Deconstructing evolution of adult phenotypes: genetic analyses of kit reveal homology and evolutionary novelty during adult pigment pattern development of Danio fishes

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The cellular bases for evolutionary changes in adult form remain largely unknown. Pigment patterns of Danio fishes are a convenient system for studying these issues because of their diversity and accessibility and because one species, the zebrafish D. rerio, is a model organism for biomedical research. Previous studies have shown that in zebrafish, stripes form by migration and differentiation of distinct populations of melanophores: early metamorphic (EM) melanophores arise widely dispersed and then migrate into stripes, whereas late metamorphic (LM) melanophores arise already within stripes. EM melanophores require the kit receptor tyrosine kinase, as kit mutants lack these cells but retain LM melanophores, which form a residual stripe pattern. To see if similar cell populations and genetic requirements are present in other species, we examined D. albolineatus, which has relatively few, nearly uniform melanophores. We isolated a D. albolineatus kit mutant and asked whether residual, LM melanophores develop in this species, as in D. rerio. We found that kit mutant D. albolineatus lack EM melanophores, yet retain LM melanophores. Histological analyses further show that kit functions during a late step in metamorphic melanophore development in both species. Interestingly, kit mutant D. albolineatus develop a striped melanophore pattern similar to kit mutant D. rerio, revealing latent stripe-forming potential in this species, despite its normally uniform pattern. Comparisons of wild types and kit mutants of the two species further show that species differences in pigment pattern reflect: (1) changes in the behavior of kit-dependent EM melanophores that arise in a dispersed pattern and then migrate into stripes in D. rerio, but fail to migrate in D. albolineatus; and (2) a change in the number of kit-independent LM melanophores that arise already in stripes and are numerous in D. rerio, but few in D. albolineatus. Our results show how genetic analyses of a species closely related to a biomedical model organism can reveal both conservatism and innovation in developmental mechanisms underlying evolutionary changes in adult form.

KEY WORDS: Zebrafish, Pigment pattern, Morphogenesis, kit, Melanophore, Evolution

INTRODUCTION

Identifying the mechanisms underlying evolutionary changes in form remains a central problem in biology. Analyses of molecular mechanisms provide insights into species and population differences (Shapiro et al., 2004; Yamamoto et al., 2004; Gompel et al., 2005; Hoekstra et al., 2006). Yet, evolutionary and developmental changes in gene activity, and their consequences for organismal form, are interpretable only in a cellular context. For many traits, this context remains poorly understood. A deeper knowledge of how genotypes are translated into phenotypes thus requires a focus on cells: how processes of morphogenesis and differentiation are orchestrated, and how these behaviors are modified within or between species (Parichy, 2005). Here, we test whether distinct cell populations underlying pigment stripe development in the zebrafish, Danio rerio, are present elsewhere in Danio, and how behaviors of these populations have changed evolutionarily.

Pigment patterns of Danio fishes provide an opportunity to study genes underlying evolutionary change, the resulting cellular consequences, and how alterations in cell behaviors affect species differences in form. Danios exhibit virtually indistinguishable embryonic and early larval pigment patterns but a diverse array of adult pigment patterns, ranging from horizontal stripes to vertical bars, and from uniform patterns to alternating spots and lines (Quigley et al., 2004; Quigley et al., 2005; Parichy, 2006). Pigment cells comprising these patterns include black melanophores, yellow xanthophores, iridescent iridophores and red erythrophores (Kelsh, 2004; Parichy et al., 2006). Because the cells are readily visible, they allow for analyses of cell behaviors — and how these behaviors differ among species – even as pigment patterns develop in the living fish.

Of the many danio adult pigment patterns, that of the zebrafish, D. rerio, is most studied: in a comparative context, the understanding of pattern-forming mechanisms in D. rerio can be used to suggest hypotheses for changes in genes and cell behaviors that may underlie pattern differences among species. Danio rerio embryos develop an early larval pigment pattern that is transformed into the adult pigment pattern beginning ~2 weeks post-fertilization. This metamorphosis involves the loss of embryonic/early larval melanophores and the appearance of ‘metamorphic’ melanophores that develop from latent precursors (Johnson et al., 1995; Parichy et al., 2000b; Parichy and Turner, 2003b; Quigley et al., 2004). After ~2 additional weeks, an early adult pigment pattern has formed, consisting of two dark ‘primary’ stripes of melanophores and a single light ‘primary interstripe’ of xanthophores and iridophores. During later growth, additional stripes and interstripes are added (Fig. 1A).

