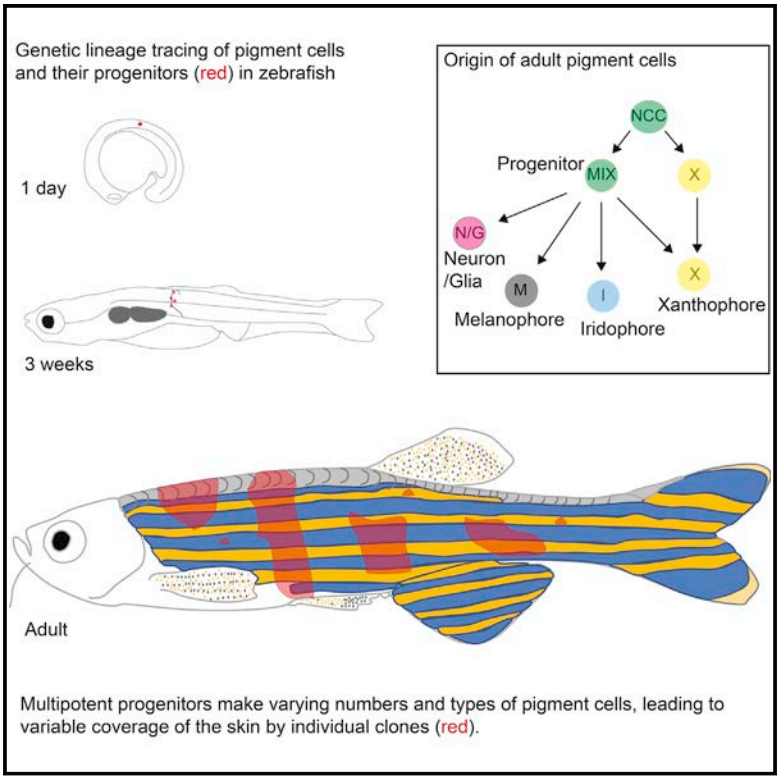
Article

Developmental Cell

Pigment Cell Progenitors in Zebrafish Remain Multipotent through Metamorphosis

Graphical Abstract



Multipotent progenitors make varying numbers and types of pigment cells, leading to variable coverage of the skin by individual clones (red).

Highlights

- Neural crest-derived progenitors of adult pigment cells remain multipotent
- The postembryonic progenitors are associated with the peripheral nervous system
- The progenitors are plastic and give rise to varying pigment cell numbers and types
- Proliferation decreases when progenitors commit to a specific pigment cell fate

Authors

Ajeet Pratap Singh, April Dinwiddie, Prateek Mahalwar, Ursula Schach, Claudia Linker, Uwe Irion, Christiane Nüsslein-Volhard

Correspondence

christiane.nuesslein-volhard@tuebingen.mpg.de

In Brief

Fish display intricate color patterns generated by specialized pigment cells. Singh et al. show that the pigment cells in zebrafish originate from neural crest-derived progenitors associated with the peripheral nervous system. These progenitors remain multipotent and plastic beyond embryogenesis and into metamorphosis, when the adult color pattern begins to develop.

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Pigment Cell Progenitors in Zebrafish Remain Multipotent through Metamorphosis

Ajeet Pratap Singh,[1] April Dinwiddie,[1] Prateek Mahalwar,[1] Ursula Schach,[1] Claudia Linker,[2] Uwe Irion,[1] and Christiane Nüsslein-Volhard[1,*]

[1]Max Planck Institute for Developmental Biology, Spemannstraße 35, Tübingen 72076, Germany
[2]Randall Division of Cell & Molecular Biophysics, King's College, London SE1 1UL, UK
*Correspondence: christiane.nuesslein-volhard@tuebingen.mpg.de
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SUMMARY

The neural crest is a transient, multipotent embryonic cell population in vertebrates giving rise to diverse cell types in adults via intermediate progenitors. The in vivo cell-fate potential and lineage segregation of these postembryonic progenitors is poorly understood, and it is unknown if and when the progenitors become fate restricted. We investigate the fate restriction in the neural crest-derived stem cells and intermediate progenitors in zebrafish, which give rise to three distinct adult pigment cell types: melanophores, iridophores, and xanthophores. By inducing clones in *sox10*-expressing cells, we trace and quantitatively compare the pigment cell progenitors at four stages, from embryogenesis to metamorphosis. At all stages, a large fraction of the progenitors are multipotent. These multipotent progenitors have a high proliferation ability, which diminishes with fate restriction. We suggest that multipotency of the nerve-associated progenitors lasting into metamorphosis may have facilitated the evolution of adult-specific traits in vertebrates.

INTRODUCTION

The neural crest is a transient, embryonic population of multipotent cells that arises from the dorsal portion of the CNS in the vertebrate embryo, and undergoes extensive migration throughout the body to give rise to diverse cell types including chondrocytes, osteocytes, gill pillar cells, neurons, glia, and pigment cells (Baggiolini et al., 2015; Dupin et al., 2007; Green et al., 2015; Mongera et al., 2013; Weston and Thiery, 2015). Some neural crest cells produce progenitors that persist and contribute to the development of adult cell types and tissues. The in vivo potential and rules of lineage segregation of these postembryonic progenitors remain poorly understood (Dupin and Sommer, 2012).

Here we use the adult color pattern of zebrafish (Figures 1A and 1A′) as a system to investigate the cell lineage and fate restrictions in the neural crest and neural crest-derived postembryonic progenitor cells. The adult coloration of zebrafish (*Danio rerio*) has emerged as a model system in which the biology of late-developing, adult-specific traits can be systematically analyzed (Irion et al., 2016; Kelsh et al., 2009; Parichy and Spiewak, 2015; Singh and Nüsslein-Volhard, 2015; Watanabe and Kondo, 2015). The layered organization of three types of neural crest-derived pigment cells—black melanophores, blue/silvery iridophores, and yellow xanthophores—generates the striped adult pattern in zebrafish, and each of these cell types can be definitively identified by its color, shape, and location in the skin (Hirata et al., 2005). The three cell types reach the skin through different routes: postembryonic progenitors associated with the peripheral nervous system (PNS) generate iridophores and melanophores (in addition to neurons and glia), and embryonic xanthophores give rise to most adult xanthophores (Budi et al., 2011; Dooley et al., 2013a; Mahalwar et al., 2014; McMenamin et al., 2014; Singh et al., 2014). The PNS-associated postembryonic progenitors in zebrafish may be comparable with the Schwann cell precursors that generate melanocytes in birds and mammals (Adameyko et al., 2009). Previously, we used Cre/loxP-mediated recombination in *sox10*-expressing, neural crest-derived progenitors in the trunk of zebrafish to induce labeled clones that allow for tracing of pigment cells through development until adulthood (Mongera et al., 2013; Singh et al., 2014). Sox10 is specifically expressed in both premigratory and migrating neural crest cells during development (Dutton et al., 2001). The pigment cell progenitors reach the skin during metamorphosis through three major routes along nerve tracts of the PNS: dorsally, laterally, and ventrally (Budi et al., 2011; Dooley et al., 2013a; Singh et al., 2014). At the onset of metamorphosis, the iridophore progenitors reach the skin along the horizontal myoseptum; they proliferate and spread dorsoventrally in the skin to contribute to most, if not all, of the light stripes along the dorsoventral axis by patterned aggregation (Singh et al., 2014). Melanophore progenitors begin to populate nerve routes to the skin at the onset of metamorphosis, and reach the skin as melanoblasts via the dorsal, horizontal, and ventral myosepta (Dooley et al., 2013a; Singh et al., 2014). Iridophores and xanthophores proliferate in the skin, whereas melanophores expand in size but rarely divide after reaching the skin. However, owing to a lack of a comprehensive lineage-tracing analysis of the pigment cell progenitors, it remains unclear if and when the pigment cell progenitors become fate restricted. Consequently, our understanding of progenitor behavior during color pattern formation and the lineage relationships between pigment cells remains ill defined.

