by *puma* mutant metamorphic melanophores. These observations and the severe reduction in melanophore births in *puma* mutants suggest two explanations for this patchy and irregular phenotype. First, *puma* may be essential for promoting the expansion of a population of dispersed melanophore precursors during pigment pattern metamorphosis. Thus, the patchiness of the *puma* phenotype would reflect clonal expansion by a limited number of residual precursor cells. Indeed, the pigment pattern defect in *puma* mutants is reminiscent of mouse mutants and chimeras in which numbers of melanophore precursors are severely reduced (e.g., Shin et al., 1999; Wilkie et al., 2002; Yoshida et al., 1996). Molecular marker analyses are consistent with this model of how *puma* normally promotes pigment pattern development (Parichy et al., 2003).

A second explanation for the *puma* mutant phenotype invokes community effects. If differentiation of precursor cells as melanophores is more likely in the presence of already differentiated melanophores, or at some critical population density of precursors, then patches of melanophores should result. By uncoupling somatic growth and pigment pattern development, our analyses of growth-dependent variation support a model of community effects among melanophores or their precursors. When *puma* mutants are reared under conditions favoring a low somatic growth rate (and at low temperature, see below), nearly wild-type pigment patterns result. By contrast, when *puma* mutants are reared to the same size and stage in conditions favoring a high growth rate, melanophore numbers do not keep pace with somatic growth, resulting in much lower melanophore densities and a failure of organized stripes to form. An increased somatic growth rate coupled with a depressed rate of precursor population expansion could result in lower effective densities for precursors at any given location, making any required cell–cell interactions less likely. These sorts of community effects already have been suggested to promote the development of amniote melanoblasts (Aubin-Houzelstein et al., 1998), as well as the normal differentiation of neural crest-derived cells as glia (Hagedorn et al., 1999; Paratore et al., 2001). Our findings thus support a model in which community effects contribute to zebrafish melanophore stripe development (Parichy et al., 2000a; Parichy and Turner, 2003). This interpretation of the *puma* mutant phenotype is complementary to *puma* having a direct role in expanding a population of melanophore precursors (above), as community effects would be expected to act synergistically with changes in the numbers of precursors to determine the total numbers and distributions of melanophores.

In addition to exhibiting a growth-dependent phenotype, our study shows that the *puma* allele we analyzed is temperature-sensitive: at a low temperature, significantly greater melanophore densities and more regular patterns develop than at a high temperature. Although different temperatures result in different developmental and growth rates, we designed our experiment to partition variation between these factors and to identify synergistic interactions. Thus, we found not only temperature effects (after controlling for differences in growth rates), but also an interaction between temperature and growth: nearly wild-type phenotypes could be rescued at low temperature but only at a low growth rate; conversely, high temperatures resulted in more severe phenotypes independent of growth rate. These data indicate that $puma^{j115el}$ is temperature-sensitive independent of growth effects. Such temperature-sensitive alleles provide a valuable tool for assessing critical periods for gene function, as the activity of gene products can be enhanced or curtailed by shifting fish between permissive and restrictive temperatures at different stages of development (e.g., Parichy and Turner, 2003; Rawls and Johnson, 2001). Current efforts are aimed at positionally cloning the *puma* gene, so the temperature-sensitivity of this allele cannot yet be related to functional characteristics of the gene product. Classically, temperature-sensitivity would be expected to reflect an amino acid substitution that destabilizes the protein product,

thereby inhibiting its function at the restrictive temperature. Nevertheless, it remains conceivable that temperature-sensitivity here reflects a less direct physiological effect (e.g., if higher temperatures result in a toxic accumulation of some metabolic byproduct).

puma mutant reveals capacity for pigment pattern regulation in adult zebrafish

It remains an open question to what extent the mechanisms of normal development are recapitulated by pattern regulatory processes that occur following genetic or environmental perturbation. This issue is most dramatically illustrated in the regeneration of entire organs or appendages by adult animals, but the phenomenon of pattern regulation is more general, particularly in vertebrate embryos in which it has been considered both an obstacle to experimental analysis and an interesting feature of development in its own right. Indeed, analyses of pattern regulation can identify otherwise cryptic developmental potential. For example, analyses of *patch* and *rump-white* mutant mice reveal a population of late-developing melanocyte precursors that allow partial recovery of pigmentation by adult stages (Jordan and Jackson, 2000). Our analyses of *puma* mutant zebrafish indicate that partial regulation of the pigment pattern occurs during terminal stages of metamorphosis and juvenile development.

