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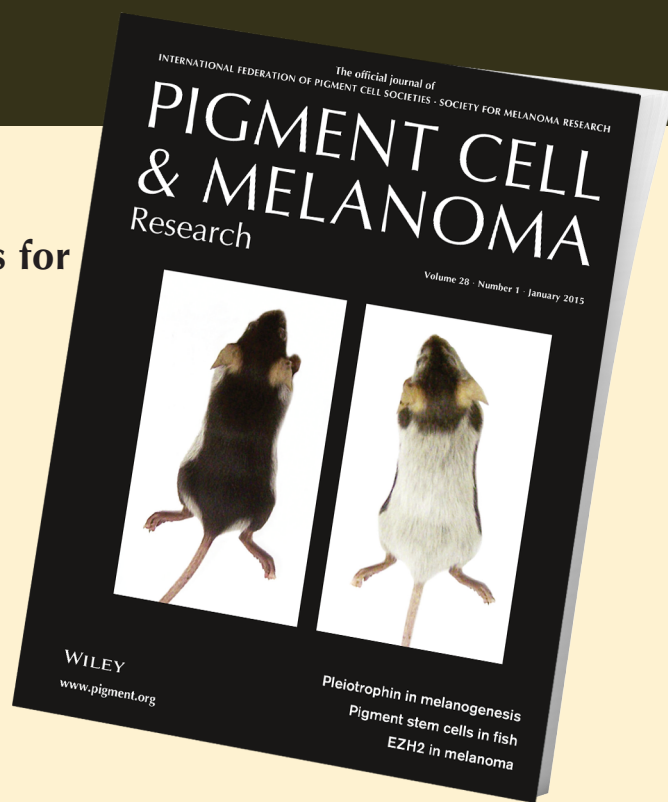
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Origins of adult pigmentation: diversity in pigment stem cell lineages and implications for pattern evolution

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Summary

Teleosts comprise about half of all vertebrate species and exhibit an extraordinary diversity of adult pigment patterns that function in shoaling, camouflage, and mate choice and have played important roles in speciation. Here, we review studies that have identified several distinct neural crest lineages, with distinct genetic requirements, that give rise to adult pigment cells in fishes. These lineages include post-embryonic, peripheral nerve-associated stem cells that generate black melanophores and iridescent iridophores, cells derived directly from embryonic neural crest cells that generate yellow-orange xanthophores, and bipotent stem cells that generate both melanophores and xanthophores. This complexity in adult chromatophore lineages has implications for our understanding of adult traits, melanoma, and the evolutionary diversification of pigment cell lineages and patterns.

Introduction

Studies of vertebrate pigmentation have provided fundamental insights into genetics (Castle and Little, 1910; Wright, 1984a), the cytoskeleton (Fujii, 2000; Kelsh et al., 2009), morphogenesis (Bonaventure et al., 2013; Kelsh et al., 2009), cell lineage diversification (Adameyko et al., 2009; Dorsky et al., 1998; Nitzan et al., 2013a; Thomas and Erickson, 2009), and modes of selection and speciation (Endler, 1980; Fan et al., 2012; Houde, 1997; Houde and Endler, 1990; Price et al., 2008; Seehausen et al., 2008; Wright, 1984b). Whereas an origin of pigment cells in the embryonic neural crest has been known for many years [(Dushane, 1934); reviewed in: (Hall and Hörstadius, 1988; Parichy et al., 2006)], the more specific developmental histories and genetic requirements of pigment cells responsible for adult pigmentation have started to be elucidated only more recently. Here, we review recent advances in understanding lineages of adult pigment cells with an emphasis on recent work in zebra fish *Danio rerio* (Figure 1), but also touching upon studies of several other fishes as well as amniotes.

Pigment cell diversity and stem cells

Adult pigmentation in amniotes depends on the patterned differentiation of melanocytes that contribute melanin to keratinocytes and, ultimately to skin, hair or feathers (Kaelin et al., 2012; Lin et al., 2013). By contrast, teleosts and other ectothermic vertebrates develop several classes of pigment cells, or chromatophores, that retain their pigments intracellularly (Bagnara and Matsumoto, 2006). Overall patterns thus reflect the numbers and arrangements of the chromatophores themselves. Certainly the most studied of these cells is the black melanophore, the melanin-containing cell homologous to the melanocyte of amniotes (and, for this reason, often referred to itself as a melanocyte). Other chromatophores receiving attention recently are yellow-orange xanthophores, having pteridines and carotenoids, iridescent iridophores, with purine-rich stacks of reflecting platelets, and shiny yellow leucophores that contain pteridines and carotenoids as well as reflective crystalline deposits. Nevertheless, the diversity of adult chromatophores is extensive and includes red erythrophores, blue cyanophores, and others (Bagnara et al., 2007; Goda and Fujii, 1995; Goda et al.,

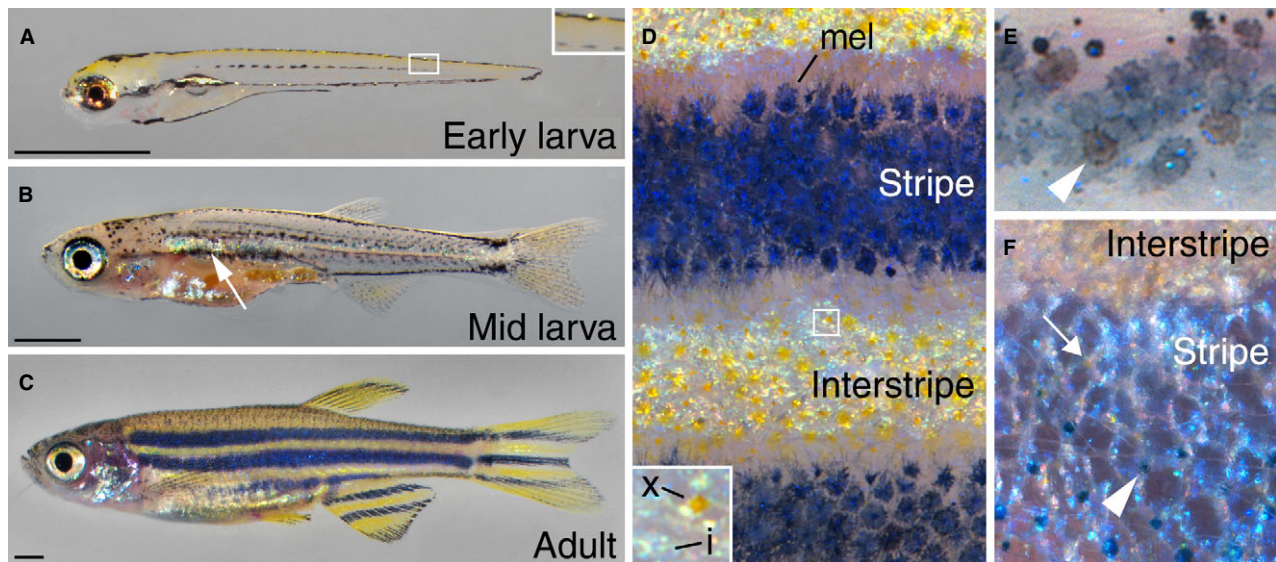


Figure 1. Zebrafish pigment cells and patterns. (A) The EL pigment pattern at 5 dpf. Note stripes of melanophores with a few shiny iridophores and the overall yellow color imparted by xanthophores. Inset, higher magnification of dorsal melanophore stripe with a single iridescent iridophore; additional EL melanophores occur scattered along the horizontal myoseptum. (B) Approximately 18 dpf larva developing its adult pattern. Interstripe iridophores have developed (arrow) and adult melanophore stripes are becoming evident. (C) Young adult pattern at approximately 2.5 months. Scale bars in A–C, 1 mm. (D) Close-up of adult pattern with dark stripes of melanophores (mel) and iridophores alternating with interstripes of iridescent iridophores and yellow-orange xanthophores. Inset, xanthophore (x) and iridophores (i). (E) In the adult, EL melanophores often have a brownish color (arrowheads), distinguishable from gray-black adult melanophores (Quigley et al., 2004). (F) Different adult iridophore morphologies in the interstripe and stripe. Treatment with epinephrine has contracted melanosomes toward melanophore cell bodies (arrowhead). Very lightly pigmented xanthophores are evident at low density as well (arrow).

2013; Khoo et al., 2012; Kimura et al., 2014; Nagao et al., 2014).

A common stem cell origin for different chromatophore classes was suggested by Bagnara et al. (1979) from the observations that cells sometimes contain pigment organelles typical of more than one class and that organelles themselves sometimes can be mosaic. In this context, ‘stem cell’ referred to a precursor in, or derived from, the neural crest, able to generate multiple differentiated cell types. Yet, stem cells are often defined as being able to self-renew while generating differentiated progeny, and, in this sense, stem cells need not be multipotent. For simplicity in this review, we use the term ‘stem cell’ in reference to latent precursors that normally give rise to adult chromatophores, and note that the degrees to which these cells can self-renew or contribute to multiple chromatophore classes remains often unclear.

Outline of zebrafish pigment pattern development

Adult chromatophore stem cell lineages have been studied most extensively in zebrafish, which exhibit two distinct patterns during their life cycle. Around the time of hatching, the fish has an embryonic/early larval (EL) pattern with stripes of melanophores along the dorsal myotomes and extending over the head, along the ventral myotomes and over the yolk sac, laterally along the

horizontal myoseptum, and ventrally under the yolk sac (Kimmel et al., 1995). Iridophores are sparsely distributed within three of these melanophore stripes (and are especially abundant over the swim bladder), whereas xanthophores are widely distributed beneath the epidermis and give an overall yellow cast to the flank (Figure 1A). This pattern likely provides camouflage while also protecting the nervous system and other tissues from UV damage in shallow water (Arunachalam et al., 2013; Engeszer et al., 2007b; Mueller and Neuhaus, 2014). Development of the EL pattern begins 15–25 h post-fertilization (hpf) (Raible et al., 1992; Vaglia and Hall, 2000) with the migration of neural crest cells, some of which differentiate as EL melanophores, xanthophores, and iridophores (Dutton et al., 2001; Kelsh et al., 1996; Odenthal et al., 1996; Raible and Eisen, 1994) that organize into the definitive EL pattern by 48 hpf through mechanisms that remain largely, though not entirely (Svetic et al., 2007), unknown.