Metamorphic melanophores in adult stripes of D. rerio appear homogeneous yet actually comprise two populations (Fig. 1B) (Johnson et al., 1995; Parichy et al., 1999; Parichy et al., 2000b). Early metamorphic (EM) melanophores appear first scattered over the flank, but then migrate to sites of stripe formation. Late metamorphic (LM) melanophores develop subsequently within the nascent stripes.
These populations are genetically distinct. EM melanophores depend on the kit receptor tyrosine kinase, which is expressed by melanophores and their precursors. Mutants for a null allele, kit\textsuperscript{R2}, completely lack EM melanophores, yet retain LM melanophores, which develop in two to three sparsely populated stripes (Fig. 1C, fish 1 versus fish 2) (Johnson et al., 1995; Parichy et al., 1999). This situation differs from mouse, in which Kit null alleles lack all melanocytes (Besmer et al., 1993; Wehrle-Haller, 2003). LM melanophores are kit-independent, yet are ablated in mutants for colony stimulating factor-1 receptor (cfsfr, previously known as fms) and endothelin receptor b1 (ednrb1). When mutants for either of these genes are combined with kit\textsuperscript{R2}, both EM and LM melanophores are lost (Johnson et al., 1995; Parichy et al., 2000a; Parichy et al., 2000b; Rawls et al., 2001) (Fig. 1C, fish 3). In D. rerio then, EM melanophores are kit-dependent whereas LM melanophores are kit-independent (though cfsfr-dependent and ednrb1-dependent).

In an evolutionary context, the discovery of EM and LM melanophores prompts two questions. First, are distinct metamorphic melanophore populations found in other danios? These could be a unique, derived feature of pigment pattern development: D. rerio has more distinctive stripes than other species, so LM melanophores that develop in these stripes might be an evolutionary innovation responsible for this phenotype. Or, distinct EM and LM melanophores could be a conserved feature of pigment pattern development among danios. Second, if EM and LM melanophores occur outside of D. rerio, have evolutionary changes in pigment patterns resulted from modifying one or the other melanophore population?

As EM and LM melanophores are defined by mutant phenotypes, one approach to testing for their presence in other species would be to isolate heterospecific mutants for the corresponding, orthologous genes. Here, we ask if both EM and LM melanophores are present in D. albolineatus, by isolating a D. albolineatus kit mutant. We chose this species because it represents a different Danio clade from D. rerio (Quigley et al., 2005) and because it exhibits a different pigment pattern of nearly uniformly dispersed melanophores that might, a priori, result from very different underlying mechanisms (Fig. 1D,E).

We can make several predictions. For example, if distinct EM and LM melanophores are not present in wild-type D. albolineatus (Fig. 1F, fish 4), then either: all melanophores are kit-dependent (as in mouse), and a kit mutant should completely lack melanophores (Fig. 1F, fish 5); or all melanophores are kit-independent and a kit mutant should resemble the wild type. As melanophores are widely dispensed in D. albolineatus, it might be anticipated that only dispersed EM melanophores would be present and LM melanophores would be absent, as the latter develop only in stripes in D. rerio. Consistent with the idea that LM melanophores might be missing in D. albolineatus, we previously showed that cfsfr may have contributed to stripe loss in this species (Parichy and Johnson, 2001; Quigley et al., 2005); kit mutant D. albolineatus might therefore resemble kit; cfsfr double-mutant D. rerio (Fig. 1C, fish 3). By contrast, if D. albolineatus has distinct EM and LM melanophores, then a kit mutant should develop some melanophores but not others. The pattern of residual melanophores should then reveal if species differences reflect evolutionary alterations to EM melanophores, LM melanophores or both (Fig. 1F, fishes 6 and 7; see below).

Our analyses demonstrate that D. albolineatus exhibit distinct populations of kit-dependent EM and kit-independent LM melanophores, suggesting that these populations may be present more generally in Danio. We find that kit mutant D. albolineatus develop LM melanophores, and that these melanophores develop in stripes – similar to kit mutant D. rerio – despite the nearly uniform melanophore pattern in adults. Nevertheless, kit mutant D. albolineatus develop fewer LM melanophores than do kit mutant D. rerio. These findings indicate that the difference between D. rerio and D. albolineatus pigment patterns evolved by the extent to which a pattern of stripes is enhanced or obscured as: EM melanophores
migrate (D. rerio) or fail to migrate (D. albolineatus); and as LM melanophores develop in large numbers (D. rerio) or in small numbers (D. albolineatus) at sites of stripe formation. In defining the cellular context for pigment pattern formation in these species, our study sets the stage for analyses of molecular mechanisms underlying evolutionary diversification, as well as the evolution of the kit function in pigment cell lineages.