In this study, we induced pigment cell clones at four time points from embryogenesis to early metamorphosis at 21 days

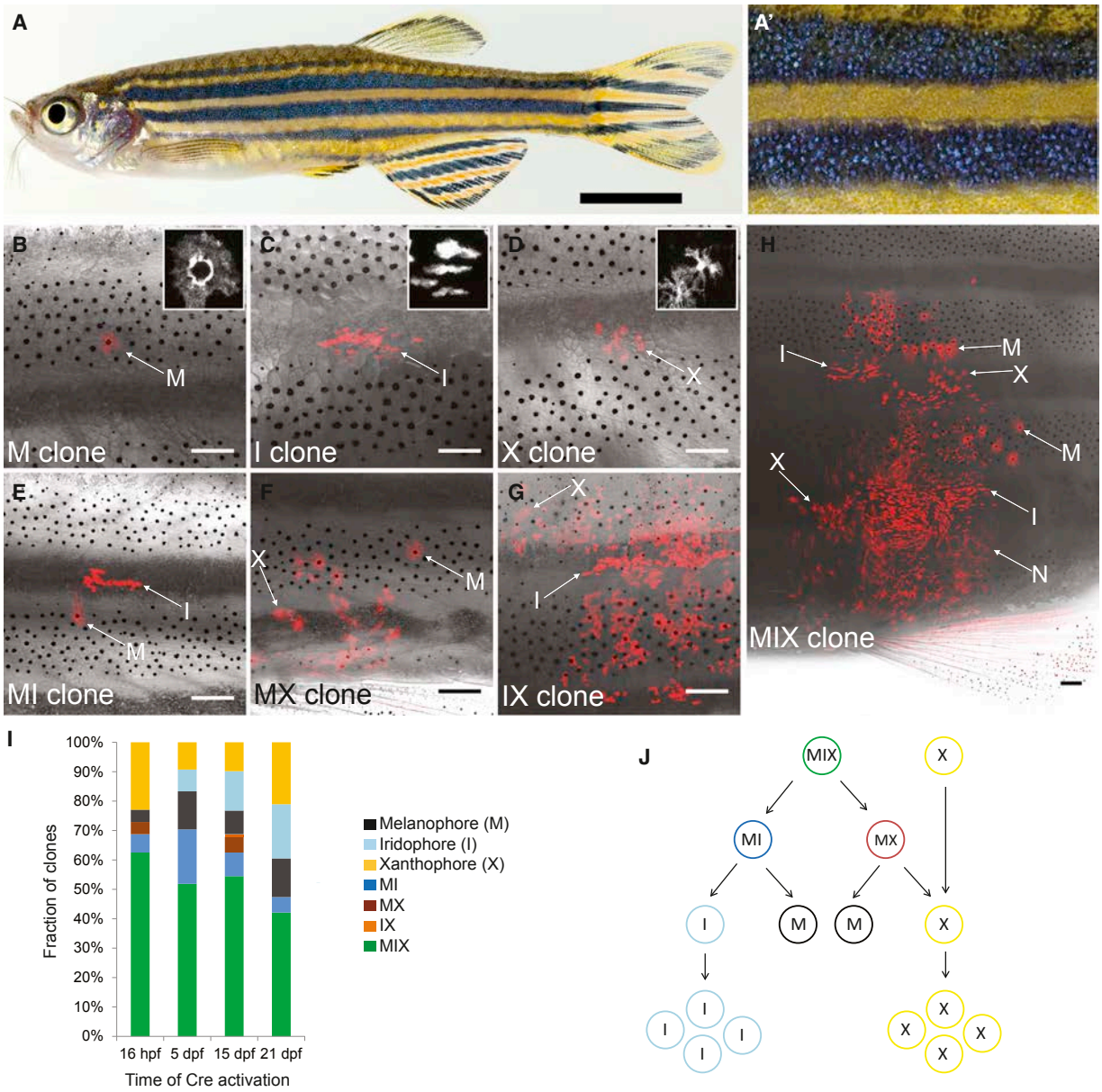


Figure 1. Clonal Association between the Three Pigment Cell Types

(A) Adult zebrafish and (A′) close-up of the striped pattern on zebrafish trunk.

(B–H) Types of pigment cell clones in young adult zebrafish carrying *Tg*(*sox10*:*ER*^{T2}-*Cre*) and *Tg*(*βactin2*:*loxP-STOP-loxP-DsRed-express*): (B) a single melanophore (M), (C) iridophores (I), (D) xanthophores (X), (E) melanophore and iridophore (MI), (F) melanophore and xanthophore (MX), (G) iridophore and xanthophore (IX; note that this is the only IX that we obtained), and (H) melanophore, iridophore, and xanthophore (MIX) clones. Representative images in (B)–(G) (DsRed/bright field) are from clones obtained from Cre activation at 15 dpf.

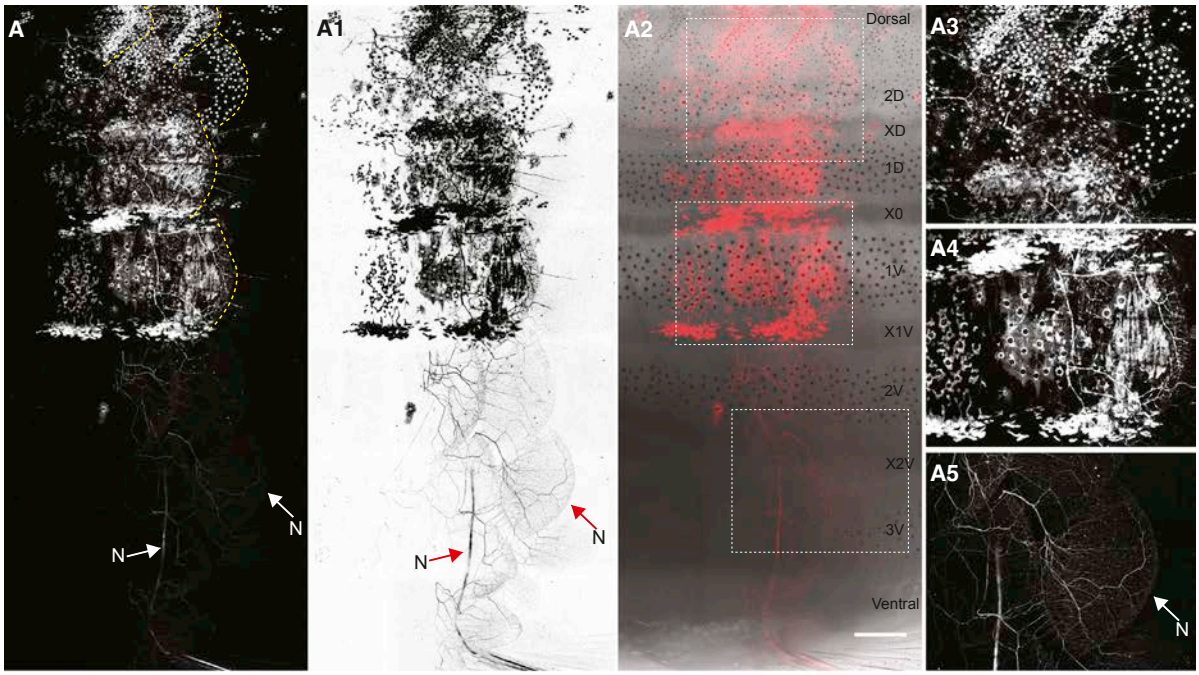
(I) Quantification of the clonal association between pigment cell types obtained by Cre activation at 16 hpf (n = 48 clones from 43 animals, median standard length [SL] at the time of image acquisition = 20.8 ± 1.84 mm); 5 dpf (n = 54 clones from 36 animals, median SL at the time of image acquisition = 24 ± 1.77 mm); 15 dpf (n = 112 clones from 74 animals, median SL at the time of image acquisition = 21 ± 1.77 mm); and 21 dpf (n = 38 clones from 23 animals, median SL at the time of image acquisition = 20 ± 2.07 mm).

