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

Margaret G. Mills*, Richard J. Nuckels* and David M. Parichy[†]

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.

Department of Biology, Institute for Stem Cell and Regenerative Medicine, University of Washington, Box 351800, Seattle WA 98195-1800, USA.

*These authors contributed equally to this work
[†]Author for correspondence (e-mail: dparichy@u.washington.edu)

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^{b5}, completely lack EM melanophores, yet retain LM melanophores, which develop in two to three sparsely populated stripes (Fig. 1C, fish

Fig. 1. *Danio* adult pigment patterns, pigment pattern metamorphosis and genetic analyses, either real or envisaged.
(**A-C**) *Danio rerio*. (A) Wild-type *D. rerio* exhibit around five melanophore stripes with light interstripes. (B) Early (left) to late (right) stages of adult pigment pattern metamorphosis. EM melanophores (light green) differentiate in a dispersed pattern then migrate (red tracks) toward developing stripes. LM melanophores (light blue) differentiate already at sites of stripe formation. Fully differentiated melanophores within adult stripes come from either EM (dark green) or LM (dark blue) melanophore populations. Other pigment cell classes are omitted for clarity. (C) EM melanophores are *kit*-dependent, whereas LM melanophores are *kit*-independent. Wild-type *D. rerio* (fish 1) with a full complement of melanophores and stripes (black). *kit* mutant *D. rerio* (fish 2) lacks EM melanophores but develops residual LM melanophores in stripes (gray) having fewer than wild-type numbers of melanophores. Fish doubly mutant for *kit* and *csf1r* or *kit* and *ednrb1* (fish 3) lack body melanophores, as both EM and LM melanophores are missing. (**D-F**) *Danio albolineatus*. (D) Wild-type *D. albolineatus* have melanophores that are nearly uniformly dispersed, although a narrow interstripe is found posteriorly (arrow). (E). Pigment pattern metamorphosis involves the differentiation of initally dispersed melanophores (gray) that show little migratory behavior. Some of these melanophores differentiate fully (black) but many more die (open cells) than in *D. rerio* (Quigley et al., 2005). (F) Wild-type *D. albolineatus* (fish 4) with uniformly distributed melanophores (black), and possible phenotypes of *kit* mutant *D. albolineatus*. If *D. albolineatus* have only *kit*-dependent EM melanophores, a *kit* mutant should lack melanophores completely (fish 5). If distinct *kit*-dependent EM and *kit*-independent LM melanophores are present, however, a *kit* mutant should develop residual melanophores (fishes 6 or 7) that are fewer than in wild-type (gray uniform pattern or gray stripes). The pattern of these cells would reveal how different populations have contributed to the difference between wild-type *D. rerio* and wild-type *D. albolineatus*: if changes only in LM melanophores have occurred, then a *kit* mutant (lacking EM melanophores) might resemble wild-type *D. albolineatus* (fish 6); or, if changes only in EM melanophores have occurred, then a *kit* mutant *D. albolineatus* (fish 7) should resemble *kit* mutant *D. rerio* (fish 3). Fish ages: ~6 months. Scale bar: in A, 4 mm for A,D.

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* (*csf1r*; previously known as *fms*) and *endothelin receptor b1* (*ednrb1*). When mutants for either of these genes are combined with kit^{b5}, 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 *csf1r*-dependent and *ednrb1*-dependent).
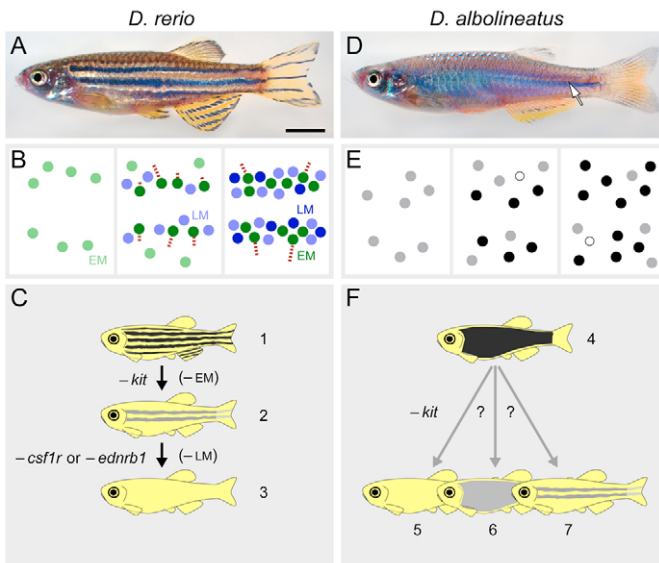
In an evolutionary context, the discovery of EM and LM melanophores prompts two questions. First, are distinct metamorphic melanophore populations found in other danios? These could be a unique, derived feature of pigment pattern development: *D. rerio* has more distinctive stripes than other species, 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 dispersed 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 *csf1r* 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*; *csf1r* 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 *kit* function in pigment cell lineages.