MATERIALS AND METHODS

Fish stocks

Fish were maintained at 26-28°C (14 hours light; 10 hours dark). D. rerio were the inbred mapping strain AB<sup>wp</sup> or kit<sup>N</sup> maintained in the AB<sup>wp</sup> background. D. albolineatus were derived from stocks originally provided by M. McClure.

F2 non-complementation screen for kit mutant D. albolineatus

To obtain D. albolineatus mutant for the kit gene, we screened mutagenized D. albolineatus by non-complementation against kit<sup>N</sup>-mutant D. rerio. Adult male D. albolineatus were mutagenized three times over 3 weeks with 3 mmol/l N-ethyl-N-nitrosourea (Sigma) (Solnica-Krezel et al., 1994). These fish were then crossed to unmutagenized D. albolineatus females and their progeny were reared to maturity. Male F1 progeny of mutagenized fish were then crossed to female kit mutant D. rerio by in vitro fertilization. The resulting F2 hybrid embryos were reared through 4 days post-fertilization (dpf) and screened for a kit mutant embryonic melanophore defect (see Results). Hybrid families with non-complementation phenotypes identified founder D. albolineatus males potentially carrying a new mutant allele of D. albolineatus kit. Founder F1 D. albolineatus males were retested against kit mutant D. rerio and outcrossed to unmutagenized D. albolineatus females for recovery of new mutants entirely within the D. albolineatus background.

Molecular methods

For PCR and sequencing, genomic DNAs were isolated from small quantities of fin tissue. Caudal fins were collected in 50 μl DNA extraction buffer (80 mmol/l KCl, 10 mmol/l Tris pH 8.0, 1 mmol/l EDTA, 0.3% Tween-20, 0.3% NP-40), heated at 95°C for 5 minutes, and cooled on ice. Samples were then digested with a final concentration of 1 μg/μl proteinase-K at 56°C for 1 hour with occasional vortexing, heated at 95°C for 10 minutes, chilled on ice and then extracted with pH 8.5 phenol:chloroform:isoamyl alcohol (25:24:1). Recovered aqueous phases were diluted 1:40 for use in PCR. For analyses of cDNAs, total RNAs were isolated from fins or embryos with Trizol (Invitrogen) as per manufacturer’s instructions and cDNAs were synthesized using Superscript II reverse transcriptase (Invitrogen) and oligo-IT primer. Sequencing used ABI BigDye v3.1 chemistry and ABI 3100 capillary sequencers. Primer sets (forward, reverse) for RT-PCR were: A1, A<sub>ATGTTTCTCCGAGTGA-AATGTA</sub>, TGGACATGGAACCTGAGTTCCCT; A2, TGTCGGACTTG-TTCCAGACGCC, CCCCCTCATAGGACAACATCTGC; A3, CCTGAG-CCTGAGCTCTGGTGC, CCACGTAGCTCCGAGAAAC.

Imaging and quantitative methods

Fish were imaged using an Olympus SZX12 stereo microscope or Zeiss Axioplan 2 compound microscope interfaced to Axiocam II digital cameras. For quantification, images were transferred to Adobe Photoshop CS2 and analyzed using FoveaPro 4 (Reindeer Graphics). For analyzing melanophore densities, we measured the height of the flank at the anterior margin of the anal fin (hia). We then defined a square area of interest with equal dimensions of 0.6 haa, an anterior boundary marked by the anterior margin of the anal fin, and a dorsal boundary just ventral to where dorsal scale melanophores occur in both D. rerio and D. albolineatus (~0.3 haa from the dorsal margin of the flank). We counted all melanophores fully within areas of interest as well as melanophores overlapping anterior or dorsal edges.

Histological analyses of melanophore development

Tyrosinase-expressing presumptive melanophore precursors were identified by incubating larvae with the melanin precursor, L-dopa (McCauley et al., 2004; Quigley et al., 2004; Quigley et al., 2005). Larvae were fixed 2 hours in 4% paraformaldehyde in phosphate buffered saline (PBS), rinsed in three changes of PBS, then placed in a solution containing 0.1% L-dopa in PBS for 1 hour to overnight.

Fin regeneration experiments

Caudal fins were amputated with a razor blade about halfway between base and distal tips. Fins were allowed to regenerate in fish system water (~27°C) containing 0.2 mmol/l phenylthiourea (PTU) to inhibit melanin synthesis (Rawls and Johnson, 2000). To reveal newly differentiated melanophores, PTU-treated fish were imaged, transferred to system water without PTU, and re-imaged ~12 hours later when regenerative melanophores had developed melanin.