The zebrafish adult pattern is very different and consists of dark stripes of melanophores with relatively small numbers of iridophores and a few pale xanthophores, alternating with light ‘interstripes’ of abundant iridophores and more heavily pigmented xanthophores (Figure 1C–F). Distinct classes of iridophores have been identified (Oshima and Kasai, 2002), including ellipsoidal cells having small reflecting platelets (S-iridophores) in both stripes and interstripes, and spindle-shaped cells having large reflect-

ing platelets (L-iridophores), associated only with stripe melanophores (Hirata et al., 2003). All of these reside in the hypodermis, between the skin and underlying myotome (Hawkes, 1974a,b; Kirschbaum, 1975; Le Guellec et al., 2004; Rakers et al., 2010), and occupy different strata within this compartment (Hirata et al., 2003, 2005). Chromatophores are also found intermingled in the dorsal hypodermis, associated with scales in the dermis, and in the fins (Hirata et al., 2005; Tu and Johnson, 2011). The adult pattern functions in shoaling (Engeszer et al., 2007a, 2008; Rosenthal and Ryan, 2005) and potentially other behaviors as well (Price et al., 2008).

The beginnings of a transition from EL to adult pattern is evident typically by approximately 8 days post-fertilization (dpf)¹: by this time, a few melanophores have been added to the lateral stripe (Hultman and Johnson, 2010; Milos and Dingle, 1978a,b; Milos et al., 1983); yellow xanthophores, though initially widespread, are no longer apparent; and adult iridophores are evident near the horizontal myoseptum, where the first adult interstripe will develop (Movie S1). Subsequently, lightly pigmented melanophores are found widely over the flank, including where the first two adult stripes will form, dorsal and ventral to the first interstripe (Figure 1B). Finally, adult interstripe xanthophores appear in association with interstripe iridophores and boundaries between stripes and interstripes become increasingly distinct: melanophores are lost from the interstripe and melanophores within stripes become larger and more stellate (Frohnhofer et al., 2013; Johnson et al., 1995; Maderspacher and Nusslein-Volhard, 2003; Mahalwar et al., 2014; McMennamin et al., 2014; Parichy and Turner, 2003b; Parichy et al., 2000b, 2009; Patterson and Parichy, 2013; Patterson et al., 2014; Singh et al., 2014; Takahashi and Kondo, 2008). A juvenile pattern forms by approximately 28 dpf and, later, new interstripes and stripes are added dorsally and ventrally.

One could posit two very different ways to make an adult pattern: by expanding and rearranging the populations of EL chromatophores, or by replacing EL chromatophores with entirely new populations of adult chromatophores. The predominant strategy used by zebrafish differs between chromatophore classes. In the following sections, we review first the development of embryonic chromatophores that give rise to the EL pattern, and then the development of adult melanophores, iridophores, and xanthophores that contribute to the body pattern, as well as chromatophores that form the normal and regenerative patterns of the fins.

Zebrafish mutants with defects in adult pattern hint at a melanophore stem cell

A distinct origin of EL and adult melanophores was first suggested by the observation that lightly pigmented melanophores appear during adult pattern development (Kirschbaum, 1975), implying *de novo* differentiation from

an unpigmented precursor. Additional support for this idea came from several mutants with defects in adult pigmentation. The first of these was *sparse* (Johnson et al., 1995), shown later to correspond to *kita* (Parichy et al., 1999), encoding a Kit receptor tyrosine kinase. In *kita* mutants, there are fewer EL melanophores initially and even these die after several days so that by mid-larval stages melanophores are completely absent (Figure 2A). By late larval stages, however, new *kita*-independent melanophores differentiate. Because such cells cannot arise by the proliferation of EL melanophores, which are missing, they must come from unpigmented cells with melanogenic potential; the same precursors might give rise to adult melanophores during normal development. A similar *kita* mutant phenotype is observed in the zebrafish relative, *D. albolineatus*, indicating an evolutionary conservation of this *kita*-independent population of melanophores (Mills et al., 2007).

A stem cell origin of adult melanophores was also suggested by the *puma* (Parichy and Turner, 2003b; Parichy et al., 2003) and *picasso* (Budi et al., 2008) mutants. In both of these, EL melanophores develop, yet most adult melanophores are missing (Figure 2B). Despite their similar phenotypes, *puma* and *picasso*

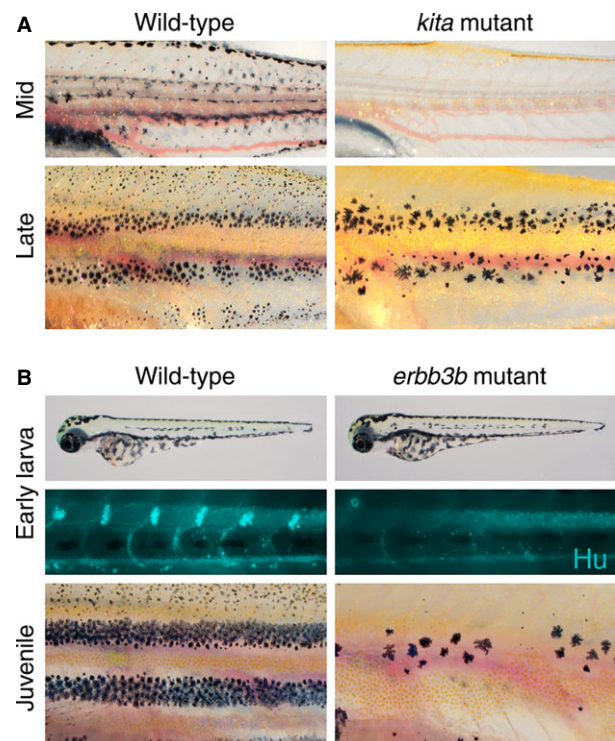


Figure 2. Mutants suggesting a stem cell origin of adult melanophores. (A) *kita* mutants completely lack melanophores at mid-larval stages but later acquire some stripe (though not dorsal scale) melanophores. (B) *erbb3b* mutants have normal EL patterns but defects in ventromedial neural crest migration and lack *Hu*⁺ dorsal root ganglia. In the juvenile, most adult melanophores fail to develop; a few large melanophores persist from the EL pattern.

affect different steps in adult melanophore development. *puma* corresponds to a mutation in *tubulin alpha-8 like-3* (*tuba8l3*) (Larson et al., 2010), which affects melanophore development autonomously (Parichy et al., 2003). Because this particular allele has a temperature-sensitive phenotype, it was possible to use temperature-shift experiments to define a critical period, corresponding to when adult melanophores normally differentiate, during which time the *puma* mutation impacted melanophore development (Parichy and Turner, 2003b; Parichy et al., 2003). This suggested that *tuba8l3* is involved in the recruitment of melanophores from a latent stem cell.

picasso corresponds to *erbb3b*, encoding an ErbB3 (EGFR-related) receptor tyrosine kinase (Budi et al., 2008; Lyons et al., 2005). In contrast to *puma*, pharmacological inhibitors of ErbB activity and transient morpholino knockdown showed that *Erbb3b* is required for adult melanophore development during embryogenesis, when neural crest cells migrate along a ventromedial migratory pathway between the somites and neural tube, and thus long before these cells actually differentiate (Budi et al., 2008; Dooley et al., 2013). Although *erbb3b* is expressed by melanophores, genetic mosaic analyses revealed both autonomous and non-autonomous requirements, indicating a role not only within the melanophore lineage but in the tissue environment of these cells as well (Budi et al., 2008). These results suggested that ErbB-dependent precursors to adult melanophores are established during neural crest migration, in parallel with precursors to EL melanophores; later, during the larval-to-adult transformation, these *erbb3b*-dependent cells are recruited as adult melanophores. Similar adult melanophore defects are observed in mutants for *neuregulin1-3*, encoding an ErbB3 ligand (Lush and Piotrowski, 2014), and *sorbs3*, encoding a scaffolding protein that functions in the ErbB pathway (Malmquist et al., 2013). Unlike adult melanophores, most EL melanophores arise independently of this pathway: when ErbB signaling is blocked in embryos, 92% of the normal complement of melanophores develop by early larval stages; most of the 'missing' approximately 8% would normally populate the lateral stripe after 3 dpf, by which time other EL melanophores have differentiated (Hultman and Johnson, 2010; Milos and Dingle, 1978a).

Both ErbB pathway and *tuba8l3* mutants also have defects in the nervous system, with drastically fewer glia and extensive defasciculation of peripheral nerves (Budi et al., 2008, 2011; Larson et al., 2010; Lyons et al., 2005; Parichy et al., 2003). In ErbB-deficient embryos, these phenotypes stem in part from defects in neural crest cell morphogenesis along the ventromedial pathway, with cells either failing to migrate, or migrating but failing to coalesce into dorsal root ganglia (DRG; Figure 2B) or sympathetic ganglia; gross defects are not apparent for dorsolateral migration, between the epidermis and the somites (Budi et al., 2008; Dooley et al., 2013; Honjo et al., 2008). All of these observations pointed to the

possibility that stem cells giving rise to adult melanophores might be established in, and recruited from, medial 'extra-hypodermal' regions and perhaps the peripheral nervous system itself.