MATERIALS AND METHODS
Fish stocks
Fish were maintained at 26-28°C (14 hours light: 10 hours dark). *Danio rerio* were the inbred mapping strain AB^{wp} or *kit^{b5}* maintained in the AB^{wp} background. *Danio 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^{b5}* 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-dT priming. Sequencing used ABI BigDye v3.1 chemistry and ABI 3100 capillary sequencers. Primer sets (forward, reverse) for RT-PCR were: A1, AGTTTTCCCCGAGTGA-AATGTA, TGGACATGAGAACTGGATTCCT; A2, TGTCCGACTTG-TTCCAGACGCG, CCCTTCATAGGACACAATCTGC; A3, CCTGAG-CCTGAGCTCTGTGAC, CCACTGTAGCTCCAGGAAAAC.

Imaging and quantitative methods
Fish were imaged using an Olympus SZX12 stereomicroscope or Zeiss Axioplan 2 compound microscope interfaced to Axiocam II digital cameras. For quantitation, 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 (haa). 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 tip. 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^{b5}* 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.

Danio 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* homozygotes, 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*
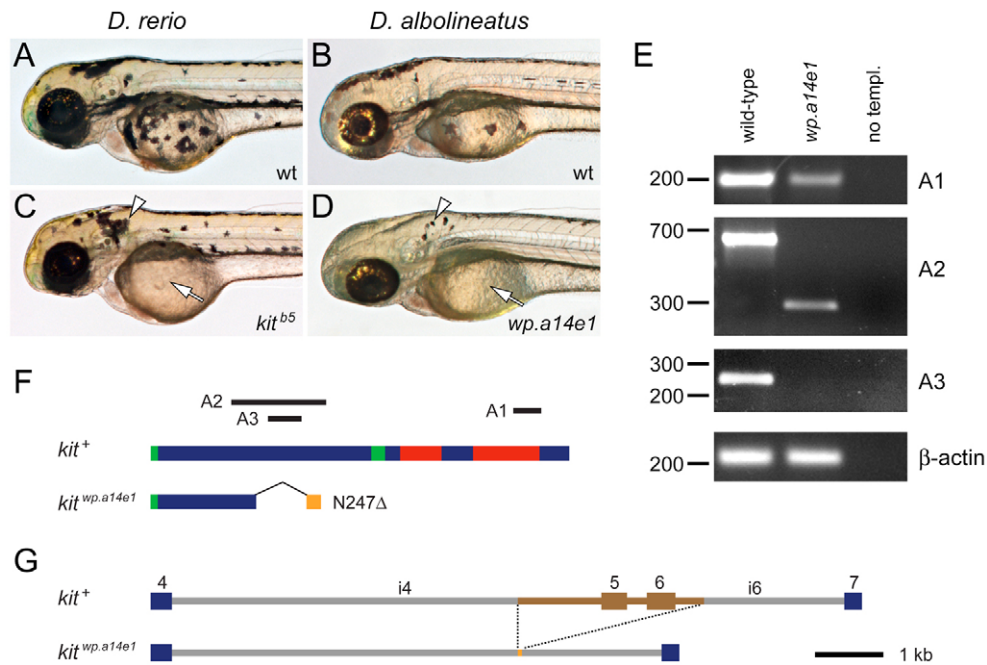
Fig. 2. Isolation and molecular characterization of a *kit* mutant *D. albolineatus* and comparison with *D. rerio*. (A-D) Melanophore phenotypes of embryos at 60 hpf. (A) Wild-type *D. rerio*. (B) Wild-type *D. albolineatus* have fewer melanophores overall compared with *D. rerio*. (C) Homozygous *kit*[b5] 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 *kit*[wp.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.

cDNAs from wild type and *wp.a14e1* homozygotes. Although an appropriately sized fragment amplified from wild type, we could not detect multiple smaller transcripts in *wp.a14e1* (data not shown).