RESULTS

Isolation and molecular characterization of D. albolineatus kit mutant

As D. rerio and D. albolineatus can be hybridized in the laboratory (Parichy and Johnson, 2001; Quigley et al., 2005), we reasoned that a kit mutant D. albolineatus could be isolated by non-complementation against kit mutant D. rerio. For screening, we used a melanophore defect in kit mutant D. rerio at 60 hpf comprising fewer embryonic melanophores (particularly over the anterior head and yolk sac) and ectopic melanophores posterior to the otocyst (Fig. 2A,C). Over the next several days, the embryonic/early larval melanophores that are present die so that fish completely lack melanophores until LM melanophores develop during metamorphosis. These defects in embryonic/early larval melanophores reflect kit requirements both for migration and for survival (Parichy et al., 1999; Rawls and Johnson, 2003). We generated ~600 F2 hybrid families by crossing mutagenized F1 D. albolineatus to homozygous kit<sup>Ps</sup> mutant D. rerio. Several families exhibited melanophore defects without lesions in kit cDNAs and were discarded. One family exhibited all the expected kit mutant phenotypes in ~50% of hybrid offspring and we recovered the mutant (wp.a14e1) entirely within the D. albolineatus background from the presumptively heterozygous, mutagenized F1 D. albolineatus founder.

D. albolineatus embryos homozygous for the wp.a14e1 mutation exhibit fewer melanophores than wild type, ectopic melanophores behind the otocyst (Fig. 2B,D), and death of all embryonic/early larval melanophores over several days (data not shown). To see whether wp.a14e1 affects kit expression, we amplified a 196 bp amplicon (A1) from kit cDNA isolated from adult fins (Fig. 2E,F). RT-PCR for A1 revealed lower transcript abundance in wp.a14e1 compared with wild type. RT-PCR for a second amplicon, A2, further showed a smaller size in mutant cDNAs (Fig. 2E,F). Sequencing wp.a14e1 kit cDNA revealed a 356 bp deletion that corresponds to exons 5 and 6, as assessed by sequence comparison with the Ensembl predicted genomic structure for D. rerio kit (kita, ENSDARG00000043317). This deletion (N247Δ) causes a frameshift with 33 novel amino acids and a premature stop codon (Fig. 2F) upstream of the transmembrane and kinase domains.

As wp.a14e1 kit cDNA lacks precisely two exons, we considered the possibility that a splicing defect might reduce, without eliminating, wild-type transcript. To test this idea, we attempted to amplify a portion of the deleted exons (A3) by RT-PCR. While A3 amplified from wild type, we could not detect amplification from wp.a14e1 homzygotes, suggesting that no wild-type transcript was present (Fig. 2F,E). We also considered the possibility that alternative splicing downstream of the deleted exons could produce in-frame transcripts that retain some residual activity. To test this idea, we attempted to amplify full-length and nearly full-length kit...
Fig. 2. Isolation and molecular characterization of a kit mutant D. albolineatus and comparison with D. rerio. (A-D) Melanophore phenotypes of embryos at 60 hpf. (A) Wild-type D. rerio. (B) Wild-type D. albolineatus have fewer melanophores overall compared with D. rerio. (C) Homozygous kit-/- mutant D. rerio exhibit fewer melanophores, especially at sites distant from their origin in the neural crest (e.g. covering the yolk, arrow). An ectopic patch of melanophores (arrowhead) occurs posterior to the otocyst. (D) Homozygous wp.a14e1 D. albolineatus have fewer peripheral melanophores (arrow) and ectopic post-otic melanophores (arrowhead), although total melanophore numbers are greatly diminished compared with kit mutant D. rerio. (E) RT-PCR for amplicons (A1-A3) from D. albolineatus kit cDNAs. A1, lower transcript abundance in wp.a14e1 compared with wild type. A2, reveals the predicted 652 bp product in wild type but a 296 bp product in wp.a14e1. A3, failure to amplify a 234 bp product demonstrates absence of wild-type kit transcript in wp.a14e1. β-actin, loading control. (F) Schematics of D. albolineatus kit cDNAs and locations of amplicons A1-A3. Green, predicted signal sequence and transmembrane domain. Red, predicted kinase domains. In kitwp.a14e1 two exons are deleted, resulting in a frameshift with novel amino acids (orange) and a premature stop codon. Sequence analyses confirm that D. albolineatus kit is orthologous to D. rerio kit (kita), rather than a second kit locus identified in D. rerio, kitb (see Discussion) (Mellgren and Johnson, 2005). (G) Genomic structure of kit in wild type and wp.a14e1 mutant D. albolineatus. Shown are exons 4-6 and intervening introns (i4-i6). The region deleted in wp.a14e1 is shown in brown, and a novel inserted sequence is shown in orange. GenBank accession number for D. albolineatus kit: EF035010.