Larval regenerative melanophores: independent evidence for a melanophore stem cell

Additional indications that zebrafish have a melanophore stem cell come from studies of larval melanophore regeneration. When EL melanophores are ablated by laser or drug treatment at 2–3 dpf, new melanophores arise from proliferative precursors and replace all but the most ventral cells that were lost (Yang and Johnson, 2006; Yang et al., 2004); if the new melanophores are themselves ablated, they too are replaced, suggesting that precursors to regenerative melanophores are self-renewing (Hultman et al., 2009; O'reilly-Pol and Johnson, 2013; Tryon et al., 2011). In principle, these regenerative melanophores might arise from 'left over' precursors to EL melanophores that simply failed to differentiate during embryogenesis. Yet, genetic and cell lineage analyses argue against this idea and instead provide evidence for a stem cell that is distinct from the EL melanophore lineage.

First, regenerative melanophores have requirements different from EL melanophores, but shared with adult melanophores. For example, regeneration fails, just as adult melanophore development fails, when ErbB signaling is blocked genetically, or pharmacologically during neural crest migration (Hultman et al., 2009) (Figure 3A).

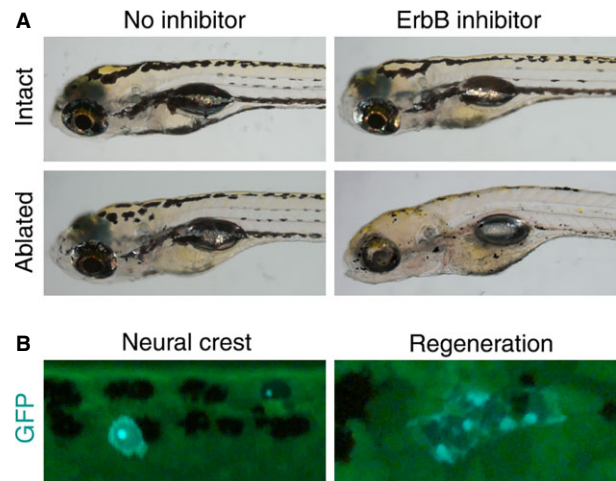


Figure 3. Larval melanophore regeneration requires stem cells. (A) Larvae in which EL melanophores have been ablated and new melanophores regenerated resemble control, unablated larvae. Yet regeneration is impaired when ErbB signaling is inhibited. (B) Transposon-based lineage analyses reveal GFP⁺ EL melanophores derived directly from neural crest, as well as GFP⁺ clones of regenerative melanophores (images courtesy of R. Tryon and S. Johnson).

Regeneration also fails in homozygous *kita* null alleles, demonstrating a requirement for Kit signaling (Yang and Johnson, 2006) similar to that exhibited by most adult melanophores (Johnson et al., 1995). Two additional genes required by regenerative but not EL melanophores have been identified by forward genetic screening for melanophore regeneration mutants: *glutamine:fructose-6-phosphate aminotransferase 2 (gfpt2)* acts within regenerative melanophores to promote terminal differentiation; the RNA helicase gene *skiv2l2* promotes the proliferation of regenerative melanophore precursors and is required for regeneration of additional tissues as well (Yang et al., 2007). Regenerative melanophores (Johnson et al., 2011) and adult melanophores (Dooley et al., 2013) also both arise independently of Microphthalmia (*Mitf*) activity during embryonic stages, when this factor is required for the specification of EL melanophores (Hou and Pavan, 2008; Levy et al., 2006; Lister et al., 1999; Opdecamp et al., 1997).

Second, evidence for distinct lineages of EL melanophores and stem cell-derived regenerative melanophores has come from transposon-based lineage analyses in which individual clones of *tyrosinase*⁺ cells were labeled during normal development and in regeneration (Tryon and Johnson, 2012; Tryon et al., 2011) (Figure 3B). Transposon integration typically occurs by early gastrulation and most *tyrosinase*⁺ clones (59%) gave rise to both EL melanophores and regenerative melanophores (Tryon et al., 2011). Nevertheless, some clones, presumably labeled slightly later, gave rise only to EL melanophores (31%) or only to regenerative melanophores (10%). These outcomes suggest a segregation of EL and regenerative fates shortly after the onset of gastrulation and by the time of neural crest dispersal. Further analyses of these data also suggested that 90–190 cells in the early larva are able to generate regenerative melanophores, or, presumably, adult melanophores. This estimate of founding stem cell number is larger than the numbers of progenitors suggested from qualitative observations of patterns in chimeras in mouse and zebrafish (Lin et al., 1992; Mintz, 1967, 1971), but not clearly different from observations based on clonal analyses in mouse (Wilkie et al., 2002).

Additional studies of larval melanophore regeneration have provided further clues to fate segregation and stem cell establishment. For example, applying transposon lineage analyses to fish heterozygous for a null allele of *kita* revealed more clones giving rise to EL melanophores and fewer clones giving rise to regenerative melanophores (O'Reilly-Pol and Johnson, 2013). This suggests that Kit signaling promotes adoption of a stem cell fate over EL melanophore fate and adds to the several roles identified for Kit in melanophore and melanocyte lineages (Besmer et al., 1993; Budi et al., 2011; Hultman et al., 2007, 2008; Mellgren and Johnson, 2004; Parichy et al., 1999; Rawls and Johnson, 2000, 2003; Wehrle-Haller, 2003). In a complementary strategy, control and ErbB-inhibited

embryos were compared for their responsiveness to transgenically supplied Kit ligand-a (*Kitlga*) (Hultman et al., 2009). Wild-type embryos overexpressing *Kitlga* developed extra melanophores derived not from the EL melanophore lineage but instead from the stem cell lineage; surprisingly, ErbB-inhibited embryos overexpressing *Kitlga* developed even more stem cell-derived melanophores than their wild-type counterparts. This unexpected result suggests that stem cell precursors persist transiently in ErbB-inhibited embryos and remain available for recruitment by *Kitlga* at these early stages. Thus, ErbB signaling seems to promote the transition of an early precursor, specified for a stem cell fate, to the definitive stem cell itself, which may be more highly regulated, and thus less responsive to *Kitlga*.

Embryonic origins, niche, and genetic requirements of melanophore stem cells

Studies of adult pigment pattern mutants and larval melanophore regeneration indicated the presence of melanophore stem cells but did not identify the cells themselves. This issue was addressed with a combination of molecular markers, fate mapping, and time-lapse imaging in wild-type and mutant backgrounds (Budi et al., 2011). Analyses of reporter expression in a transgenic line, *Tg(mitfa:GFP)^{w47}*, during early stages of adult pattern formation revealed proliferative extra-hypodermal GFP⁺ cells, often associated with peripheral nerves (Figure 4A–C). Although zebrafish *mitfa* reporters and *mitfa* transcript can be detected in other chromatophore lineages and in glia (Dooley et al., 2013; Eom et al., 2012; Parichy et al., 2000b), the melanogenic potential of at least some peripheral nerve-associated cells was confirmed by post-embryonic overexpression of *Kitlga*, which resulted in ectopic melanophore development (Figure 4D). Consistent with such cells being descendants of an ErbB-dependent stem cell established in the embryo, nerve-associated *mitfa:GFP*⁺ cells as well as ectopic *Kitlga*-induced melanophores were missing in the *erbb3b* mutant. To test if extra-hypodermal *mitfa:GFP*⁺ cells contribute to hypodermal chromatophores, two approaches were used (Figure 4E, F). First, fate mapping using the vital dye, Dil, showed that labeling of extra-hypodermal cells was followed several days later by the occurrence of labeled melanophores in the hypodermis. Second, time-lapse imaging revealed *mitfa:GFP*⁺ cells entering the hypodermis from extra-hypodermal locations, after migrating over the dorsal or ventral edges of the myotomes, or via the horizontal or vertical myosepta (Movies S2 and S3).

The earliest localization of melanophore stem cells has been examined as well (Dooley et al., 2013). Consistent with previous inferences, *mitfa:GFP*⁺ cells were found within the ventromedial neural crest migratory pathway associated with DRG and were later found more broadly along spinal nerves. By transplanting cells from embryos