Pattern regulation in *puma* mutants depends on melanophores that are recovered only after melanophore stripes are largely completed in wild-type fish. These very late-appearing melanophores could come from: (1) the same populations of cells that normally contribute to stripe development during pigment pattern metamorphosis; or (2) a distinct population of cells that does not normally contribute to stripe development, but can be recruited to do so during regulation of the pigment pattern. Results of this and the accompanying study do not exclude the first possibility, but are consistent with the latter possibility. In particular, *puma* mutants recovered many melanophores in dorsal regions of the flank at a time when numerous melanophores appear over the dorsal surface of the flank in wild-type fish. Although many of these cells presumably populate developing scales (Sire et al., 1997), some may be recruited into stripes in the *puma* mutant. Consistent with this idea, fish that are doubly mutant for *puma* and *kit*, which ablates dorsal scale melanophores, almost completely lack melanophores and stripes (Parichy et al., 2003). Collectively, these data together with studies of fin stripe regeneration (Rawls and Johnson, 2000, 2001) and analyses of earlier development (Raible and Eisen, 1996; Vaglia and Hall, 1999, 2000) reveal the extensive regulatory abilities of zebrafish neural crest and pigment cell lineages.

Acknowledgments

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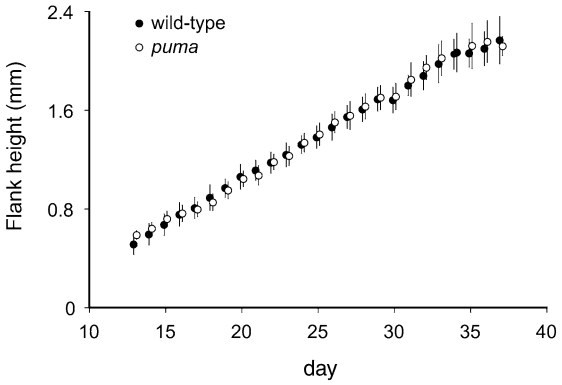
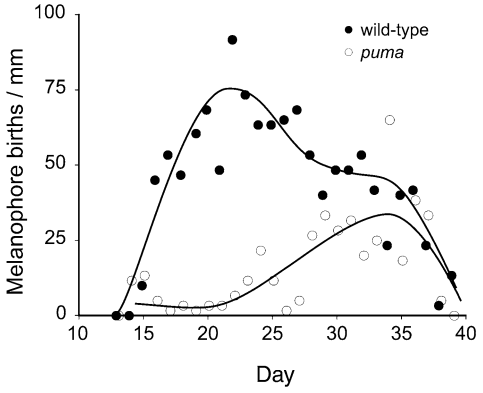
Appendix



Suppl. Fig. 1. Somatic growth is similar between wild-type and *puma* mutant larvae reared in isolation. Shown is the mean ± SD dorsal–ventral height of the flank at the posterior margin of the anal fin. Flank height did not differ between wild-type and *puma* mutants ($P = 0.5$), after controlling for differences in growth rates among individuals ($P < 0.0001$).



Suppl. Fig. 2. *puma* is essential for normal numbers of melanophore births. Shown are mean numbers of newly appearing melanophores per day in wild-type (closed circles) and *puma* mutants (open circles). Wild-type larvae exhibit an increase in melanophore births until a peak at ~22 dpf, followed by a decline during later stages of metamorphosis and juvenile development. In contrast, *puma* mutants exhibit low melanophore birth rates through middle metamorphic stages, and only reach a peak of melanophore births during terminal stages of pigment pattern metamorphosis. Fitted lines are smoothing splines estimated by the JMP Statistical Package (see Statistical methods). Error bars are omitted for clarity, though variability in birth rates among *puma* mutants was greater than among wild-type individuals.

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