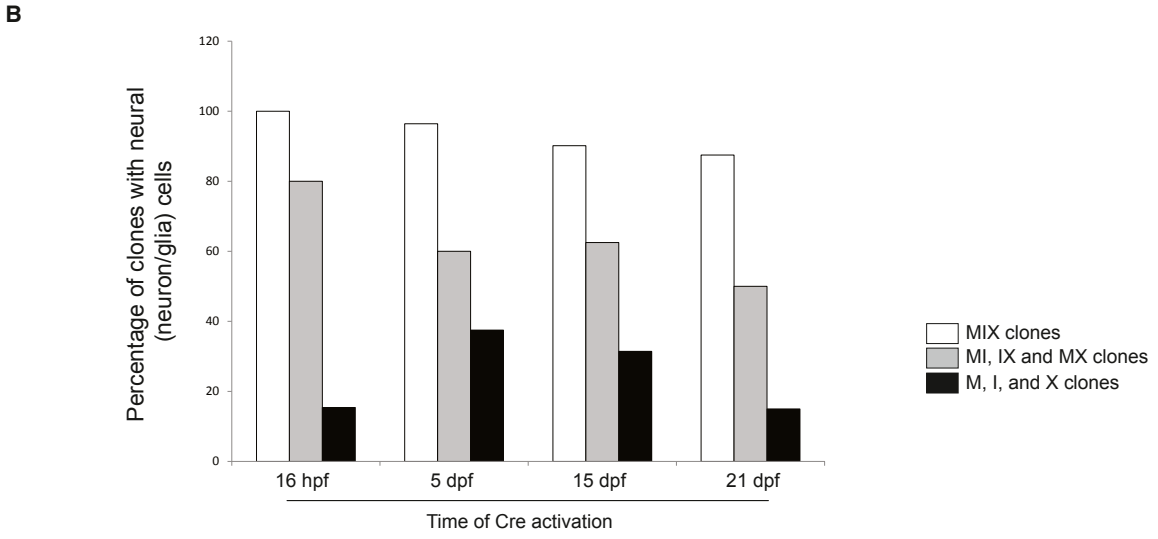
(J) Schematic representation of the segregation of pigment cell fates.

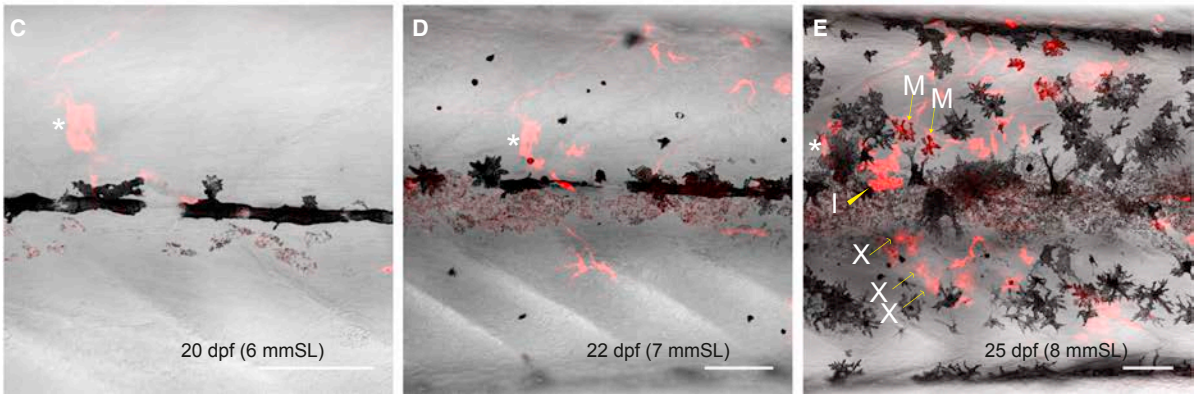
N, neuron; M, melanophore; I, iridophore; X, xanthophore.

Insets in (B)–(D) are blow-ups. Scale bars represent 5 mm (A) and 250 μm (B–H). See also Figures S1 and S2 and Tables S1 and S2.

post fertilization (dpf), when the adult color pattern begins to develop. We analyzed clone size and composition in 2- to 3-month-old fish. We focused on the trunk region of zebrafish that includes the pigment cells forming the color pattern in the hypodermis and the scales (Figures 1A and 1A′). Our results show that neural crest-derived progenitors remain multipotent, at least until metamorphosis, and disperse in close association with nerve tracts along the dorsoventral axis. Many clones







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contain neurons as well as the three pigment cell types, which generate the color pattern in the skin and the scales, but clones also may be restricted to one or two cell types. Importantly, at all stages the progenitor cells are not stereotypic in terms of the types and numbers of cells that they generate: there is variability in the amount and extent of clonally derived pigment cells along the dorsoventral axis. Individual clones induced in the embryo may give rise to the entire complement of pigment cells of one hemisegment or to only a few pigment cells, suggesting that a variable number of multipotent stem cells (one to a few) are laid down in each hemisegment during embryogenesis. The stem cells produce progenitors that multiply and disperse along the branching PNS so that at later stages a single progenitor will generate a smaller number of cells. Furthermore, multipotent progenitors produce larger clones, suggesting that commitment to a specific pigment cell lineage reduces the ability to proliferate. This effect is most striking for the melanophores: clones with only labeled melanophores tend to be restricted to one or two cells, whereas iridophore-only or xanthophore-only clones are larger, yet still smaller than clones in which all three pigment cell types are labeled. In addition, analysis of clones in mutants of signaling systems that regulate the development of a single pigment cell type suggests that the establishment of multipotent progenitors along the three dorsoventral routes is not affected in the mutants, but that the activity of these genes is required in the committed precursors and differentiating pigment cells.

RESULTS

Progenitors of Adult Pigment Cells Remain Multipotent from Embryogenesis to Early Metamorphosis

To obtain DsRed-labeled clones of neural crest and their progenitor-derived cells in the trunk of zebrafish, we induced Cre/loxP-mediated recombination in fish carrying both the transgenes *Tg*(*sox10*:*ER*^{T2}-*Cre*) (Mongera et al., 2013) and *Tg*(*βactin2*:*loxP-STOP-loxP-DsRed-express*) (Bertrand et al., 2010). Cre was activated in four sets of fish at the following stages of development: embryonic migratory neural crest stage (16 hr post fertilization [hpf]); larval stage (5 dpf); prior to the onset of metamorphosis (15 dpf); and during metamorphosis (21 dpf, upon the appearance of iridophore clusters in the skin). All clones (a total of 252) that contained labeled pigment cells in the trunk of young adult fish (2–3 months post fertilization) were imaged and analyzed. All fish that were positive for pigment cell clones had, on average, fewer than two clones. For example, from Cre activation at 15 dpf, 262 fish were screened, and we obtained 74 fish carrying 112 labeled clones. In a separate experiment, imaging of clones 1 day after Cre activation revealed labeling of one cell per clone (Cre activation at 15 dpf; imaging at 16 dpf; eight clones obtained after screening 101 fish; Figure S1). This indicates that each clone arose from a single recombination event (for the exact sample sizes and methodology, see figure legends, Table S1, and Experimental Procedures). Clones varied in pigment cell composition, and a number of clones contained only one or two pigment cell types (Figures 1B–1G, quantification in Figure 1I; for pigment cell identification, see Figure S2). Significantly, each set contained a large fraction of clones with all three pigment cell types labeled: from 62% of clones obtained from Cre activation at 16 hpf, to 42% of all clones obtained from Cre activation at 21 dpf (Figure 1H, quantification in Figure 1I; Table S2). We term these clones MIX clones for melanophore-, iridophore-, and xanthophore-containing clones. Interestingly, IX clones (containing iridophores and xanthophores) were observed only once (Figure 1G), while clones containing melanophores and iridophores (MI clones, 24 clones), or melanophores and xanthophores (MX clones, eight clones) were more frequent (Figure 1I). This suggests constraints on lineage segregation, and that the multipotent progenitors differentiate into progenitors, which have the potential to generate either melanophores and iridophores, or melanophores and xanthophores (Figure 1J). The IX clones were uncommon, which suggests that progenitors for iridophores and xanthophores may split off at early time points.