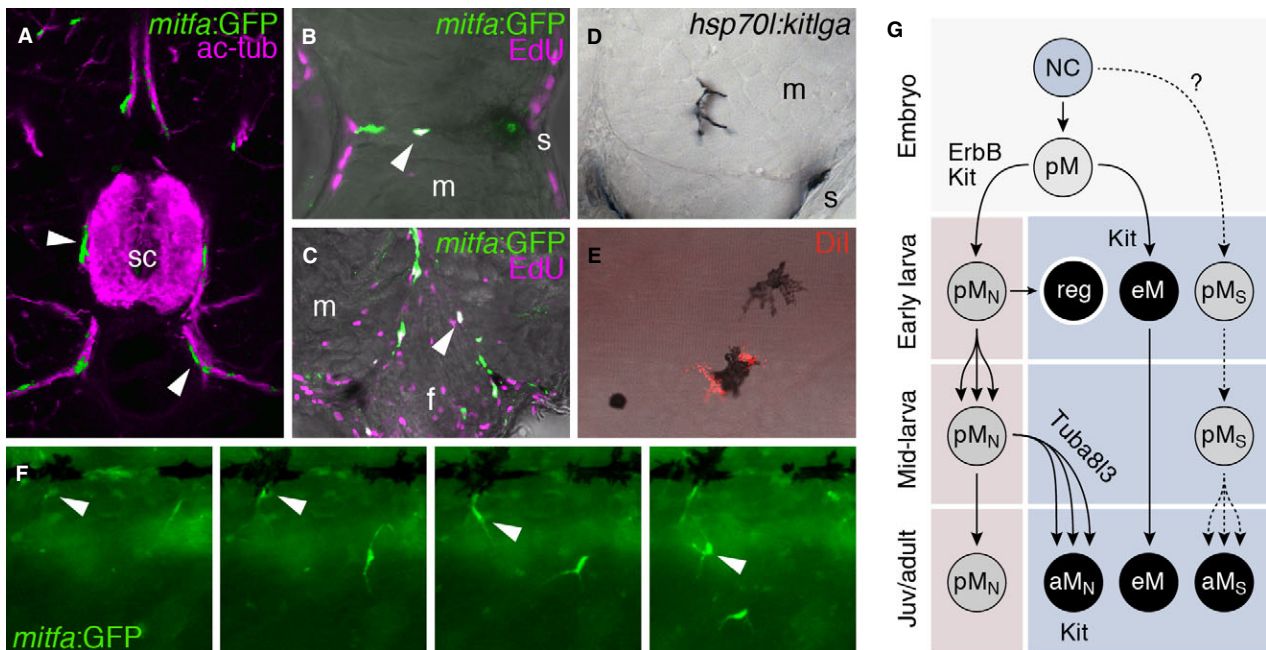


Figure 4. Melanophore stem cells. (A) *mitfa:GFP*⁺ cells (green; arrowheads) associated with peripheral nerves (ac-tub; magenta) at an early stage of adult pattern development. sc, spinal cord. (B, C) Proliferative *mitfa:GFP*⁺ cells in the horizontal myoseptum (B) and near the base of the fin (C) are indicated with white nuclei (arrowheads) in overlays with staining for EdU (magenta). m, myotome; s, skin; f, fin base. (D) Ectopic expression of *Kitlga* using the heat-shock inducible promoter of *hsp70l* results in melanophore development in the vicinity of peripheral nerves coursing through the myotomes. (E) Injection of Dil in extra-hypodermal regions results in later Dil-labeled melanophores in the hypodermis. (F) Time-lapse frames showing *mitfa:GFP*⁺ cells entering the hypodermis (e.g., arrowhead). (G) Model for melanophore lineages showing approximate stages (top to bottom) and later tissue compartments (pink, extra-hypodermal; blue, hypodermal). Progenitors specific for particular chromatophore classes are preceded with a 'p'. Neural crest (NC) cells generate a melanophore progenitor (pM) in the embryo that generates ErbB-dependent, peripheral nerve-associated melanophore stem cells (pM_N) as well as embryonic/early larval melanophores (eM). If eM are ablated, pM_N are recruited to the skin as regenerative larval melanophores (reg), but during normal development pM_N remain associated with peripheral nerves where they proliferate and ultimately, during adult pigment pattern development, migrate to the hypodermis to generate adult melanophores (aM_N). This recruitment fails in *tuba8l3* mutants. Some eM persist into the adult though many are lost, particularly from the interstripe. Kit signaling has several roles, promoting establishment of pM_N and also the migration, survival, and differentiation of eM and aM_N. An additional lineage (pM_S) able to generate adult melanophores and possibly other chromatophore classes is hypothetical (see text).

depleted of EL melanophores to *albino* hosts, it was further possible to correlate donor, DRG-associated *mitfa:GFP*⁺ cells with later patches of melanized melanophores in the adult. This same study identified the *sparse-like* mutant as allelic to *kitlga* and demonstrated that in this background *mitfa:GFP*⁺ cells fail to localize at the DRG, consistent with roles for Kit signaling in establishing the melanophore stem cell (O'Reilly-Pol and Johnson, 2013; Yang and Johnson, 2006). Further studies will need to determine whether the peripheral nerve-associated *mitfa:GFP*⁺ cells identified at later stages by Budi et al. (2011) correspond to the progeny of early *mitfa:GFP*⁺ cells identified by Dooley et al. (2013), or whether additional sources of such cells and melanophores are present.

Whether or not the cells identified so far in zebrafish represent a single population, the existence of peripheral nerve-associated precursors to adult melanophores is reminiscent of peripheral nerve-associated cells that give rise to skin melanocytes in amniotes (Adameyko et al., 2009) and raises the possibility of an evolutionarily ancient origin of this lineage. In chick, these cells express Sox10,

an early marker of both melanocyte and glial lineages, and FoxD3 – a marker of multipotent neural crest and glia – represses the melanocyte fate; in mouse, nerve-associated melanocyte precursors initially express the promoter of proteolipid protein (PLP), which is active in Schwann cells and their precursors (Adameyko et al., 2009; Nitzan et al., 2013b). In zebrafish, PLP does not mark peripheral glia (Brosamle and Halpern, 2002) but analyses thus far indicate that *mitfa:GFP*⁺ cells co-express Sox10 (as would be expected) and also can express Foxd3; interestingly the *mitfa:GFP*⁺; Foxd3⁺ population is especially proliferative (Budi et al., 2011). In principle, these multiply labeled cells could represent multipotent precursors adopting a melanophore fate, cells in which an initial melanogenic potential is being repressed, or another lineage entirely. Additional fate mapping should resolve these issues and may provide new insights into the homology of zebrafish and amniote peripheral nerve-associated precursors.

Once melanophore stem cells are established in zebrafish, several pathways are required for the recruitment of

hypodermal adult melanophores. For example, recruitment fails in *tuba8/3* mutants, which exhibit fewer *mitfa*:GFP⁺ cells overall; those cells that do reach the hypodermis are more likely than wild-type cells to differentiate, suggesting a compensatory response to their overall depletion (Budi et al., 2011). *kita* mutants, however, exhibit numerous *mitfa*:GFP⁺ cells during adult pattern development, in contrast to the deficiency of these cells at very early stages (Dooley et al., 2013). The later *mitfa*:GFP⁺ cells fail to melanize and are more likely to die than those of wild-type, suggesting additional roles for Kit during terminal differentiation (Budi et al., 2011; Mills et al., 2007) as has been inferred in other contexts (Mellgren and Johnson, 2004; Rawls and Johnson, 2000). The few adult melanophores that do differentiate in *kita* mutants proliferate extensively, likely explaining the late-arising pattern of residual stripes (Figure 2A) (Budi et al., 2011). Post-embryonic development of *mitfa*:GFP⁺ cells or melanophores also requires the gap junction protein Connexin41.8 (Johnson et al., 1995; Maderspacher and Nusslein-Volhard, 2003; Watanabe and Kondo, 2012; Watanabe et al., 2006), the potassium channel Kcnj13 (Haffter et al., 1996; Iwashita et al., 2006; Maderspacher and Nusslein-Volhard, 2003), the cell adhesion molecule Immunoglobulin superfamily 11 (Eom et al., 2012), Endothelin receptor b1a (Budi et al., 2011; Johnson et al., 1995; Parichy et al., 2000a), cell surface scaffolding protein Tetraspanin 3c (Inoue et al., 2014), and Notch signals (Hamada et al., 2014). Finally, additional factors, including Basonuclin-2 (Bnc2) (Lang et al., 2009; Patterson and Parichy, 2013), Colony stimulating factor-1 (Csf1) receptor (Budi et al., 2011; Maderspacher and Nusslein-Volhard, 2003; Parichy and Turner, 2003a; Parichy et al., 2000b; Patterson and Parichy, 2013) and others (Amsterdam et al., 2009; Frohnhofner et al., 2013; Kawakami et al., 2000; Krauss et al., 2013; Lopes et al., 2008; Patterson and Parichy, 2013) act – or are very likely to act – through other cell types to promote adult melanophore development.

Together, these analyses suggest a model for the establishment, maintenance, and recruitment of melanophore stem cells (Figure 4G) in which early progenitors, that can generate EL melanophores or melanophore stem cells, become restricted for the latter fate and migrate along the ventromedial neural crest migratory pathway, requiring ErbB signaling for the development of DRG at which they localize, in a manner that also requires *Kita*. These same cells can be recruited as regenerative melanophores when EL melanophores are ablated. During the larval-to-adult transformation, DRG-associated melanophore stem cells begin to proliferate, populate the peripheral nerves more extensively, and they or their progeny migrate to the hypodermis along peripheral nerves and through the myosepta, or after detaching from peripheral nerves and traveling over the edges of the myotomes. Once in the hypodermis, the cells begin to differentiate as melanophores. Meanwhile,

some EL melanophores persisting from the early pattern join developing adult stripes, whereas others die or are lost from view as they are covered by iridophores (Budi et al., 2008; Parichy and Turner, 2003b; Patterson and Parichy, 2013; Quigley et al., 2004; Takahashi and Kondo, 2008).

Comparative analyses of adult melanophore development have provided additional insights into EL and stem cell melanophore lineages. Stem cell-derived melanophores make the major contribution to adult patterns of zebrafish and most of the zebrafish relatives that have been examined (Mills et al., 2007; Quigley et al., 2004, 2005). Yet the spotted danio, *D. nigrofasciatus*, exhibits an evolutionarily derived condition with far fewer stem cell-derived melanophores. Instead, stripes that are superficially similar to those of zebrafish arise largely from EL melanophores, which persist and reorganize in the adult. Genetic mosaic analyses demonstrate the species difference has arisen through evolutionary changes non-autonomous to melanophore lineages (Quigley et al., 2004).

Among more distantly related species, flounders and other flatfishes provide exciting opportunities to examine pigment stem cells in the context of a metamorphosis more dramatic than that of zebrafish. In these fishes, a bilaterally symmetric larva – with a bilaterally symmetric pigment pattern – metamorphoses into an asymmetric adult having an upper (ocular) side with a distinct adult pigment pattern, and a lower (blind) side that is relatively unpigmented. In flounder, vital dye labeling and histological analyses suggest that precursors to adult melanophores colonize hypodermis on both upper and lower sides, via the myosepta and also from the bases of the fins (Washio et al., 2013; Watanabe et al., 2008; Yamada et al., 2010). Despite the migration of precursors to both sides, adult melanophores differentiate only on the upper side, a difference that correlates with lower dorsal than ventral levels of a factor known to inhibit melanization, Agouti-signaling protein 1 (Asip1) (Cerda-Reverter et al., 2005; Guillot et al., 2012).