kit mutant reveals kit-dependent and kit-independent melanophores in D. albolineatus

Isolation of a kit mutant D. albolineatus allowed us to test whether genetically distinct metamorphic melanophores are present. We find that D. albolineatus resembles D. rerio in having distinct kit-dependent and kit-independent metamorphic melanophores. During early pigment pattern metamorphosis (Fig. 3; ~15-35 dpf), wild-type D. rerio developed new, metamorphic melanophores (although total melanophore densities changed relatively little, presumably due to countervailing effects of overall somatic growth). Wild-type D. albolineatus showed small increases in melanophore densities during this period. Simultaneously, kit mutants of both species completely lacked melanophores. Thus, D. albolineatus exhibits a population of early metamorphic kit-dependent melanophores; we designate these EM melanophores, and we infer that these cells correspond to the EM melanophores of D. rerio.

During late pigment pattern metamorphosis (Fig. 3; ~36-48 dpf), wild-type D. rerio exhibited a sharp but transient increase in melanophore density, whereas wild-type D. albolineatus showed a continued slow increase. kit mutants of both species developed residual kit-independent melanophores during this period. Thus, D. albolineatus exhibits late kit-independent metamorphic melanophores; we designate these cells LM melanophores, presumably corresponding to LM melanophores of D. rerio.

Pigment pattern evolution by changes in EM and LM melanophores

By subtracting away EM melanophores, and revealing a residual pattern of LM melanophores, kit mutants should provide a glimpse into the relative roles of these melanophore populations during pigment pattern evolution: if this species difference results principally from changes in LM melanophores, then kit mutant D. rerio and kit mutant D. albolineatus should have very different pigment patterns of residual LM melanophores (Fig. 1C,F; fish 2...
metamorphosis, wild-type *D. rerio* developed distinctive stripes (Fig. 4E); wild-type *D. albloineatus* developed a mostly uniform pattern of melanophores, although melanophores were absent from an ‘interstripe region’ that was more distinctive at these stages (Fig. 4F) than in the adult (Fig. 1D). Simultaneously, *kit* mutant *D. rerio* developed LM melanophores in residual stripes adjacent to the primary interstripe (Fig. 4G); likewise, *kit* mutant *D. albloineatus* developed LM melanophores in a stripe dorsal to the interstripe region, and sometimes ventrally as well (Fig. 4H), despite the more widely dispersed arrangement of melanophores in wild-type *D. albloineatus*. In *kit* mutants of both species, residual LM melanophores developed already within stripes and did not migrate from more dispersed locations on the flank. These events can be observed in movies of individual larvae (see Movie 1 in the supplementary material). The similar pigment patterns of *kit* mutants implicate *kit*-dependent EM melanophores in the species difference: EM melanophores migrate into stripes in *D. rerio* but fail to do so in *D. albloineatus*.

Further comparison of wild types and *kit* mutants reveals evolutionary changes in LM melanophores as well. In both *D. rerio* and *D. albloineatus*, *kit* inactivation caused a similar drop in total melanophore densities, corresponding to the loss of *kit*-dependent EM melanophores (Fig. 3). By contrast, LM melanophores that developed in *kit* mutants were far fewer in *D. albloineatus* than in *D. rerio* (Fig. 3, Fig. 4G,H). Together, these observations show that pigment pattern differences between wild-type *D. rerio* and wild-type *D. albloineatus* involve: (1) changes in the morphogenesis of *kit*-dependent EM melanophores; and (2) changes in the population size of *kit*-independent LM melanophores.

**Late *kit* requirement in metamorphic melanophore lineage development**

To better understand when *kit* is required within metamorphic melanophores, and whether this requirement is similar in *D. rerio* and *D. albloineatus*, we examined the distribution of late-stage melanoblasts, which are competent to produce melanin when supplied with the melanin precursor, L-dopa (Quigley et al., 2004). If *kit* is required during a late step in melanophore differentiation, L-dopa+ melanoblasts should be observed in *kit* mutants in regions where melanophores develop in wild type but not in *kit* mutants. Conversely, if *kit* is required at early steps of melanophore differentiation or specification, L-dopa+ melanoblasts should be absent from such regions in *kit* mutants. As *kit* mutants of both species lack melanophores over the dorsal, we examined this region at the end of pigment pattern metamorphosis. Following L-dopa incubation, very few newly melanized cells were observed in wild-type *D. rerio* (Fig. 5A,A'), suggesting that most melanoblasts had already differentiated as melanophores. In *kit* mutant *D. rerio*, larger numbers of L-dopa+ melanoblasts were present (Fig. 5B,B') than in wild type; these cells may die or simply fail to differentiate.