Furthermore, several of the pigment cell clones labeled neural cells (Figures 2A–2A5; neurons and/or glia; 75% of clones obtained from Cre activation at 16 hpf, 72% of clones obtained from Cre activation at 5 dpf, 68% of clones obtained from Cre activation at 15 dpf, and 47% of all clones obtained from Cre activation at 21 dpf). A larger fraction of MIX clones labeled neural cells in comparison with single cell clones (histogram in Figure 2B). From these data, we conclude that neural crest-derived progenitors are multipotent, up to at least 3 weeks post fertilization, and can continue to give rise to all three pigment cell types of the body and scales, as well as peripheral nerves and glia.

Xanthophores Have a Dual Cellular Origin

It was recently shown that adult xanthophores in zebrafish originate from existing embryonic/larval xanthophores, which start to proliferate at the onset of metamorphosis (Mahalwar et al., 2014; McMenamin et al., 2014). Indeed, in agreement with these findings, we obtained a number of large, xanthophore-only clones at all stages (Figure 1I). However, adult xanthophores can develop after the ablation of embryonic/larval xanthophores (McMenamin et al., 2014; Walderich et al., 2016), indicating that a second source of adult xanthophores must exist. Our clonal analysis shows that many of the PNS-associated pigment cell progenitors are still multipotent at the onset of metamorphosis, and that the MIX and MX clones include variable numbers of xanthophores (Figure 1I). Repeated imaging of labeled clones during metamorphosis showed that clones that developed neural cells, iridophores, and melanophores during metamorphosis also produced xanthophores (Figures 2C–2E, 3, and S3; n = 11

Figure 2. Shared Multipotent Progenitors for Pigment Cells and Neural Cells

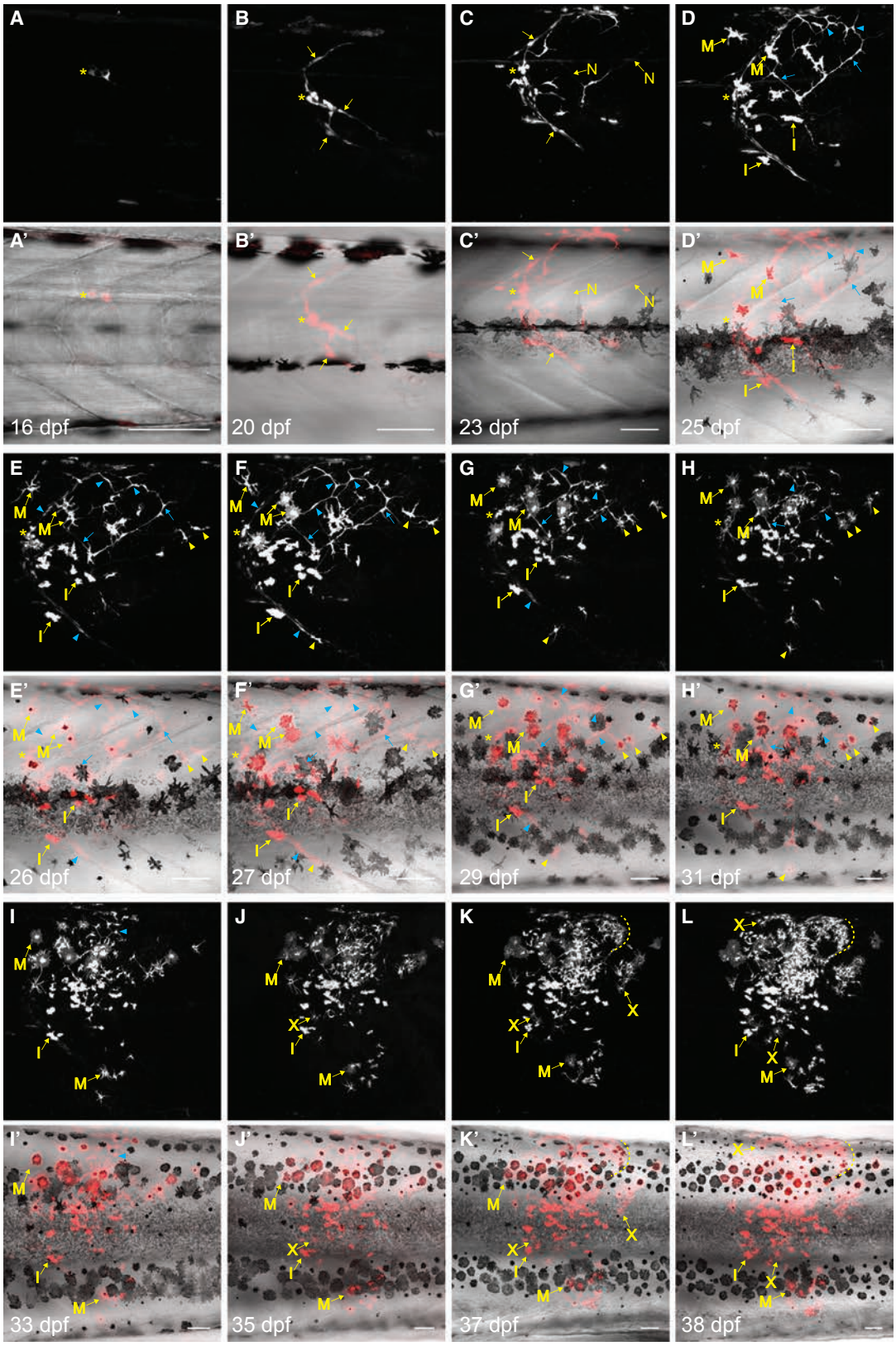
(A–A5) Example of a clone with labeled neurons and pigment cells in the trunk skin, scales, and dorsum. Dashed curves in (A) show scale pigmentation. Dashed squares in (A2) show regions enlarged in (A3)–(A5). Scale bar, 500 μm.

(B) Quantification of the clonal association between pigment cells and neural cells.

(C and D) A clone in the skin of 20-dpf zebrafish with DRG (asterisk) and neurons. Scale bar, 100 μm.

(E) Xanthophores, iridophores, and melanophores appear in this clone at 25 dpf. Asterisk indicates the DRG. Scale bar, 100 μm.

N, neuron; M, melanophore; I, iridophore; X, xanthophore.



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clones). Overall, we conclude that xanthophores have a dual cellular origin: initially from existing embryonic/larval xanthophores, and subsequently also from multipotent postembryonic progenitors.