A stem cell origin of adult iridophores

Although melanophores have been most studied, it is iridophores that are the first adult chromatophores to differentiate in zebrafish (Figure 5A), and these cells play critical roles in establishing the position, and delimiting the width, of subsequently developing melanophore stripes (Frohnhofner et al., 2013; Parichy et al., 2009; Patterson and Parichy, 2013; Patterson et al., 2014; Singh et al., 2014). Given the interplay between iridophore and melanophore patterning, it might be anticipated that earlier development of these cells could be coordinated as well. Indeed, mutants with defects in adult melanophore stem cell establishment have iridophore deficiencies (Figure 5B) and fate mapping has indicated an extra-hypodermal origin of adult iridophores similar to that of melanophores (Budi et al., 2011).

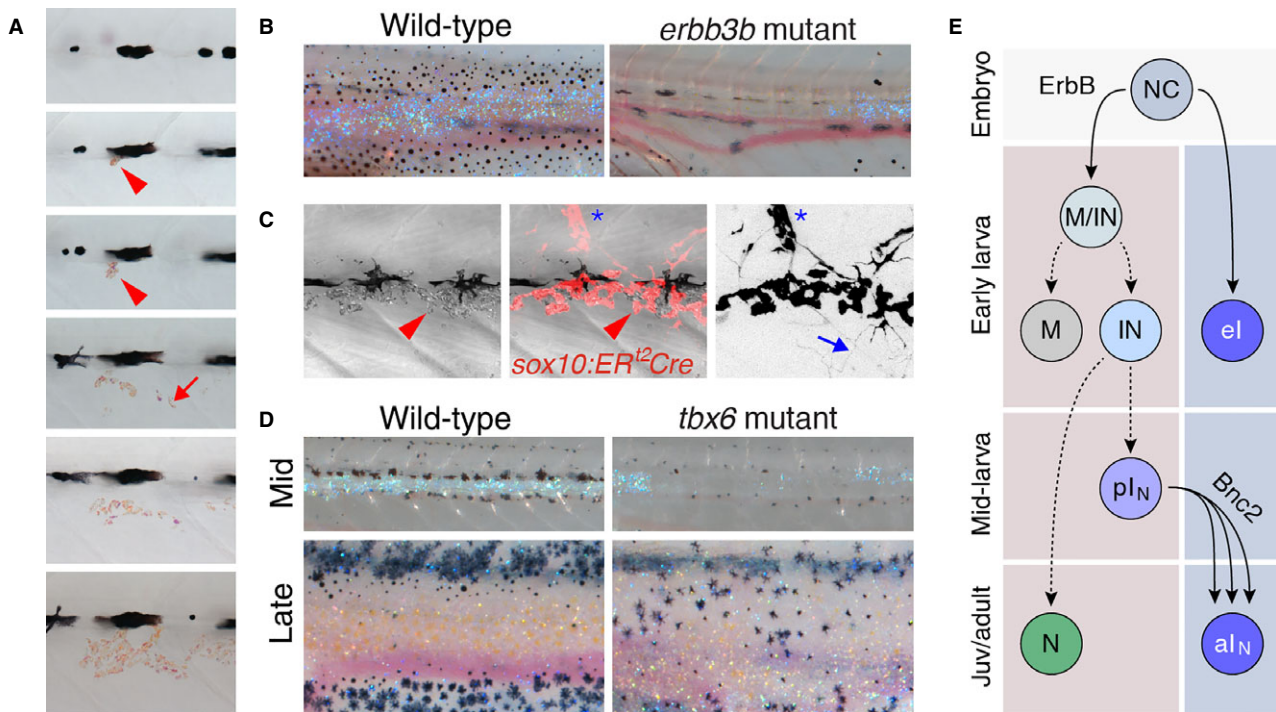


Figure 5. Adult iridophore origins. (A) The first adult iridophores to appear (arrowheads) arise at the horizontal myoseptum and then increase in number (arrow) to form the first interstripe. Shown is a single individual on consecutive days. (B) Iridophores, evident by blue reflectance, are reduced in *erbb3b* mutants that lack DRG. (C) Genetic labeling at early stages reveals the clonal expansion of iridophores in the hypodermis (arrowhead) and a shared lineage with neurons of the DRG (asterisk); arrow, neuronal arbor (right image inverted to better show neural labeling; courtesy of A. Singh, P. Mahalwar, C. Nüsslein-Volhard). (D) Myosepta provide routes by which iridophore precursors reach the skin as illustrated by defects in iridophore (and melanophore) abundance and patterning in *tbx6* mutants. (E) Model for iridophore lineages. NC cells give rise to a few iridophores of the EL pattern (el) as well as multipotent cells in peripheral nerves (MIN) that can initially generate melanophores, iridophores, neural derivatives but subsequently undergo further restriction (M versus IN). It is not clear whether M correspond to pM_N in Figure 4G, nor whether there exists a progenitor restricted to the iridophore fate alone (pl_N), though these seem likely.

Confirming that adult iridophores originate from extra-hypodermal stem cells and providing further insights into this lineage are recent analyses using genetic labeling with ER^{T2}-Cre, driven by the promoter of the very early lineage marker *sox10* (Singh et al., 2014). Clones labeled after EL stages in the vicinity of the DRG gave rise to adult iridophores and, interestingly, also generated neurons or glia (Figure 5C), and small but variable numbers of melanophores. This suggests an initially multipotent progenitor that becomes further restricted for melanophore versus iridophore/neural fates (Figure 5D). In bipotent, directly neural crest-derived cells of the embryo, an EL iridophore fate is specified over an EL melanophore fate by the transcription factor Foxd3 (Curran et al., 2010), and it will be interesting to learn whether Foxd3 acts similarly during these post-embryonic stages [and whether such cells might correspond to the multiply labeled cells described by Budi et al. (2011)].

An additional factor important to adult iridophore development is Leukocyte tyrosine kinase (Ltk) which acts autonomously to the iridophore lineage (Lopes et al., 2008). *Ltk* is expressed by iridophores and their precursors

and in the *Ltk* mutant, *shady*, EL iridophore specification fails. The several *shady* alleles also have reduced complements of adult iridophores ranging in severity from complete absence to only a minor deficiency, though it is unclear precisely when and how *Ltk* promotes the development of this adult stem cell-derived population.

Presumably during or after their specification, adult iridophore precursors migrate to the hypodermis via the horizontal myoseptum (Singh et al., 2014). Mutants with defects in myoseptum development, including *choker* (Frohnhofer et al., 2013; Svetic et al., 2007) and *fused somites* (*tbx6*) (Nikaido et al., 2002; Windner et al., 2012), have corresponding defects in iridophore colonization of the flank as well as later pattern formation (Figure 5D). Once iridophores have arrived in the hypodermis, the cells proliferate to form dense aggregations at the first interstripe but also spread further dorsally and ventrally, ultimately initiating additional interstripes as well (Patterson et al., 2014; Singh et al., 2014). In mutants for the highly conserved zinc finger protein, Basonuclin-2, the iridophore population fails to expand; adults lack most of their body iridophores as well as

melanophores and xanthophores (Lang et al., 2009; Patterson and Parichy, 2013). Although mutant phenotypes identify several additional loci required by adult iridophores (Amsterdam et al., 2009; Haffter et al., 1996; Kawakami et al., 2000; Krauss et al., 2013), including genes of the Endothelin signaling pathway (Krauss et al., 2014; Parichy et al., 2000a), the precise molecular mechanisms by which iridophore stem cells are established and recruited to the adult pattern remain to be elucidated.

Origins of adult xanthophores and their relationships to other lineages

EL xanthophores initially over the lateral flank disappear before the adult pattern develops, whereas interstripe xanthophores are the last adult pigment cells on the flank to differentiate (McMenamin et al., 2014; Patterson and Parichy, 2013; Patterson et al., 2014) (Figure 6A). From results on melanophores and iridophores, it might be anticipated that adult xanthophores also arise from stem cells, perhaps associated with peripheral nerves. Yet, two recent studies show this is not the case, and that, instead, many adult xanthophores are derived directly from the EL xanthophore lineage (Mahalwar et al., 2014; McMenamin et al., 2014). In one analysis, early markers or neural crest cells (*sox10*) or xanthophores [*pax7* or *colony stimulating factor-1 receptor*] were used with cell transplantation or Cre-recombination to label cells in the hypodermis at early larval stages. These same groups of cells increased in number and differentiated as xanthophores in the adult interstripe and also were present in lower numbers within the melanophore stripes (Mahalwar et al., 2014). In the other analysis (McMenamin et al., 2014), EL xanthophores were shown to give rise directly to adult xanthophores by fate mapping cells that had already differentiated using nuclear-localizing photoconvertible EosFP (nEosFP) driven by the promoter of *aldehyde oxidase 3* (*aox3*), which encodes a pteridine synthesis enzyme (Parichy et al., 2000b; Ziegler et al., 2000): EL xanthophores marked at 4–5 days lost their pigment yet persisted and proliferated, and then many, localizing in the interstripe, re-acquired pigment during adult pattern development (Figure 6A); others, localizing in the interstripe or the stripe, failed to do so. This approach also revealed some adult xanthophores that initiated *aox3*:nEosFP expression only after 4–5 days, suggesting they had not yet differentiated by the time of photoconversion; these cells may have been resident initially in the hypodermis as well (and presumably would have been labeled by the earlier lineage markers used by Mahalwar et al., 2014) (Figure 6B). An *aox3* reporter line further showed that cryptic *aox3*:GFP⁺ cells are widespread during adult pattern development, becoming densely packed and deeply pigmented in the interstripe, but also occurring in the stripes by juvenile stages. Results of both recent studies are thus consistent with

earlier observations that lightly pigmented xanthophores occur at low density even within stripes of older, adult fish (Hirata et al., 2003; Parichy and Turner, 2003a). *aox3*:GFP⁺ cells also are found extra-hypodermally, but are not clearly associated with peripheral nerves (McMenamin et al. 2014). Although there is currently no evidence that extra-hypodermal cells migrate to the hypodermis to differentiate as xanthophores in zebrafish, xanthophore precursors are believed to migrate from extra-hypodermal locations to the skin during flounder metamorphosis (Washio et al., 2013).