To identify the lesion corresponding to the *wp.a14e1* mutation, we sequenced genomic DNAs within the *kit* locus (Fig. 2G). These analyses revealed a deletion extending from intron 4 to intron 6 with the resulting loss of exons 5 and 6, as well as a 28 bp insertion of unknown origin. Together, these data indicate that *wp.a14e1* corresponds to a deletion within *kit* and is very likely to be a null allele. For simplicity, we refer to *kit*[wp.a14e1] and *kit*[b5] as '*kit*' for *D. albolineatus* and *D. rerio*, respectively.

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
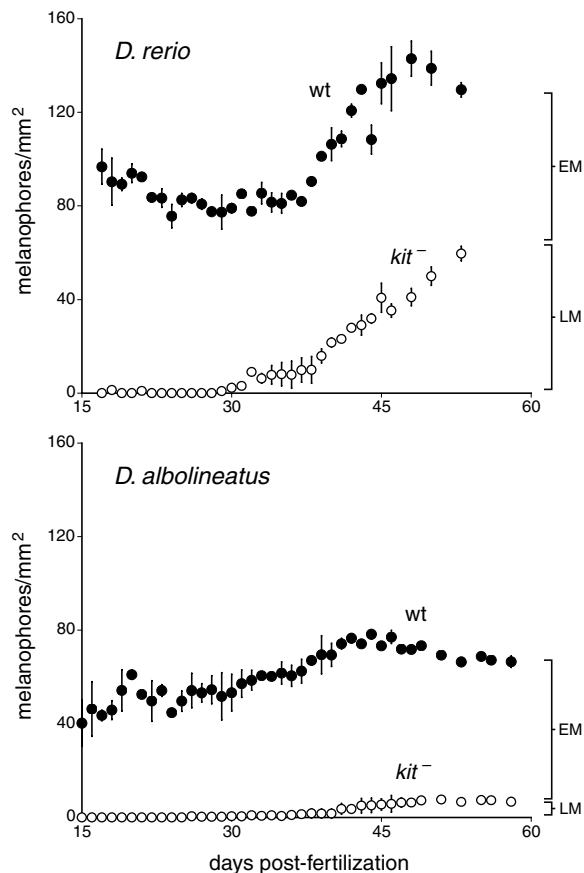
Fig. 3. Quantitative analyses of melanophore accumulation in wild-type *D. rerio* and *D. albolineatus* and their respective *kit* mutants. In *D. rerio* (upper plot) wild-type melanophore densities (means±1 s.e.) are relatively constant during the first half of pigment pattern metamorphosis (~15-36 dpf) but then increase dramatically during the second half of pigment pattern metamorphosis (~37-49 dpf), corresponding to the appearance of new melanophores within stripes. Note that total melanophore numbers (as opposed to densities) increase throughout metamorphosis. In *kit* mutants there are no melanophores until late metamorphosis when residual LM melanophores develop. In *D. albolineatus* (lower plot), wild-type melanophore densities increase more slowly through metamorphosis and never reach the same maximum as in wild-type *D. rerio*. *kit* mutant *D. albolineatus* completely lack melanophores during early pigment pattern metamorphosis, then recover a few residual LM melanophores during late pigment pattern metamorphosis. Vertical bars to the right of plots indicate the inferred final contributions of EM and LM melanophores to the different pigment patterns. The timing of pigment pattern metamorphosis was somewhat delayed in these analyses compared with previous studies, presumably owing to slight differences in rearing conditions.

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).

The *kit* mutant *D. albolineatus* 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. albolineatus* exhibited mostly dispersed EM melanophores, and *kit* mutants of both species completely lacked melanophores (Fig. 4A-D). During late pigment pattern

metamorphosis, wild-type *D. rerio* developed distinctive stripes (Fig. 4E); wild-type *D. albolineatus* 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. albolineatus* 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. albolineatus*. 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. albolineatus*.