Scale Pigmentation Shares a Lineage with Skin Pigmentation

Our analysis shows that scales, the mesoderm-derived dermal skeletal elements that develop during metamorphosis (Lee et al., 2013; Mongera and Nüsslein-Volhard, 2013), are populated by pigment cells originating from multipotent progenitors; in fact, nearly half of the total clones induced at 15 dpf contributed to scales, in addition to body pigmentation (59 of 112 clones; Figures 2A–2A2, close-up in Figures 2A3 and 2A4). Scale pigmentation is readily distinguished from body pigmentation by the characteristic inverted-C-shaped organization and the distinct shapes of scale pigment cells (dashed lines in Figures S3K–S3O′). Neurons derived from these progenitors innervate the skin of the trunk as well as the scales (Figures 2A–2A2, close-up in Figure 2A5). Imaging clones induced at 4–5 dpf over several days revealed events that lead to the development of the three pigment cell types on the body and scales from shared progenitors (Figures 3 and S3). Figure 3 shows progenitors located at the dorsal root ganglion (DRG) that will give rise to neurons, iridophores, and melanophores over the next several days (Figures 3A–3D′). At first, many undifferentiated cells appear along the newly developing neuronal arbors (yellow arrows in Figures 3C and 3C′ indicate thin neuronal arbors, and corresponding blue arrows in Figures 3D and 3D′ indicate cells that appear along these nerve branches). In a few cases, we were able to follow undifferentiated cells as they differentiated into pigment cells (yellow arrowheads in Figures 3E–3H′). Xanthophores and melanophores of the scales appear in this clone over the next few days (Figures 3I–3L′, dashed curves indicate newly formed scales and their pigment cells). A detailed development of scale pigmentation is presented in Figure S3, and shows the appearance of cells in close association with peripheral nerves. These cells expand in number and subsequently differentiate into melanophores, xanthophores, and iridophores of the scales. Therefore, scale and body pigment cells originate from common multipotent progenitors.

Pigment Cell Clones Are Distributed along the Dorsoventral Body Axis

Although a large fraction of pigment cell progenitors remain multipotent (Figure 1I), we observed variability of individual clones in terms of their span along the dorsoventral axis, and in terms of their cell types and cell numbers (Figures 4A–4D). Figure 4 shows the span of clones in young adult fish that were obtained from Cre activation at 15 dpf. Quantification revealed variability in the extent to which individual clones contributed to pigment cells along the dorsoventral axis: the first class of clones included the pigment cells of the dorsum and dorsal body stripes (Figure 4A), a second class primarily spanned laterally without reaching either the dorsal- or ventral-most regions of the body (Figure 4B), and a third class primarily covered ventral regions (Figure 4C, quantification in Figure 4D; n = 112 clones). Interestingly, the clones that were fate restricted to one or two pigment cell types predominantly belonged to the second class, and spanned a region along the first light stripe. Such clones, mainly of iridophores and melanophores, are presumably derived from committed precursors directly migrating through the horizontal myoseptum. By comparison, MIX clones, in which all three pigment cell types are labeled, spanned a larger area along the dorsoventral axis. This suggests that the ability of progenitors to proliferate is reduced when they become fate restricted (quantification in Figure 4D).

To refine our analysis of progenitor contribution along the dorsoventral axis, we specifically focused on melanophores, which can be easily recognized and quantified due to their distinct black pigment. In adult zebrafish, there are four to five dark stripes and four light stripes that can be identified based on their location with respect to anatomical landmarks in the body (text in Figures 4A–4C refers to standard zebrafish stripe nomenclature [Frohnhöfer et al., 2013]). We counted the number of labeled melanophores that develop in each clone in the dark stripes 1D, 1V, and 2V. Stripes 1D and 1V are the first dark stripes that appear at the dorsal and ventral side of the first light stripe, X0; stripe 2V is subsequently added ventrally. Stripes 2D and 3V were not included in this analysis as these stripes are present only in the anterior part of the trunk. Moreover, stripe 2D is masked by the melanophores of the dorsum, making it difficult to unambiguously count the number of labeled melanophores. The analysis revealed a distribution of melanophores consistent with clone span, and this suggested that there are dorsoventrally restricted multipotent progenitors. Dorsally prominent MIX clones contributed primarily to the melanophores of the dorsal stripes, whereas ventrally prominent MIX clones contributed primarily to the melanophores of the ventral stripes (quantification in Figure 4E; blue, stripe 1D; brown, stripe 1V; green, stripe 2V).

Pigment Cell Progenitors Do Not Display Stereotypic Cell-Fate Restrictions

To obtain an overview of progenitor behavior in transition from embryogenesis to metamorphosis, we performed a similar quantitative analysis of clone span and melanophore count from clones obtained by Cre activation at 16 hpf, 5 dpf, and 21 dpf (Figure 5). In clones induced at 16 hpf, a time point when the neural crest cells as such are still present and start to migrate, a large fraction of the MIX clones span the entire dorsoventral axis, and melanophores contribute to most, if not all, dark stripes. Surprisingly, however, there is considerable variability in span and pigment cell number of these clones (Figures 5A and 5A′, quantification in Figures 5D and 5G). This variability continues during the larval and metamorphic stages: clones obtained from Cre activation at 5 and 21 dpf are variable in their span, pigment

Figure 3. Long-Term Imaging of Progenitors that Make Neurons and Three Pigment Cell Types

(A–L′) Repeated imaging of a clone induced at 5 dpf in zebrafish carrying *Tg*(*sox10*:*ER*^{T2}-*Cre*) and *Tg*(*βactin2*:*loxP-STOP-loxP-DsRed-express*). Grayscale in (A)–(L): clone; Red/gray scale in (A′)–(L′): clone/bright field. Asterisks indicate the DRG. Yellow arrows indicate nerve routes, unless indicated otherwise; blue arrows and arrowheads show undifferentiated progenitors; yellow arrowheads in (E)–(H′) show undifferentiated progenitors that make melanophores; dashed curves indicate pigment cells of a scale. Scale bars, 100 μm. See also Figure S3.