Several pathways have been studied for roles in promoting adult xanthophore development. First is signaling by colony stimulating factor-1 (Csf1), the receptor for which, Csf1r, is expressed by xanthophores and their precursors from very early stages and required for their development (Maderspacher and Nusslein-Volhard, 2003; Parichy and Turner, 2003a; Parichy et al., 2000b; Patterson and Parichy, 2013). Csf1 misexpression drives ectopic xanthophore differentiation, and during adult pattern development, this factor is expressed by the skin and at high levels by interstripe iridophores, thereby promoting differentiation and probably also increased density of xanthophores in the interstripe (Patterson and Parichy, 2013). In *csf1r* mutants (*pfegger* or *panther*), xanthophore development mostly fails in the embryo and adult (Haffter et al., 1996; Maderspacher and Nusslein-Volhard, 2003; Parichy et al., 2000b); the absence of EL xanthophores may be directly responsible for the absence of adult xanthophores, as would be predicted by *aox3*:nEosFP fate mapping. Indeed, transgenic ablation of EL xanthophores causes adult xanthophore deficiency as well (McMenamin et al., 2014). In a comparative context, signaling through Csf1r is also required for xanthophore development in the guppy *Poecilia reticulata* (Kottler et al., 2013), whereas increased Csf1 expression has contributed to a marked increase in xanthophore numbers in the pearl danio, *D. albolineatus* (Patterson et al., 2014).

A second factor required by adult xanthophores is thyroid hormone (TH), known for roles in amphibian and flatfish metamorphosis and for tissue-specific effects in both ectotherms and amniotes (Horn and Heuer, 2009; Laudet, 2011; Mcmenamin and Parichy, 2013; Shi, 2000; Sirakov et al., 2013). Fish that are deficient for TH, either because they have had their thyroids ablated or because of mutations affecting thyroid-stimulating hormone receptor (Tshr), lack adult xanthophores owing to a failure of terminal differentiation (Figure 6C) (McMenamin et al., 2014). Conversely, excess TH, either applied directly or resulting from a hyperactivating mutation of Tshr, results in precocious xanthophore differentiation and increased xanthophore proliferation, generating a dramatic increase in xanthophore numbers. Roles for TH in promoting xanthophore development are accompanied by repressive effects on melanophore population expansion; it remains to be determined whether TH effects on

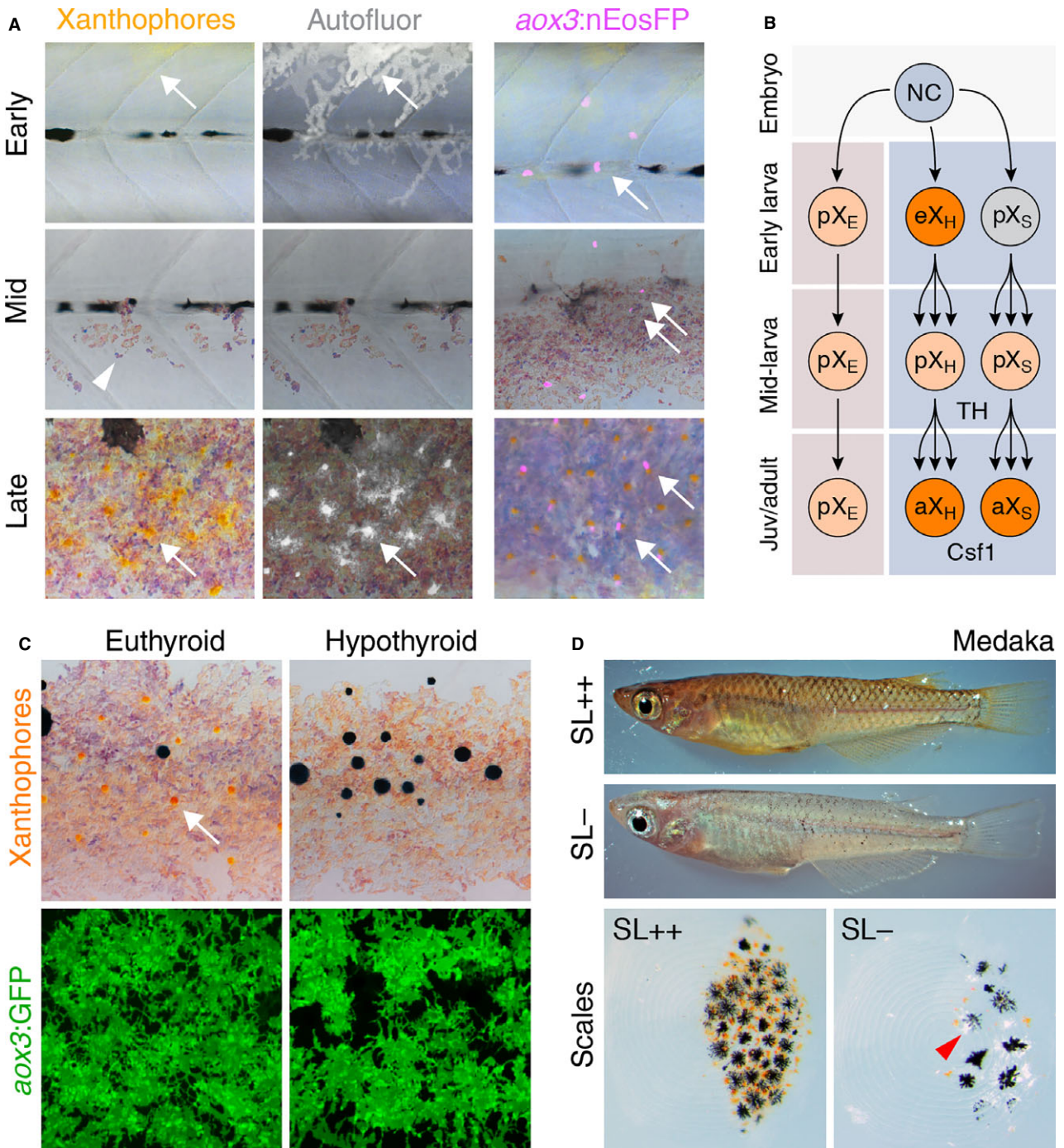


Figure 6. Xanthophore lineage and requirements. (A) Left, Autofluorescent xanthophores (arrows) are present in the early larva, but are not visible on the lateral flank during middle larval stages (though some remain visible along the dorsum; not shown). Arrowhead indicates iridophores. By late larval stages, adult xanthophores are visible in the interstripe. Right, EL xanthophores marked with photoconvertible nEosFP (arrow), persist into the adult pattern. Fish are treated with epinephrine, to contract pigment toward cell centers, adjacent to nuclei. (B) In the embryo, NC cells differentiate as early larval xanthophores in the hypodermis (eX_H), which then lose their pigment and proliferate, and ultimately re-differentiate as adult xanthophores. Additional $aox3^+$ cells are found extra-hypodermally (pX_E) and can differentiate in response to excess TH in zebrafish, or during normal development in other species. Other cells, presumed to arise in the hypodermis (pX_S), do not fully differentiate as EL xanthophores, yet do differentiate as adult xanthophores (aX_S). (C) In hypothyroid fish, adult xanthophores (arrow) fail to differentiate although $aox3:GFP^+$ precursors remain. (D) In medaka, SL promotes xanthophore development in a *ci* mutant expressing SL constitutively (upper) whereas *ci* mutants lacking SL have dramatically fewer xanthophores, evident by reduced yellow coloration, as well as increased numbers of leucophores (middle). Melanophore numbers are not significantly reduced in *ci* mutants compared to wild-type (Fukamachi et al., 2004). (E) Lower, Close-up of scales showing modulation of xanthophore and leucophore (arrowhead) abundances in response to SL (images in D courtesy S. Fukamachi).

xanthophores and melanophores are direct or mediated through other cell types or factors.

Additional clues into adult xanthophore development have come from studies of medaka, *Oryzias latipes*, which has the same classes of pigment cells as zebrafish but also shiny yellow and white leucophores. An extensive collection of embryonic and adult pigment mutants exists for medaka (Fukamachi et al., 2001; Kelsh et al., 2004; Lamoreux et al., 2005; Tomita, 1975) and these are providing valuable insights complementary to those from zebrafish mutants. For example, the medaka *color interfere* (*ci*) mutant exhibits dramatically fewer adult xanthophores and correspondingly more adult leucophores as compared to the wild-type. Positional cloning of *ci* revealed its correspondence to *somatolactin*, encoding a pituitary-derived endocrine factor, SL, similar to growth hormone (Figure 6D) (Fukamachi et al., 2004, 2006, 2009). Interestingly, cobalt variant carp have a similar pigment phenotype associated with reduced SL production (Kaneko et al., 1993).