In wild-type *D. albloineatus*, L-dopa incubation revealed numerous previously unmelanized melanoblasts (Fig. 5C,C'). Many of these cells die without reaching a melanized stage, revealing a late block in melanophore development that contributes to the reduced total melanophore number in this species, as reported previously (Quigley et al., 2005). If *kit* functions at a similar step in melanophore development in *D. albloineatus*, then *kit* mutant *D. albloineatus* should have similar or greater numbers of L-dopa+ cells. Consistent with this prediction, L-dopa+ melanoblasts in *kit* mutant *D. albloineatus* were at least as numerous (Fig. 5D,D') as melanoblasts and melanophores in wild-type *D. albloineatus*. These results suggest that: (1) *kit* is required during a late step in metamorphic melanophore development; and (2) this requirement is similar between species.

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The *kit* mutant *D. albloineatus* phenotype shows that evolutionary changes in both EM melanophores and LM melanophores have occurred. During early pigment pattern metamorphosis, wild-type *D. rerio* and *D. albloineatus* exhibited mostly dispersed EM melanophores, and *kit* mutants of both species completely lacked melanophores (Fig. 4A-D). During late pigment pattern versus fish 6); or, if the species difference results principally from changes in EM melanophores, then the mutants should have similar pigment patterns of residual LM melanophores (Fig. 1C,F: fish 2, fish 7).

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Differential kit-dependence of regeneration melanophores and other lineages

In *D. rerio*, amputation of the fin is followed by regeneration of the fin and its pigment pattern (Goodrich and Nichols, 1931). An early population of ‘primary’ regeneration melanophores requires kit for its development, whereas a later population of ‘secondary’ regeneration melanophores develops independently of kit (Rawls and Johnson, 2000). We tested whether similar kit-dependent and kit-independent regenerative melanophores are present in *D. albolineatus*. In wild-type *D. albolineatus*, large numbers of regenerative melanophores develop by 10 days post-amputation (dpa) (Fig. 6A). In kit mutant *D. albolineatus*, there were far fewer melanophores even in the unamputated fin, and new regenerative melanophores failed to develop by 10 dpa (Fig. 6B); after longer periods (≥ 20 dpa) a few kit-independent regenerative melanophores were observed (Fig. 6C). Thus, kit-independent regenerative melanophores are found in both species. Finally, mammalian kit mutants have defects in hematopoiesis and primordial germ cell development (Russell, 1949; Besmer et al., 1993) that are not found in *D. rerio* kit mutants (Parichy et al., 1999). We observed no gross defects for hematopoiesis or fertility in kit mutant *D. albolineatus*.

**DISCUSSION**

We tested the hypothesis that genetically distinct populations of kit-dependent and kit-independent melanophores underlying stripe development in *D. rerio* also occur elsewhere in *Danio*. To this end, we isolated a presumptive null allele of kit in *D. albolineatus*, allowing us to dissect pigment pattern development genetically. Our analyses revealed distinct kit-dependent and kit-independent melanophores in *D. albolineatus*, as in *D. rerio*.
Additionally, our comparison of pigment patterns between wild types and kit mutants of the two species provided novel insights into evolutionary changes in cell behaviors promoting stripe formation in D. rerio, and an absence of stripes in D. albolineatus, with different changes affecting kit-dependent and kit-independent melanophores. These findings have implications for: (1) the cellular bases of pigment pattern formation within species; and (2) evolutionary transformations underlying these and other pigment pattern differences between species.

**Evolutionarily conserved melanophore populations**

The finding that D. rerio has both kit-dependent and kit-independent melanophores was surprising given the absence of kit-independent melanocytes in mouse (Johnson et al., 1995; Parichy et al., 1999). We found that D. albolineatus also has kit-independent melanophores. While the development of residual melanophores in kit mutant D. albolineatus might, in principle, indicate residual activity through kit<sup>pop.al14e1</sup>, this allele seems most likely to be null, given the nature of the lesion and our inability to detect either wild-type kit transcript, or alternative variants with intact open reading frames in kit<sup>pop.al14e1</sup> homozygotes. Thus, we infer that D. albolineatus has kit-independent LM melanophores, a discovery that is somewhat surprising given the mostly uniform pigment pattern of this species: one might have assumed a priori that LM melanophores, which develop only in stripes in D. rerio, would be completely absent in D. albolineatus. For instance, severe alleles of leopard mutant D. rerio have a uniform pattern (Asai et al., 1999) similar to D. albolineatus, yet leopard mutants lack LM melanophores (Johnson et al., 1995). This disparity in the development of superficially similar phenotypes illustrates the importance of manipulative experimental approaches, particularly in comparative studies.