Further comparison of wild types and *kit* mutants reveals evolutionary changes in LM melanophores as well. In both *D. rerio* and *D. albolineatus*, *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. albolineatus* 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. albolineatus* 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. albolineatus*, 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 dorsum, 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. albolineatus*, 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. albolineatus*, then *kit* mutant *D. albolineatus* should have similar or greater numbers of L-dopa+ cells. Consistent with this prediction, L-dopa+ melanoblasts in *kit* mutant *D. albolineatus* were at least as numerous (Fig. 5D,D′) as melanoblasts and melanophores in wild-type *D. albolineatus*. These results suggest that: (1) *kit* is required during a late step in metamorphic melanophore development; and (2) this requirement is similar between species.
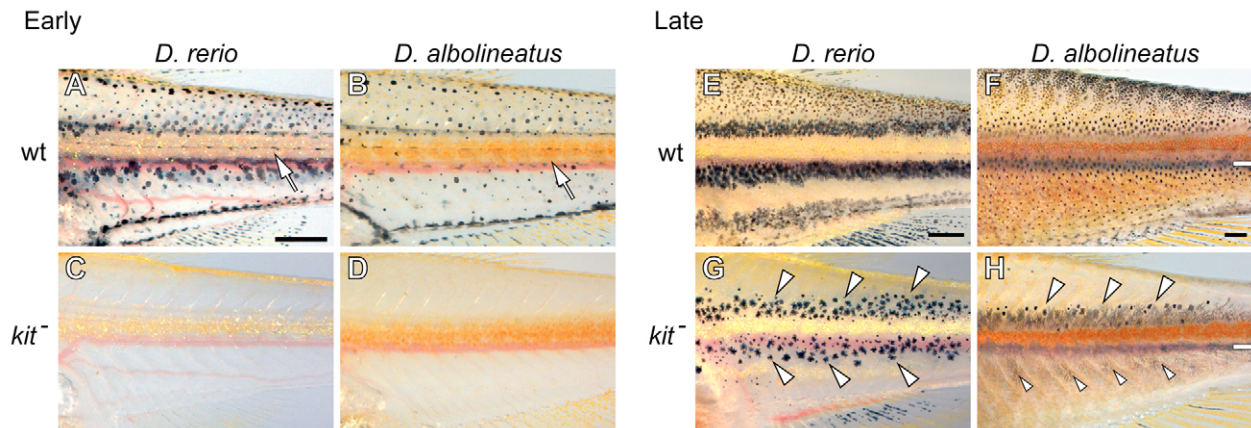
Fig. 4. Pigment patterns at early metamorphosis and after late metamorphosis. (**A**) Wild-type *D. rerio* with initially dispersed metamorphic melanophores as well as nascent stripes bounding the primary interstripe (arrow). (**B**) Wild-type *D. albolineatus* has dispersed melanophores, which are largely absent from a corresponding interstripe region (arrow). (**C,D**) *kit* mutants of both species lack melanophores at early metamorphosis. (**E**) Wild-type *D. rerio* with a juvenile pigment pattern of two primary stripes bounding the primary interstripe and a third stripe developing further ventrally. (**F**) Wild-type *D. albolineatus* with melanophores relatively uniformly dispersed except for the interstripe region. Melanophores adjacent to the interstripe tend to be larger and darker. White bar indicates darkness owing to internal structures (aorta, vertebrae, etc.) rather than melanophores. (**G**) *kit* mutant *D. rerio* develop residual LM melanophores in stripes (large arrowheads) dorsal and ventral to the interstripe. (**H**) *kit* mutant *D. albolineatus* develop residual LM melanophores primarily in a stripe dorsal to the interstripe (large arrowheads), although a few melanophores are also found ventral to the interstripe (small arrowheads). Ages, A and C, 31 dpf; B and D, 28 dpf; E and G, 53 dpf; F and H, 60 dpf. Scale bars: in A, 500 µm for A-D; in E, 500 µm for E,G; in F, 500 µm for F,H.

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*.
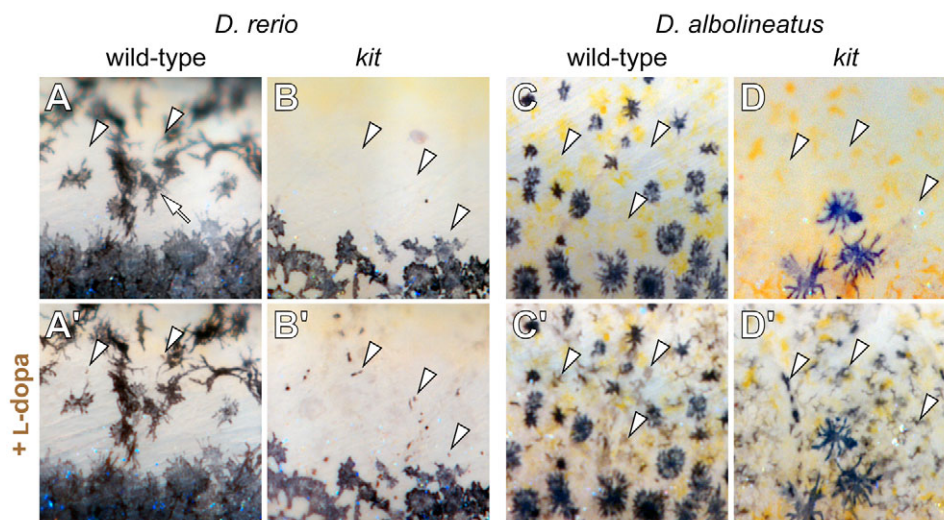