Consistent with the reciprocal changes in adult xanthophore and leucophore abundance in response to SL, lovely recent analyses of several additional medaka mutants imply a common xanthophore–leucophore progenitor at embryonic stages (Kimura et al., 2014; Nagao et al., 2014). These have identified a critical role for the Pax7a transcription role in development of both cell types, a role consistent with that identified for Pax7 in zebrafish embryonic xanthophore development (Minchin and Hughes, 2008) as well as a genetic association between *pax7* allelic variation and a variant xanthophore phenotype in adult cichlids (Roberts et al., 2009). The recent medaka studies further showed that in this species, the Sox5 transcription factor specifies the xanthophore over leucophore fate in embryos. A downstream transporter, Slc2a15b, promotes leucophore differentiation, whereas both Slc2a15b and Slc2a11 promote yellow pigmentation in xanthophores and leucophores. It is not known whether these same cells persist into the adult, or whether additional stem cells for adult xanthophores and leucophores exist in medaka.

Stem cells contributing to fin pigment pattern and regeneration

The preceding discussion has been limited to pigment cell lineages on the body. Yet, chromatophores on the fins are of considerable interest for at least two reasons. First, some mutants exhibit a decoupling of body and fin patterns, with one developing normally despite defects in the other (Lang et al., 2009; Mellgren and Johnson, 2006; Parichy et al., 2000a) (Figure 7A, B). This suggests either that body and fin micro-environments differentially regulate chromatophore development, or that body and fins are populated by different chromatophore precursors having distinct genetic requirements. Second, the fin is a

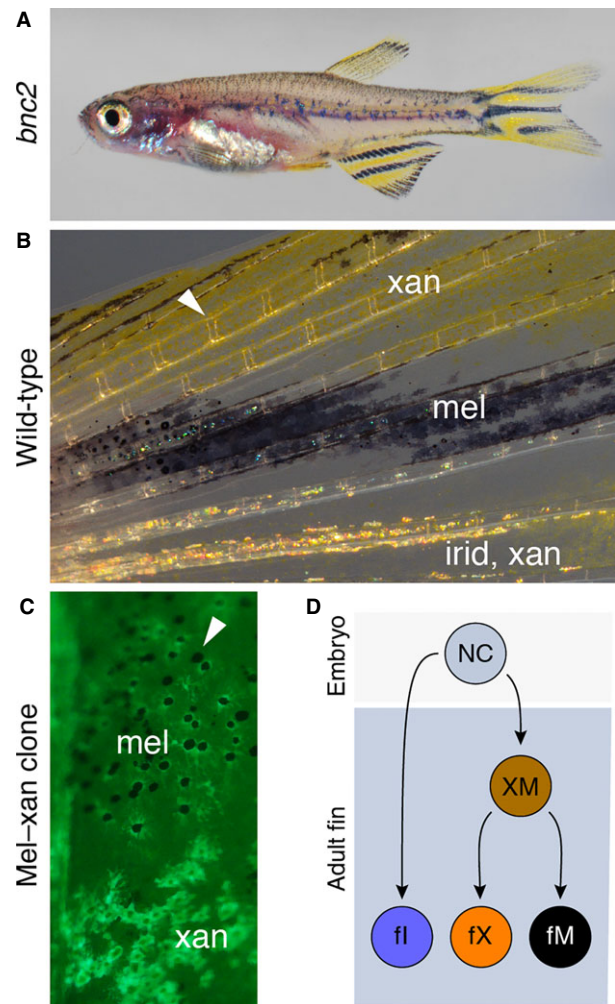


Figure 7. Fin chromatophore patterns and lineages. (A) Despite a severe deficiency in body chromatophores, the *bnc2* mutant has normal fin chromatophores and patterns. (B) Detail of fin chromatophores. Arrowhead indicates joint between fin bones. (C) Example of transposon-based lineage tracing that resulted in a single GFP⁺ clone in the anal fin, stretching from proximal (top) to distal (bottom), and contributing to both melanophores and xanthophores. Arrowhead indicates one of several unlabeled melanophores from an additional unmarked clone (image courtesy of R. Tryon and S. Johnson). (D) NC cells giving rise to fin chromatophores are likely to segregate an adult fin iridophore (fl) lineage initially, followed by a bipotent stem cell (XM) able to generate fin xanthophores (fX) and fin melanophores (fM), that also persists into the adult, in which it can generate regenerative xanthophores and melanophores as well (not shown).

convenient system for studying regeneration: upon amputation, fin bones (lepidotrichia), vasculature, nerves, skin, and pigment pattern are regenerated rapidly, and such cycles of amputation/regeneration can be repeated indefinitely (Goodrich and Nichols, 1931; Morgan, 1900; Tornini and Poss, 2014). Knowing more about the cells that make this possible could provide insights into mechanisms of regeneration and growth control more generally.

During normal development, transposon-based lineage analyses using a *tyrosinase* reporter showed that developing adult fins are likely to be colonized by fewer than ten precursor cells that give rise to all of the fin melanophores (Tu and Johnson, 2010). Some melanophore clones populated only proximal regions of the fin, whereas others extended from proximally to the distal tip where growth occurs. Interestingly, clones that generated melanophores invariably generated xanthophores, and vice versa (Tu and Johnson, 2010, 2011) (Figure 7C). This result indicates that founding cells that colonize the fins are not restricted for one or the other fate at the time of labeling, reminiscent of the multiple chromatophore fates sometimes generated by embryonic neural crest cells (Curran et al., 2010; Dutton et al., 2001; Raible and Eisen, 1994) and contrasting with the apparently distinct lineages of body melanophores and xanthophores characterized so far (Budi et al., 2011; Dooley et al., 2013; Mahalwar et al., 2014; McMenamin et al., 2014). The same analyses revealed only very few clones that also produced fin iridophores, suggesting an earlier segregation of this fate. Together, these results strongly suggest the existence of one or more populations of stem cells in the fin distinct from those that have been identified so far on the body (Figure 7D).

During regeneration, melanophores (and xanthophores) rapidly repopulate fin tissue and generate a normal pattern (Goodrich and Nichols, 1931; Goodrich et al., 1954; O'Reilly-Pol and Johnson, 2008), a process that requires the *de novo* differentiation of melanophores from unpigmented stem cells, rather than proliferation of existing melanophores (Rawls and Johnson, 2000). Moreover, the same clones that give rise to melanophores and xanthophores also give rise to regenerative melanophores and xanthophores (Tu and Johnson, 2010, 2011).

Analyses of *kita* null and temperature-sensitive alleles showed that melanophore regeneration requires Kit signaling, which acts not to establish a fin chromatophore stem cell during embryonic stages but instead to promote the terminal differentiation of regenerating melanophores in the fin itself; in the absence of Kit signaling some melanophores regenerate but they are markedly delayed (Rawls and Johnson, 2000, 2001). These observations led to the classification of fin regenerative melanophores as either primary, *kita*-dependent cells or secondary, *kita*-independent cells. Whereas primary melanophores differentiate distally near the front of regenerating tissue, secondary melanophores differentiate more proximally near the plane of amputation. Although melanophore classes having different Kit dependencies might indicate the existence of two distinct populations of stem cells, this hypothesis was excluded by elegant transposon-based lineage analyses using a temperature-sensitive *kita* allele, in which it was possible to challenge cells to regenerate successively at either restrictive or permissive temperature: single clones could regenerate both primary, *kita*-dependent and secondary, *kita*-independent

regeneration melanophores, implying that a single stem cell relies on Kit signaling for specific cellular processes (Tu and Johnson, 2010). These cells may, for instance, require Kit both for proliferation and migration toward the distal tip of the regenerate; in the absence of such signaling, the numbers of regenerative melanophores would be reduced and their distribution restricted to the vicinity of the stem cell niche(s).

Even more stem cells?

As the preceding sections show, several distinct lineages of direct developing and stem cell-derived chromatophores have now been identified as contributing to the adult pigment pattern of zebrafish. There may be still more lineages. For example, the existence of bipotent melanophore and xanthophore stem cells in the fin (Tu and Johnson, 2010, 2011) suggest such cells might be present on the body as well. If so, these could be the same cells that generate some adult xanthophores inferred from fate mapping (McMenamin et al., 2014). Indeed, extensive regeneration after even repeated laser ablations of adult cells suggest the existence of widespread precursors of still-unknown origin (Nakamasu et al., 2009; Takahashi and Kondo, 2008; Yamaguchi et al., 2007).

A candidate marker for such stem cells or their immediate progeny in adults is *mitfa* (Lister et al., 1999). Although classically considered a marker of the melanocyte or melanophore lineage, *mitfa* expression is detectable in cells expressing markers of the xanthophore lineage in the embryo (Parichy et al., 2000b). Later, during development of the adult pattern, *mitfa* transgenes are typically expressed in the hypodermis by spindle-shaped and highly motile precursors corresponding to melanoblasts, and at lower levels in stellate, slower moving xanthophores and xanthoblasts (Budi et al., 2011; Eom et al., 2012; McMenamin et al., 2014). In the adult, *mitfa* reporters are expressed in melanophores, xanthophores, and unpigmented cells (Figure 8A). Although not a substitute for lineage analysis, these expression domains raise the possibility that cells expressing *mitfa* arise from bipotent or even multipotent stem cells. It will be interesting to learn whether even earlier markers of the neural crest lineage (Luo et al., 2001) identify such stem cells.