Our demonstration that D. albolineatus has kit-independent melanophores shows that these cells are not unique to D. rerio and may be present more widely among danios. Whether these cells have a broader phylogenetic distribution remains uncertain; emerging transgenic technologies as well as genetic screens offer the prospect of testing for kit-independent melanophores in additional model organisms (Kelsh et al., 2004; Chapman et al., 2005; Goda et al., 2006; Sobkow et al., 2006). Whatever the phylogenetic distribution of these cells, our genetic deconstruction of pigment pattern evolution illustrates a powerful approach to identifying homology and novelty in the evolution of developmental mechanisms more generally.

While our data reveal kit-dependent EM melanophores and kit-independent LM melanophores, the reasons for the different genetic requirements of these populations remain obscure. At least three possibilities can be suggested. First, duplication of an ancestral kit gene with subsequent partitioning of daughter gene activities between EM and LM melanophores would seem an attractive explanation, given the history of such events for receptor tyrosine kinases (Braasch et al., 2006; Grassot et al., 2006). Nevertheless, a paralogous kit locus in D. rerio, kith, is not detectably expressed in either embryonic melanophores (Mellgren and Johnson, 2005) or metamorphic melanophores (D.M.P., unpublished).

Second, differential kit-dependence might reflect particular morphogenetic activities. For instance, mouse melanoblasts require kit during proliferative and migratory phases, yet become independent of kit transiently after reaching the dermis, and again once they reach the hair follicle (Yoshida et al., 1996). In D. rerio embryonic melanophores, kit is required initially for migration and, afterwards, transiently for survival (Rawls and Johnson, 2003). Conceivably EM and LM melanophores differ in their kit-dependence because they execute different morphogenetic behaviors. While it is tempting to associate kit-dependence with the migration of EM melanophores into stripes (Parichy et al., 2000b; Parichy and Turner, 2003b), the corresponding cells in D. albolineatus do not migrate substantially (Quigley et al., 2005) yet still require kit (this study), suggesting that other morphogenetic differences would have to explain differential kit-dependence of EM melanophores and LM melanophores.

Third, differential kit-dependence could result from compensatory function by another genetic pathway. Such a pathway would presumably be activated sufficiently only in LM melanophores, perhaps owing to their microenvironment. For example, Kit and Ednrb exhibit some functional redundancy in melanocytes (Hou et al., 2004; Aoki et al., 2005), and might have similar overlap in
melanophores. Consistent with this idea, ednrbl mutation ablates residual LM melanophores in kit mutant D. rerio (Johnson et al., 1995; Parichy et al., 2000a). This model predicts that endothelins, or ligands for other candidate receptors, should be present near LM melanophores but not EM melanophores.

Finally, our study provides new insights into the functions of kit in kit-dependent melanophores, and the extent to which these functions are conserved across species and developmental contexts. kit has been implicated in survival, proliferation, differentiation and migration in melanophore or melanocyte lineages, with different studies emphasizing different primary roles depending on the particular stage and system (Reid et al., 1995; Wehrle-Haller and Weston, 1995; Bernex et al., 1996; Langtimm-Sedlak et al., 1996; Yoshida et al., 1996; Mackenzie et al., 1997; Parichy et al., 1999; Kelsh et al., 2000). Recent studies have demonstrated requirements for zebrafish kit in embryonic melanophore migration and survival (Rawls and Johnson, 2003), for population expansion during larval melanophore regeneration (Yang et al., 2004; Yang and Johnson, 2006), and during terminal differentiation of regenerating fin melanophores as well as larval melanophores when their development is delayed experimentally (Rawls and Johnson, 2000; Mellgren and Johnson, 2004). Our finding of numerous melanoblasts in kit mutants of D. rerio and D. alblolineatus indicates that kit is required during late steps in metamorphic melanophore development as well, presumably to promote survival or differentiation. The various functions for kit suggest the flexible roles this signaling pathway plays during melanophore and melanocyte development, and the differential sensitivity of these functions to perturbations across developmental contexts.

**Cellular bases for species differences**

An examination of phenotypes within Danio suggests that a pattern including horizontal stripes is likely to be ancestral, whereas the especially distinctive stripes of D. rerio and the more uniform pattern of D. alblolineatus are both derived (Parichy and Johnson, 2001; Quigley et al., 2005). What are the cellular bases for these novel phenotypes? Our analyses indicate that species differences reflect changes in both EM and LM melanophores, yet these populations have been affected in different ways.