Fig. 5. *kit* is required during a late step in melanophore development. Dorsal regions of late larval fish are shown before (A-D) and after (A'-D') incubation with L-dopa. Arrowheads, sites of melanoblasts before and after L-dopa treatment. (**A,A'**) In wild-type *D. rerio*, previously melanized melanophores are present within the dorsal melanophore stripe and on dermal scales (arrow). Very few L-dopa+ melanoblasts are revealed (arrowheads). (**B,B'**) *kit* mutant *D. rerio* exhibit more undifferentiated melanoblasts than wild-type *D. rerio*. (**C,C'**) In wild-type *D. albolineatus*, many more L-dopa+ melanoblasts are present than in wild-type *D. rerio*. (**D,D'**) *kit* mutant *D. albolineatus* have similar numbers of L-dopa+ melanoblasts to wild-type *D. albolineatus*.

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^{wp.a14e1}*, 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^{wp.a14e1}* 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*, *kitb*, 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,
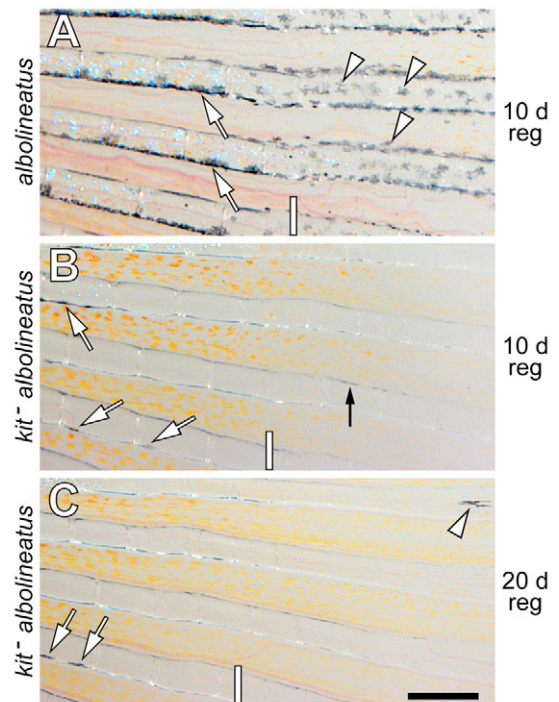

Fig. 6. *kit*-dependent and *kit*-independent regenerative melanophores in *D. albolineatus*. (**A**) In wild-type *D. albolineatus*, many regenerative melanophores (arrowheads) develop by 10 dpa and ultimately form a pattern indistinguishable from that of the unamputated fin. These cells differentiate de novo and have not migrated in from unamputated regions, as revealed by initially rearing fish in the melanin synthesis inhibitor phenylthiourea, allowing new and previously differentiated melanophores to be distinguished from one another (data not shown) (see Rawls and Johnson, 2000). Vertical bars, amputation planes. Arrows, melanophores already present in the unamputated fin. (**B**) In *kit* mutant *D. albolineatus*, regenerative melanophores do not develop by 10 dpa. Dark regions along edges of lepidotrichia are shadows (e.g. black arrow), not melanophores. (**C**) After longer periods (≥20 dpa) a few *kit*-independent regenerative melanophores differentiate (arrowhead), although fins never recover melanophore numbers equivalent to wild-type regenerates. Scale bar: in C, 500 μm for A-C.