Beyond molecular markers, several phenotypes are consistent with an undocumented source of adult pigment cells as well. As noted above, for example, *kita* mutants develop adult melanophores despite their defect in establishing nerve-associated melanophore stem cells (Dooley et al., 2013; O'Reilly-Pol and Johnson, 2013) and the complete absence of melanophores at middle larval stages (Johnson et al., 1995; Mills et al., 2007) (Figure 2A). Similar *kita*-independent melanophores are present in adult guppies and include cells that contribute to the elaborate pattern 'ornaments'

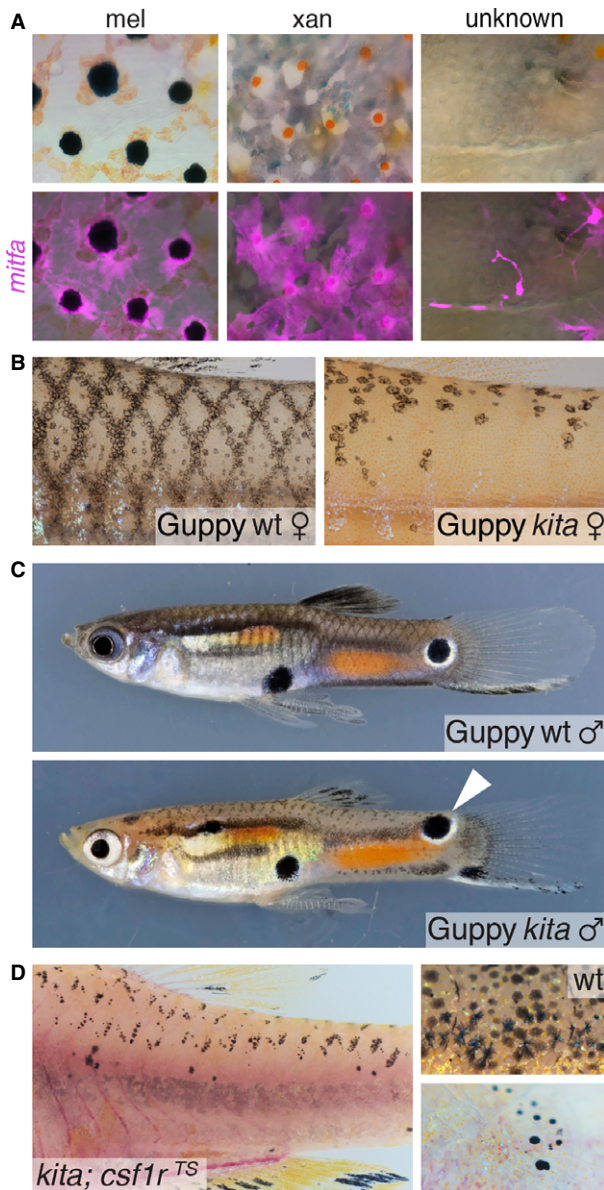


Figure 8. Additional lineages may be present. (A) *mitfa* transgenes mark adult melanophores, xanthophores, and unpigmented cells in the adult. (B) Kita-independent melanophores persist on scales of guppies. (C) Kita-independent melanophore-containing ornaments (e.g., arrowhead) persist in the beautiful patterns of male guppies. (D) In zebrafish, *kita*-independent melanophores develop on scales following *csf1r*-dependent ablation and regeneration (images in B and C courtesy of V. Kottler and C. Dreyer).

unique to males (Kottler et al., 2013) (Figure 8B, C). Moreover, genetic ablations of chromatophores in zebrafish doubly mutant for a null allele of *kita* and a temperature-sensitive allele of *csf1r* result in the development of scale xanthophores and melanophores (Budi et al., 2011) (Figure 8D) even though scale melanophores are not present normally in *kita* mutants, suggesting that an additional population of stem cells

may have been uncovered in this context. Finally, in both *tuba8l3* mutants and ErbB-inhibited fish, most adult melanophores are initially lacking but new melanophores eventually develop (Budi et al., 2008; Parichy and Turner, 2003b; Parichy et al., 2003). All of these observations raise the possibility of additional stem cells competent to generate melanophores and perhaps other chromatophore classes.

Conclusions: implications for development, translational biology and evolution

Work so far indicates a surprising diversity of embryonic and adult chromatophore lineages in teleosts, with perhaps more lineages to be discovered. Such analyses add substantially to our understanding of neural crest fates and complement recent studies of other neural crest sublineage contributions (or lack thereof) to traits of teleosts and other species (Espinosa-Medina et al., 2014; Mongera and Nusslein-Volhard, 2013; Mongera et al., 2013; Shimada et al., 2013). It will also be interesting to learn how many other adult traits depend substantially on post-embryonic stem cells, as opposed to cells that have already acquired their definitive phenotypes during embryogenesis.

Another area in which studies of adult pigment cell lineages seem poised to make a contribution is our understanding of melanoma onset and progression. Melanoma remains one of the deadliest cancers, and its incidence has continued to increase (Balch et al., 2001, 2010; Howlader et al., 2014; Jemal et al., 2009). Several studies of amniotes suggest that the invasive and metastatic potential of melanoma cells is attributable, at least in part, to the re-expression of genes used during early stages of neural crest and melanocyte lineage development (Bailey et al., 2012; Gupta et al., 2005; Kulesa et al., 2013). Despite such realizations, and the association of melanoma cells with a neural crest–melanocyte lineage, it remains unclear to what extent melanomas arise from cells at different steps in this lineage (e.g., latent stem cells versus transit amplifying cells versus differentiated melanocytes) (Bennett, 2003; Grichnik et al., 2006). Several teleosts have been used for studying melanoma (Meierjohann and Schartl, 2006; Patton et al., 2010; Schartl et al., 2012), and in recent years, the zebrafish has become a tractable model, in which melanoma can be induced using the same oncogenic mutation (BRAF^{V600E}) responsible for many human melanomas (Patton et al., 2005). Studies using this system have provided important new insights into genomic alterations in melanoma cells and their metastatic potential and are aiding in drug discovery (Ceol et al., 2011; Lister et al., 2014; Michailidou et al., 2009; White et al., 2011; Yen et al., 2013). Interestingly, ErbB-inhibited fish remain susceptible to melanoma (Santoriello et al., 2012), adding to the notion that additional stem cell populations may yet be found in fish that contribute to

melanoma, and such findings would likely have great translational relevance to human.

In an evolutionary context, studies of teleost pigment cell lineages invite interesting comparisons with other phylogenetic groups. Although the precise origins of adult chromatophores in other ectothermic vertebrates have yet to be elucidated, it seems likely that lineages homologous or analogous to those of teleosts will be found, particularly in amphibians, given the ontogenetic changes in pigment patterns often observed in this group (Niu and Twitty, 1950; Parichy, 1998; Parichy et al., 2006; Stearner, 1946). In a broader phylogenetic context, a potential homology of peripheral nerve-associated precursors to teleost melanophores and amniote melanocytes has been cited already, and it is conceivable that teleosts will be found to have adult stem cells homologous to melanocyte stem cells present in hair (Nishimura, 2011; Nishimura et al., 2002; Tanimura et al., 2011) or feather (Lin et al., 2013) follicles as well. Although xanthophores are not found in amniotes, their pigments – pteridines and carotenoids – are found in avian feathers and in the avian iris, and orthologues of a gene required for xanthophore differentiation in medaka (*slc2a11b*) persist in birds, though they have been lost from mammals, which lack such pigmentation (Kimura et al., 2014). Xanthophore-like cells also have been identified in some species of ascidians (Jeffery, 2006; Jeffery et al., 2004, 2008); it will be exciting to learn if these cells depend on a gene regulatory network similar to that of vertebrate xanthophores, and whether or not such a network reflects shared ancestry, or an independent recruitment to such a function in vertebrates and non-vertebrate chordates.

Finally, studies of adult pigment cell lineages also suggest clues to the evolutionary diversification of pigment patterns themselves. Development of adult pigment patterns requires interactions among different pigment cell classes, and changes in these interactions and the timing of pigment cell differentiation are likely to explain some species differences (Kondo and Asai, 1995; Miyazawa et al., 2010; Patterson et al., 2014; Quigley et al., 2005; Watanabe and Kondo, 2012). Diversification of teleost pigment patterns has likely been facilitated by extra genes persisting from whole-genome duplication and the attendant opportunities for subfunctionalization (Braasch et al., 2006, 2009a,b; Force et al., 1999; Hultman et al., 2007). The existence of multiple chromatophore lineages in teleosts – having different developmental origins and genetic requirements – seems likely to have added another dimension to pattern diversification, in providing additional sources of variation upon which selection can act and additional possibilities for cell-type specialization and complexity. Together, these several factors seem likely to have contributed to the often striking pattern differences across life cycle stages within species (Booth, 1990; Quigley et al., 2004), and stunning pattern diversity across species (Baldwin, 2013; Fan et al., 2012; Greenwood et al., 2012; Kottler et al., 2014; Mills and

Patterson, 2008; Seehausen et al., 2008), including the evolution of species-specific ornaments and other pigimentary adaptations (Hughes et al., 2013; Kemp et al., 2009; Kottler et al., 2014; Martin et al., 2014; Santos et al., 2014). All of these possibilities demand further experimental analyses that are likely to be very interesting, whatever their ultimate conclusions might be.

Acknowledgements

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Note

¹Days post-fertilization are used here for heuristic purposes only. In practice, developmental rates differ markedly between fish strains and laboratories, so age is not an adequate indicator of developmental stage. Normal stages of post-embryonic zebrafish development based on measures of size and attainment of developmental milestones have been described and specific stage reporting conventions have been recommended to account for genetic and environmental variation (Parichy et al., 2009).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Movie S1. Adult pigment pattern development in zebra-fish over approximately 3 weeks, beginning with the early larval pattern and ending with the juvenile pattern. Animation highlights changes in melanophore pattern and is compiled from repeated daily imaging of a

single individual, with frames aligned and rescaled to control for growth (Quigley et al., 2005).

Movie S2. *mitfa*:GFP⁺ cells (e.g., arrows) migrating to the hypodermis from the dorsal aspect of the myotome (Budi et al., 2011).

Movie S3. *mitfa*:GFP⁺ cells (e.g., arrow) enter the hypodermis via the myoseptum during adult pattern development (Budi et al., 2011).