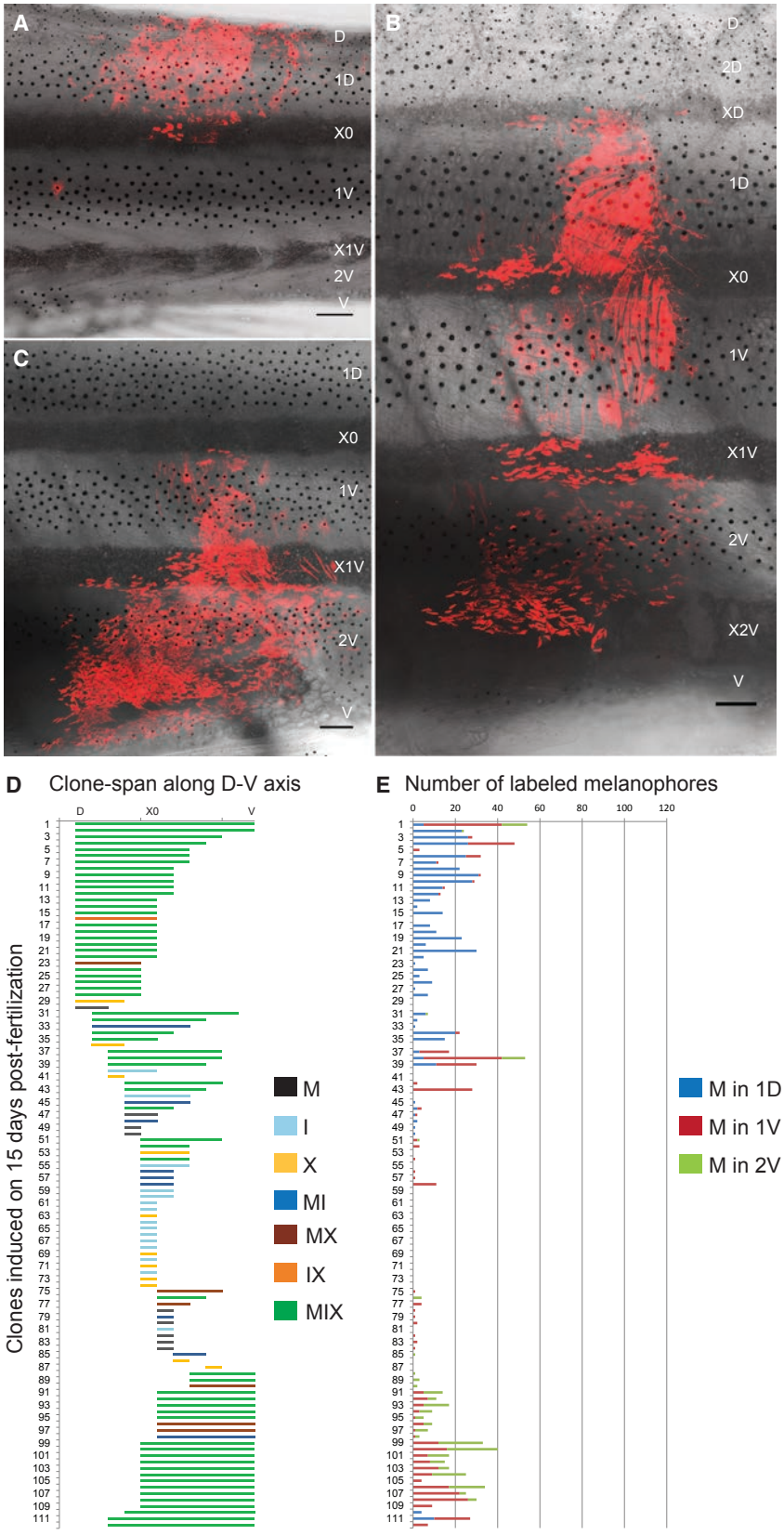


Figure 4. Regional Distribution of Clonally Derived Pigment Cells

(A–D) Clones in young adult animals obtained from Cre activation at 15 dpf (genotype *Tg*(*sox10*:*ER*^{T2}-*Cre*)/+; *Tg*(*βactin2*:*loxP-STOP-loxP-DsRed-express*)/+) show variability in clone span along the dorsoventral body axis: the clonally derived pigment cells may predominantly contribute to pigment cells of the (A) dorsal, (B) lateral, or (C) ventral regions. Scale bars represent 250 μm. (D) Quantification of the extent of the clones along the dorsoventral body axis; each line represents the dorsoventral span of a single clone. Color code indicates clone type according to pigment cell composition. MIX clones tend to be larger than clones containing only a single pigment cell type. D, dorsal; V, ventral; X0, first light stripe.

(E) Quantification of the number of labeled melanophores in each clone; each line represents the number of melanophores in an individual clone. Color code indicates the dark stripe: blue, 1D; brown, 1V; green, 2V.