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, *ednrb1* 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. albolineatus* 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. albolineatus* 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. albolineatus*, 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. albolineatus*. These cells migrate to join developing stripes in *D. rerio*, but migrate little in *D. albolineatus*, 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. albolineatus* 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.*

albolineatus 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. albolineatus* 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. albolineatus*. Whereas *D. rerio* develops numerous LM melanophores in its nascent stripes, *D. albolineatus* 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 kitb 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. albolineatus*. In *D. rerio*, *csf1r* promotes xanthophore development and LM melanophore development (Parichy et al., 2000b), whereas *D. albolineatus* 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. albolineatus*, 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. albolineatus* 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. albolineatus* reveal latent stripe-forming potential that is, nevertheless, somewhat predicted by the phenotype of wild-type larval *D. albolineatus*, 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. albolineatus* (Quigley et al., 2004) (e.g. *D. dangila*; D.M.P., unpublished, and movies at http://protist.biology.washington.edu/dparichy). 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.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/6/1081/DC1

References

Aoki, H., Motohashi, T., Yoshimura, N., Yamazaki, H., Yamane, T., Panthier, J. J. and Kunisada, T. (2005). Cooperative and indispensable roles of endothelin 3 and KIT signalings in melanocyte development. *Dev. Dyn.* **233**, 407-417.

Asai, R., Taguchi, E., Kume, Y., Saito, M. and Kondo, S. (1999). Zebrafish leopard gene as a component of the putative reaction-diffusion system. *Mech. Dev.* **89**, 87-92.

Bernex, F., De Sepulveda, P., Kress, C., Elbaz, C., Delouis, C. and Panthier, J. J. (1996). Spatial and temporal patterns of c-kit-expressing cells in W^lacZ/+^ and W^lacZ^/W^lacZ^ mouse embryos. *Development* **122**, 3023-3033.

Besmer, P., Manova, K., Duttlinger, R., Huang, E. J., Packer, A., Gyssler, C. and Bachvarova, R. F. (1993). The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Dev. Suppl.* **1993**, 125-137.

Braasch, I., Salzburger, W. and Meyer, A. (2006). Asymmetric evolution in two fish-specifically duplicated receptor tyrosine kinase paralogons involved in teleost coloration. *Mol. Biol. Evol.* **23**, 1192-1202.

Chapman, S. C., Lawson, A., Macarthur, W. C., Wiese, R. J., Loechel, R. H., Burgos-Trinidad, M., Wakefield, J. K., Ramabhadran, R., Mauch, T. J. and Schoenwolf, G. C. (2005). Ubiquitous GFP expression in transgenic chickens using a lentiviral vector. *Development* **132**, 935-940.

Goda, T., Abu-Daya, A., Carruthers, S., Clark, M. D., Stemple, D. L. and Zimmerman, L. B. (2006). Genetic screens for mutations affecting development of Xenopus tropicalis. *PLoS Genet.* **2**, e91.

Gompel, N., Prud'homme, B., Wittkopp, P. J., Kassner, V. A. and Carroll, S. B. (2005). Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in Drosophila. *Nature* **433**, 481-487.

Goodrich, H. B. and Nichols, R. (1931). The development and the regeneration of the color pattern in *Brachydanio rerio*. *J. Morphol.* **52**, 513-523.

Grassot, J., Gouy, M., Perriere, G. and Mouchiroud, G. (2006). Origin and molecular evolution of receptor tyrosine kinases with immunoglobulin-like domains. *Mol. Biol. Evol.* **23**, 1232-1241.

Hoekstra, H. E., Hirschmann, R. J., Bundey, R. A., Insel, P. A. and Crossland, J. P. (2006). A single amino acid mutation contributes to adaptive beach mouse color pattern. *Science* **313**, 101-104.

Hou, L., Pavan, W. J., Shin, M. K. and Arnheiter, H. (2004). Cell-autonomous and cell non-autonomous signaling through endothelin receptor B during melanocyte development. *Development* **131**, 3239-3247.

Iwashita, M., Watanabe, M., Ishii, M., Chen, T., Johnson, S. L., Kurachi, Y., Okada, N. and Kondo, S. (2006). Pigment pattern in jaguar/obelix zebrafish is caused by a Kir7.1 mutation: Implications for the regulation of melanosome movement. *Plos Genet.* **2**, 1861-1870.

Johnson, S. L., Africa, D., Walker, C. and Weston, J. A. (1995). Genetic control of adult pigment stripe development in zebrafish. *Dev. Biol.* **167**, 27-33.

Kelsh, R. N. (2004). Genetics and evolution of pigment patterns in fish. *Pigment Cell Res.* **17**, 326-336.