Despite the different pigment patterns of wild-type D. rerio and D. alblolineatus, we find similar melanophore stripes in kit mutants of both species. This implies that species differences depend in part on changes in the distribution of EM melanophores, which are subtracted away in the kit mutants. During normal development, EM melanophores arise dispersed over the flank in D. rerio and in D. alblolineatus. These cells migrate to join developing stripes in D. rerio, but migrate little in D. alblolineatus, remaining in their dispersed arrangement. Why should these cells not migrate? In D. rerio, melanophore movement into stripes requires interactions between melanophores and xanthophores (Parichy et al., 2000b; Parichy and Turner, 2003a), as well as interactions between melanophores themselves (Maderspacher and Nusslein-Volhard, 2003; Watanabe et al., 2006). The lack of EM melanophore migration in D. alblolineatus might reflect changes in these cellular interactions, a possibility supported by interspecific hybridization studies (Quigley et al., 2005). A variety of candidate patterning molecules (Nishimura et al., 1999; Santiago and Erickson, 2002; Iwashita et al., 2006; Watanabe et al., 2006) could contribute to such interactions and are currently being tested for such roles. Nevertheless, factors other than cell–cell interactions could explain this species difference as well. For example, the kit pathway is itself a candidate: both kit mutant D. rerio embryos and wild-type D. alblolineatus adults have fewer melanophores, reduced melanophore migration, increased melanophore death and numerous melanophores and melanoblasts in the epidermis (Quigley et al., 2005). While the kit mutant phenotype of D. alblolineatus shows that kit retains a function in this species, these data do not exclude more subtle evolutionary changes in kit or its pathway.

Our analyses also show that kit-independent LM melanophores have contributed to the species difference between D. rerio and D. alblolineatus. Whereas D. rerio develops numerous LM melanophores in its nascent stripes, D. alblolineatus develops far fewer of these cells. If, as we surmise, no kit activity is present in either kit mutant, we can ask how LM melanophore populations have changed to make stripes more or less conspicuous. If LM melanophores develop due to kit activity or compensatory activity by a different genetic pathway (e.g. endothelin signaling), then such loci would be good candidates for contributing to the species difference. However, LM melanophores of D. rerio also require csf1r, and this pathway has itself been implicated in generating the different pigment patterns of D. rerio and D. alblolineatus. In D. rerio, csf1r promotes xanthophore development and LM melanophore development (Parichy et al., 2000b), whereas D. alblolineatus has more xanthophores and fewer LM melanophores than D. rerio (this study) (Quigley et al., 2005). This might be seen as a csf1r-dependent change in the allocation of cells, perhaps from a common precursor, toward xanthophores and away from LM melanophores. Nevertheless, our histological analyses show that late-stage melanoblasts are plentiful in D. alblolineatus, arguing against this model. Further dissection of LM melanophore development in D. rerio should clarify the roles of csf1r and other pathways, and should suggest additional hypotheses for species differences.

This study indicates that a mostly uniform pigment pattern in D. alblolineatus arose in part by obscuring an ancestral stripe pattern: through a failure of EM melanophores to migrate into stripes, and by a reduced number of LM melanophores constituting the stripes themselves. The residual stripes that form in kit mutant D. alblolineatus reveal latent stripe-forming potential that is, nevertheless, somewhat predicted by the phenotype of wild-type larval D. alblolineatus, in which melanophores adjacent to the primary interstripe tend to be larger and darker (Fig. 1D). A similar pattern comprising primary melanophore stripes and a primary interstripe occurs in other juvenile danios, some of which then develop adult pigment patterns very different from either D. rerio or D. alblolineatus (Quigley et al., 2004) (e.g. D. dangilar; D.M.P., unpublished, and movies at http://protist.biology.washington.edu/ dparchy). Conceivably, the simple juvenile pattern of stripes and interstripe is a ‘groundplan’ that is modified in different ways in different Danio species. An analogous groundplan is present in larval salamanders, in which the lateral lines have a conserved role in initiating melanophore stripe formation: stripes have been enhanced by additional stripe-forming mechanisms in one species, and obscured by changes in pigment cell numbers and behaviors in other species (Parichy, 1996b; Parichy, 1996a). Pigment pattern groundplans also have been described for butterflies (Nijhout, 1991). Our findings illustrate how mechanistic dissection of phenotypes can provide novel insights into evolutionarily conserved and derived features, and how the modularity of pigment patterns can generate diversity through alterations in some but not other pattern elements.

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