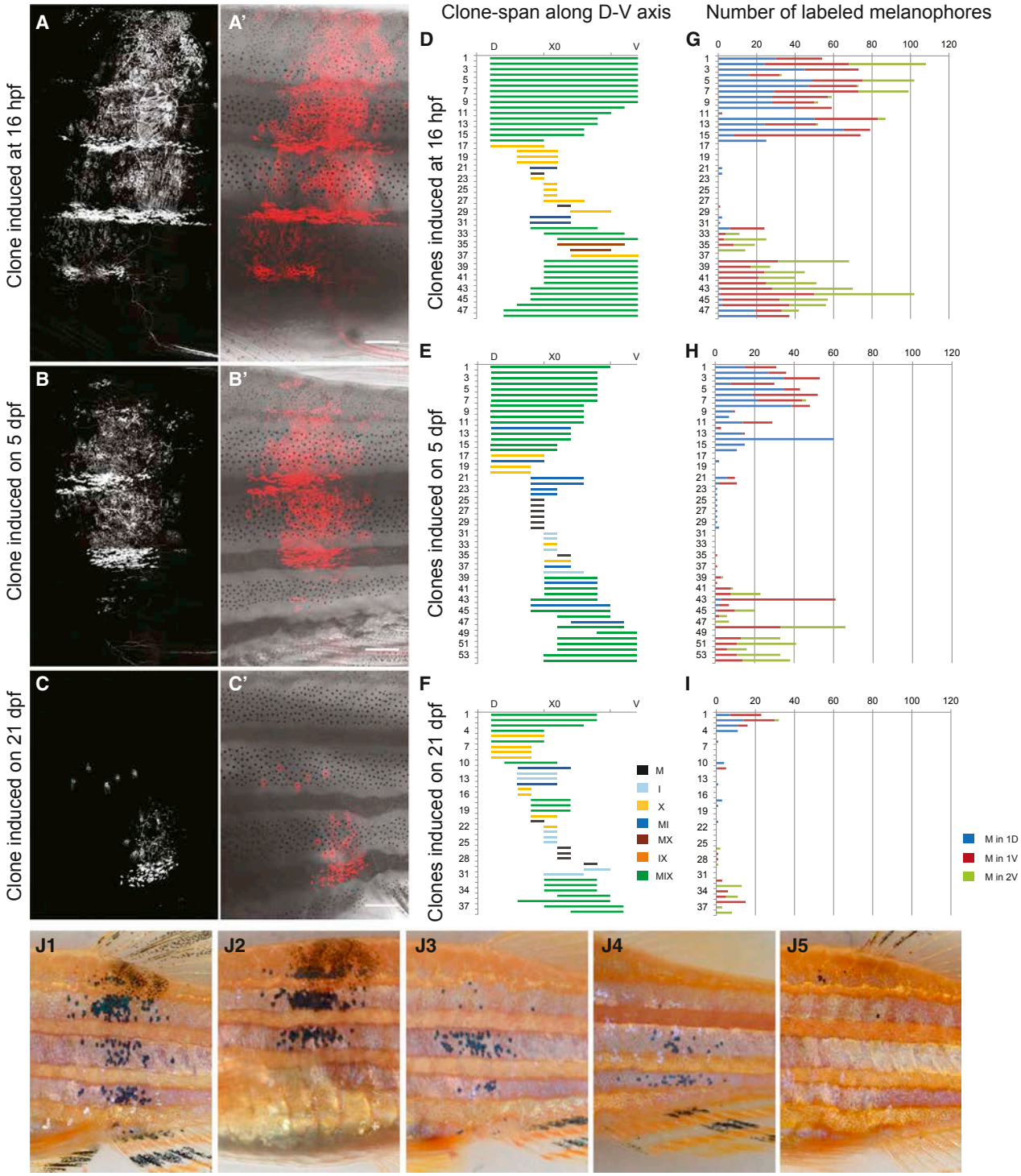


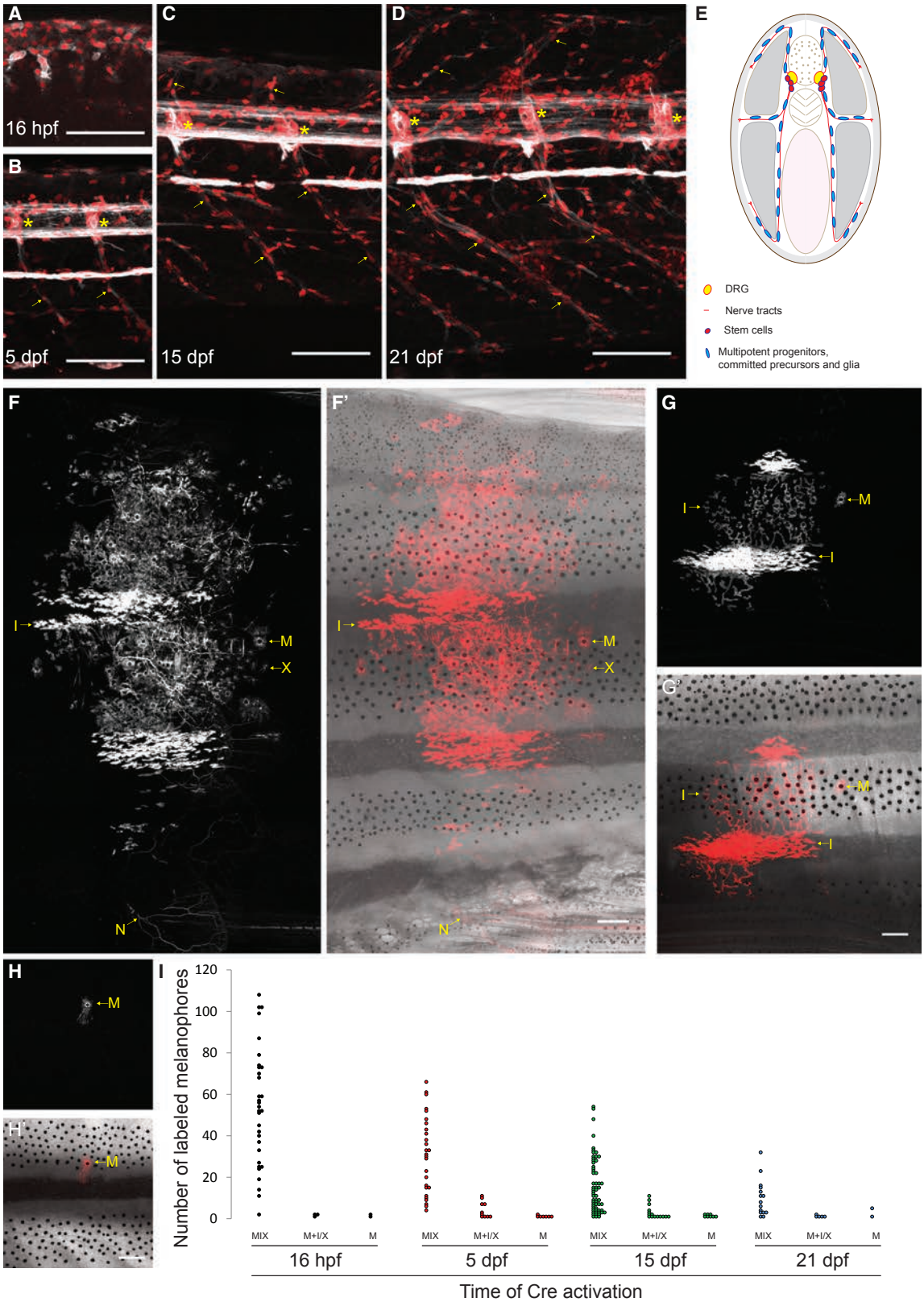
Figure 5. Neural Crest-Derived Progenitors are Multipotent but Do Not Have Stereotypic Outcome

(A–F) Clones in young adult animals obtained from Cre activation at (A, A′, D, G) 16 hpf, (B, B′, E, H) 5 dpf, and (C, C′, F, I) 21 dpf (genotype *Tg*(*sox10*:*ER*^{T2}-*Cre*)/+; *Tg*(*βactin2*:*loxP-STOP-loxP-DsRed-express*)/+) reveal a progressive reduction in the size and span of the clonally derived pigment cells, albeit several clones remain MIX, suggesting existence of multipotent progenitors at all time points. (D–F) Quantification of the extent of the clones along the dorsoventral body axis; each line represents dorsoventral span of a single clone. Color code shows clone type according to pigment cell composition.

(G–I) Quantification of the number of labeled melanophores in each clone; each line represents the number of melanophores in an individual clone. Color code indicates the dark stripe: blue, 1D; brown, 1V; green, 2V.

(J1–J5) Skin of F_0 adults from embryos injected with *albino* knockout CRISPR.

Scale bars in (A)–(C′) represent 500 μm. See also Figure S4.



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cell number, and composition, and at all time points 40%–60% are MIX clones (Figures 5B–5C′, quantification in Figures 5E–5F, bar graph in Figure 1I). This suggests that there is an inherent variability in the number and type of cells that are derived from individual progenitors, and that this variability exists in the embryonic neural crest cells as well as the neural crest-derived progenitors of larval and early metamorphic stages. An additional factor contributing to the variability is the expression of *sox10* promoter in various progenitors, which are at different stages of differentiation. In conclusion, despite some fate-restricted clones, most neural crest-derived cells remain multipotent and do not undergo stereotypic cell-fate restrictions. Quantification of the clone span shows that the fate-restricted clones are smaller than the MIX clones (Figures 5D–5F), demonstrating a link between proliferation ability and cell-fate restriction. At all time points, fate-restricted clones are predominantly laterally situated.

Pigment Cell Progenitors Increase in Number from Embryogenesis to Metamorphosis

Clones obtained from Cre activation at earlier stages can cover much larger areas in the skin and generate more pigment cells compared with clones that are obtained from Cre activation at later larval and metamorphic stages (Figures 5D–5I). The quantification of melanophore numbers in these clones also reveals a clear inverse correlation between the number of labeled cells and the timing of Cre activation; whereas 50% of the clones obtained from Cre activation at 16 hpf produced 30 or more labeled melanophores, this fraction dropped to ~28% at 5 dpf, ~10% at 15 dpf, and ~2.5% at 21 dpf (Figures 4E and 5G–5I). Furthermore, three clones obtained from Cre activation at 16 hpf generated more than 100 melanophores, nearly the full complement of the melanophores per hemisegment in stripes 1D, 1V, and 2V (quantification in Figures 5G and S4). Clones obtained by Cre activation at 16 hpf, a time when *Tg*(*sox10*:*ER^{T2}-Cre*) drives Cre expression in the neural crest cells (Mongera et al., 2013), indicate that a variable, very small number of neural crest cells, as few as one, can produce the complete complement of all pigment cells per hemisegment along the dorsoventral axis. Thus, a single neural crest cell can produce more than one pigment cell stem cell. The maximum clone size is the complement of one hemisegment, although pigment cells are not segmentally restricted in the adult skin, and there is lateral mixing of the pigment cells derived from the progenitors in the neighboring segments (Singh et al., 2014; Walderich et al., 2016). Proliferation of pigment cells and the dorsoventral restriction of the clones are regulated by homotypic competition between cells of neighboring segments (Fadeev et al., 2016; Walderich et al., 2016).

To confirm the observed variability, in terms of cell number and clone span, of labeled clones, we used the CRISPR/Cas9 system to knock out the *albino* locus in somatic embryonic cells and analyzed the mosaic F_0 adults; the mutations of the *albino* gene render melanophores unpigmented, and the remaining few wild-type melanophores can be recognized by their distinct black pigment (Dooley et al., 2013b; Irion et al., 2014b). This experiment offers two crucial advantages in testing our conclusions: first, there is a very high knockout efficiency (Irion et al., 2014b) and second, the CRISPR acts very early within the first few hours of embryonic development. Hence, the wild-type, black melanophores of a given cluster in this experiment must originate from rare early embryonic cells that were not affected by the *albino* CRISPR, and are most likely clonally related. Strikingly, we observe variability in the dorsoventral span and melanophore numbers similar to that in the Cre-induced clones (examples in Figures 5J1–5J5). Similar clusters were obtained when blastomeres were transplanted from wild-type donor embryos into *albino* recipients (Walderich et al., 2016).

Next, we corroborated the increase in neural crest-derived cells between 16 hpf and 21 dpf by visualizing the *sox10*-expressing nuclei in *sox10mG* transgenic fish (Richardson et al., 2016; Figures 6A–6D). In this line, expression of nuclear mCherry and membrane-bound GFP is driven by the same *sox10*-promoter as in *Tg*(*sox10*:*ER^{T2}-Cre*). During embryogenesis, *sox10*-positive cells are observed in migrating streams (Figure 6A), and by 5 dpf these can be seen at the DRGs and along the ventral nerve tracts; few cells are detected along the dorsal nerve tracts at this stage (Figure 6B). At 15 and 21 dpf, *sox10*-positive cells are obvious along the dorsal branches as well along the DRGs and the ventral nerve tracts (Figures 6C–6D, nerve tracts that exit via the horizontal myoseptum are not visible in this plane of view; schematic in Figure 6E). The increase in *sox10*-positive cells is evident along the entire nerve tracts. We quantified the number of *sox10*-positive nuclei in the DRGs between 5 and 21 dpf, and observed a clear increase in cell numbers (5 dpf: 10.2 ± 1.5 cells, ten DRGs from five animals; 15 dpf: 21 ± 6.4 cells, 12 DRGs from six animals; 21 dpf: 67.4 ± 15.7 cells, eight DRGs from four animals; average ± SD).