Kelsh, R. N., Schmid, B. and Eisen, J. S. (2000). Genetic analysis of melanophore development in zebrafish embryos. *Dev. Biol.* **225**, 277-293.

Kelsh, R. N., Inoue, C., Momoi, A., Kondoh, H., Furutani-Seiki, M., Ozato, K. and Wakamatsu, Y. (2004). The Tomita collection of medaka pigmentation mutants as a resource for understanding neural crest cell development. *Mech. Dev.* **121**, 841-859.

Langtimm-Sedlak, C. J., Schroeder, B., Saskowski, J. L., Carnahan, J. F. and Sieber-Blum, M. (1996). Multiple actions of stem cell factor in neural crest differentiation *in vitro. Dev. Biol.* **174**, 345-359.

Mackenzie, M. A., Jordan, S. A., Budd, P. S. and Jackson, I. J. (1997). Activation of the receptor tyrosine kinase Kit is required for the proliferation of melanoblasts in the mouse embryo. *Dev. Biol.* **192**, 99-107.

Maderspacher, F. and Nusslein-Volhard, C. (2003). Formation of the adult pigment pattern in zebrafish requires leopard and obelix dependent cell interactions. *Development* **130**, 3447-3457.

McCauley, D. W., Hixon, E. and Jeffery, W. R. (2004). Evolution of pigment cell regression in the cavefish *Astyanax*: a late step in melanogenesis. *Evol. Dev.* **6**, 209-218.

Mellgren, E. M. and Johnson, S. L. (2004). A requirement for kit in embryonic zebrafish melanocyte differentiation is revealed by melanoblast delay. *Dev. Genes Evol.* **214**, 493-502.

Mellgren, E. M. and Johnson, S. L. (2005). *kitb*, a second zebrafish ortholog of mouse *Kit*. *Dev. Genes Evol.* **215**, 1-8.

Nijhout, H. F. (1991). *The Development and Evolution of Butterfly Wing Patterns*. Washington, DC: Smithsonian Institution Press.

Nishimura, E. K., Yoshida, H., Kunisada, T. and Nishikawa, S. I. (1999). Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. *Dev. Biol.* **215**, 155-166.

Parichy, D. M. (1996a). Pigment patterns of larval salamanders (Ambystomatidae, Salamandridae): the role of the lateral line sensory system and the evolution of pattern-forming mechanisms. *Dev. Biol.* **175**, 265-282.

Parichy, D. M. (1996b). When neural crest and placodes collide: interactions between melanophores and the lateral lines that generate stripes in the salamander *Ambystoma tigrinum tigrinum* (Ambystomatidae). *Dev. Biol.* **175**, 283-300.

Parichy, D. M. (2005). Variation and developmental biology: prospects for the future. In *Variation: A Hierarchical Examination of a Central Concept in Biology* (ed. B. Halgrimsson and B. K. Hall), pp. 475-498. New York: Academic Press.

Parichy, D. M. (2006). Evolution of danio pigment pattern development. *Heredity* **97**, 200-210.

Parichy, D. M. and Johnson, S. L. (2001). Zebrafish hybrids suggest genetic mechanisms for pigment pattern diversification in *Danio*. *Dev. Genes Evol.* **211**, 319-328.

Parichy, D. M. and Turner, J. M. (2003a). Temporal and cellular requirements for Fms signaling during zebrafish adult pigment pattern development. *Development* **130**, 817-833.

Parichy, D. M. and Turner, J. M. (2003b). Zebrafish *puma* mutant decouples pigment pattern and somatic metamorphosis. *Dev. Biol.* **256**, 242-257.

Parichy, D. M., Rawls, J. F., Pratt, S. J., Whitfield, T. T. and Johnson, S. L. (1999). Zebrafish sparse corresponds to an orthologue of c-kit and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. *Development* **126**, 3425-3436.

Parichy, D. M., Mellgren, E. M., Rawls, J. F., Lopes, S. S., Kelsh, R. N. and Johnson, S. L. (2000a). Mutational analysis of *endothelin receptor b1* (*rose*) during neural crest and pigment pattern development in the zebrafish *Danio rerio*. *Dev. Biol.* **227**, 294-306.

Parichy, D. M., Ransom, D. G., Paw, B., Zon, L. I. and Johnson, S. L. (2000b). An orthologue of the *kit*-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, *Danio rerio*. *Development* **127**, 3031-3044.

Parichy, D. M., Reedy, M. V. and Erickson, C. A. (2006). Regulation of melanoblast migration and differentiation. In *The Pigmentary System: Physiology and Pathophysiology.* 2nd edn (ed. J. J. Nordland, R. E. Boissy, V. J. Hearing, R. A. King, W. S. Oetting and J. P. Ortonne). New York: Oxford University Press.

Quigley, I. K., Turner, J. M., Nuckels, R. J., Manuel, J. L., Budi, E. H., Macdonald, E. L. and Parichy, D. M. (2004). Pigment pattern evolution by differential deployment of neural crest and post-embryonic melanophore lineages in *Danio* fishes. *Development* **131**, 6053-6069.

Quigley, I. K., Manuel, J. L., Roberts, R. A., Nuckels, R. J., Herrington, E. R., Macdonald, E. L. and Parichy, D. M. (2005). Evolutionary diversification of pigment pattern in *Danio* fishes: differential *fms* dependence and stripe loss in *D. albolineatus*. *Development* **132**, 89-104.

Rawls, J. F. and Johnson, S. L. (2000). Zebrafish *kit* mutation reveals primary and secondary regulation of melanocyte development during fin stripe regeneration. *Development* **127**, 3715-3724.

Rawls, J. F. and Johnson, S. L. (2003). Temporal and molecular separation of the kit receptor tyrosine kinase's roles in zebrafish melanocyte migration and survival. *Dev. Biol.* **262**, 152-161.

Rawls, J. F., Mellgren, E. M. and Johnson, S. L. (2001). How the zebrafish gets its stripes. *Dev. Biol.* **240**, 301-314.

Reid, K., Nishikawa, S. I., Bartlett, P. F. and Murphy, M. (1995). Steel factor directs melanocyte development *in vitro* through selective regulation of the number of c-kit⁺ progenitors. *Dev. Biol.* **169**, 568-579.

Russell, E. S. (1949). Analysis of pleiotropism at the *W*-locus in the mouse: relationship between the effects of *W* and *W^v* substitution on hair pigmentation and erythrocytes. *Genetics* **34**, 708-723.

Santiago, A. and Erickson, C. A. (2002). Ephrin-B ligands play a dual role in the control of neural crest cell migration. *Development* **129**, 3621-3632.

Shapiro, M. D., Marks, M. E., Peichel, C. L., Blackman, B. K., Nereng, K. S., Jonsson, B., Schluter, D. and Kingsley, D. M. (2004). Genetic and developmental basis of evolutionary pelvic reduction in threespine sticklebacks. *Nature* **428**, 717-723.

Sobkow, L., Epperlein, H. H., Herklotz, S., Straube, W. L. and Tanaka, E. M. (2006). A germline GFP transgenic axolotl and its use to track cell fate: dual origin of the fin mesenchyme during development and the fate of blood cells during regeneration. *Dev. Biol.* **290**, 386-397.

Solnica-Krezel, L., Schier, A. F. and Driever, W. (1994). Efficient recovery of ENU-induced mutations from the zebrafish germline. *Genetics* **136**, 1401-1420.

Watanabe, M., Iwashita, M., Ishii, M., Kurachi, Y., Kawakami, A., Kondo, S. and Okada, N. (2006). Spot pattern of *leopard Danio* is caused by mutation in the zebrafish connexin41.8 gene. *EMBO Rep.* **7**, 893-897.

Wehrle-Haller, B. (2003). The role of Kit-ligand in melanocyte development and epidermal homeostasis. *Pigment Cell Res.* **16**, 287-296.

Wehrle-Haller, B. and Weston, J. A. (1995). Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. *Development* **121**, 731-742.

DEVELOPMENT

Yamamoto, Y., Stock, D. W. and Jeffery, W. R. (2004). Hedgehog signalling controls eye degeneration in blind cavefish. *Nature* **431**, 844-847.

Yang, C. T. and Johnson, S. L. (2006). Small molecule-induced ablation and subsequent regeneration of larval zebrafish melanocytes. *Development* **133**, 3563-3573.

Yang, C. T., Sengelmann, R. D. and Johnson, S. L. (2004). Larval melanocyte regeneration following laser ablation in zebrafish. *J. Invest. Dermatol.* **123**, 924-929.

Yoshida, H., Kunisada, T., Kusakabe, M., Nishikawa, S. and Nishikawa, S. I. (1996). Distinct stages of melanocyte differentiation revealed by analysis of nonuniform pigmentation patterns. *Development* **122**, 1207-1214.