Taken together, these data confirm that a substantial number of neural crest-derived progenitors remain uncommitted. We suggest that the numbers and types of cells generated by a multipotent progenitor are not fixed. In addition, intermediate progenitors may represent a heterogeneous pool of *sox10*-positive cells. These data show that although the neural crest progenitors remain multipotent throughout embryonic, larval, and early metamorphic stages, they increase in number, and with time each progenitor gives rise to a smaller number of cells.

Most Melanophores Originate from Undifferentiated Progenitors

The analysis of clone span and melanophore numbers suggests that fate restriction is concomitant with a reduction in the

Figure 6. Progenitors Increase in Number between Embryogenesis and Metamorphosis; Proliferation Ability Diminishes with Fate Restriction

(A–D) *Tg*(*sox10mG*) expression in zebrafish trunk at (A) 16 hpf, (B) 5 dpf, (C) 15 dpf, and (D) 21 dpf. Asterisk indicates DRG; arrows point to *sox10*-positive cells along the nerve tracts. Gray, membrane-tagged GFP; red, nuclear RFP.

(E) Schematic representation of the association between pigment cell progenitors and peripheral neurons.

(F–I) Committed melanoblasts generate a small number of melanophores. Melanophore distribution in (F) MIX, (G) MI, and (H) M clones. (I) Quantification of the number of labeled melanophores; each dot represents the number of labeled melanophores in an individual clone.

Scale bars represent 100 μm in (A)–(D) and 250 μm in (F)–(H). See also Figures S5 and S6.

progenitor's ability to proliferate: MIX clones tend to be larger than the fate-restricted clones (Figures 5D–5I). To understand the quantitative differences in the potential of multipotent progenitors and committed precursors, we counted the melanophores produced in clones that generated all three pigment cell types (MIX clones), clones that generated melanophores and one additional pigment cell type (MI or MX clones), and the clones in which only melanophores were produced (M clones) (examples from Cre activation at 5 dpf in Figures 6F–6H; and at 16 hpf, 15 dpf, and 21 dpf in Figure S5). Melanophore quantification was done for the clones obtained from Cre activation at all four stages (Figure 6I). Progenitors that give rise to the MIX clones generate a large number of melanophores, whereas progenitors that give rise to the clones belonging to the other two categories (MI/MX clones or M clones) make only a small number of melanophores (Figure 6I). The clones containing only melanophores make the fewest melanophores (rarely more than two cells). This indicates that committed melanophore precursors rarely undergo cell divisions before terminal differentiation into mature melanophores.

Committed xanthophores and iridophores are known to exhibit local proliferation (Mahalwar et al., 2014; Singh et al., 2014). Consistent with this, clones containing only iridophores can extend up to two light stripes and contain many iridophores (Figures S6A1–S6C2). Similarly, the committed xanthophores can generate several xanthophores (Figures S6D–S6H). Interestingly, fate-restricted clones of iridophores frequently spanned the first light stripe, X0, and fate-restricted clones of melanophores were associated with the first two dark stripes, 1D and 1V (Figures 4D and 5D–5F). This may reflect the developmental sequence of events. During metamorphosis, iridophores initially appear along the horizontal myoseptum and organize the first light stripe. Similarly, melanophores that appear via the horizontal myoseptum during early metamorphosis are incorporated in the first two dark stripes.

The dorsoventrally restricted organization of clones persists in mutants that are compromised in a single pigment cell type (Figure 7). We analyzed the clonal organization of pigment cells in *sparse/kita* mutants (Parichy et al., 1999) that lack a subset of melanophores (Figures 7A–7C), in *shady/leukocyte tyrosine kinase* (*ltk*) mutants (Lopes et al., 2008) that lack iridophores (Figures 7D–7F), and in *pfeffer/fms/colony stimulating factor 1receptor a* (*csf1ra*) mutants (Parichy et al., 2000b) that lack xanthophores (Figures 7G–7I). We found MIX clones in *sparse*, MX clones in *shady*, and MI clones in *pfeffer*, which show a similar distribution along the dorsoventral axis as in wild-type. Furthermore, developmental profiling of clones in *shady* and *pfeffer* mutants shows qualitatively normal behavior of pigment cell progenitors in generating the remaining pigment cell types (Figure S7). This is consistent with the notion that the genes affected in these mutants do not have an influence on the organization of the multipotent progenitors but are required to promote the differentiation of a particular cell type.

DISCUSSION

Our clonal analysis of the adult pigment cell progenitors in zebrafish shows that the majority of neural crest-derived progenitors remain multipotent for several weeks after fertilization. The progenitors generate neurons contributing to the PNS, and all three pigment cell types that make up the adult color pattern on the body and scales. The progenitors may either be plastic or there may be multiple progenitors at different stages of differentiation throughout metamorphosis. This is reflected in lack of stereotypy in clones: clones of varying sizes and shapes are obtained at all four time points studied here.

Cell-Fate Plasticity and Commitment in the Pigment Cell Progenitors

In the context of the larval pigment cells that originate directly from the neural crest, it is known that several of the genes required in a pigment cell-specific manner are initially widely expressed in neural crest cells and are subsequently preferentially restricted to a particular pigment cell type. For example, *mitfa*, a gene that is primarily required in melanophores, shows neural crest-wide expression during embryogenesis (Dooley et al., 2013a; Lister et al., 1999). Similar observations have been made for the progressive restriction of expression of *ltk* and *csf1ra* in iridophores and xanthophores, respectively (Lopes et al., 2008; Parichy et al., 2000b). In fact, some of the pigment cell-specific genes, such as *mitfa* and *csf1ra*, overlap in their expression pattern in neural crest cells (Curran et al., 2010; Parichy et al., 2000b). These observations may offer an explanation for the lack of lineage commitment in neural crest cell-derived progenitors. In principle, the progenitors may express a diverse set of cell-specific factors that promote a split into separate MX and MI lineages (Figure 1J). Commitment to a given pigment cell fate would then depend upon the availability of specific ligands in the immediate environment of the undifferentiated progenitors.

The cell numbers required to generate the adult stripe pattern are not identical along the anterior-posterior body axis; in the anterior region of the trunk there are four to five dark stripes and four light stripes, and only three dark and two light stripes extend to the posterior regions. Similarly, along the dorsoventral axis, pigment cell numbers and composition varies in body, viscera, and scales (Ceinos et al., 2015; Fadeev et al., 2016). Given that the pigment cells share a lineage along the dorsoventral axis, it might be advantageous to have progenitors that are not fate restricted: this will allow an efficient local regulation of specific cell fates during development and regeneration. The progenitors may become fate restricted depending upon the environmental cues. The environment, in turn, might be defined by the ligands for pigment cell-specific signaling systems such as Kita for melanophores, Ltk and Ednrb for iridophores, and Csf1ra for xanthophores (Dooley et al., 2013a; Fadeev et al., 2016; Frohnhöfer et al., 2013; Lopes et al., 2008; Parichy et al., 1999, 2000a, 2000b). Consistent with a role for the tissue environment, other genes have been identified that affect pigment cell fate non-cell autonomously, and in a body region-specific manner (Ceinos et al., 2015; Krauss et al., 2014a; Lang et al., 2009). Expression of the ligands may lead to a specific pigment cell fate by interactions between specific ligand-receptor systems, although it is also possible that there are other mechanisms that prime the progenitors toward specific cell fates. During metamorphosis, the three pigment cell types depend upon one another for differentiation, migration, proliferation, and survival, and in

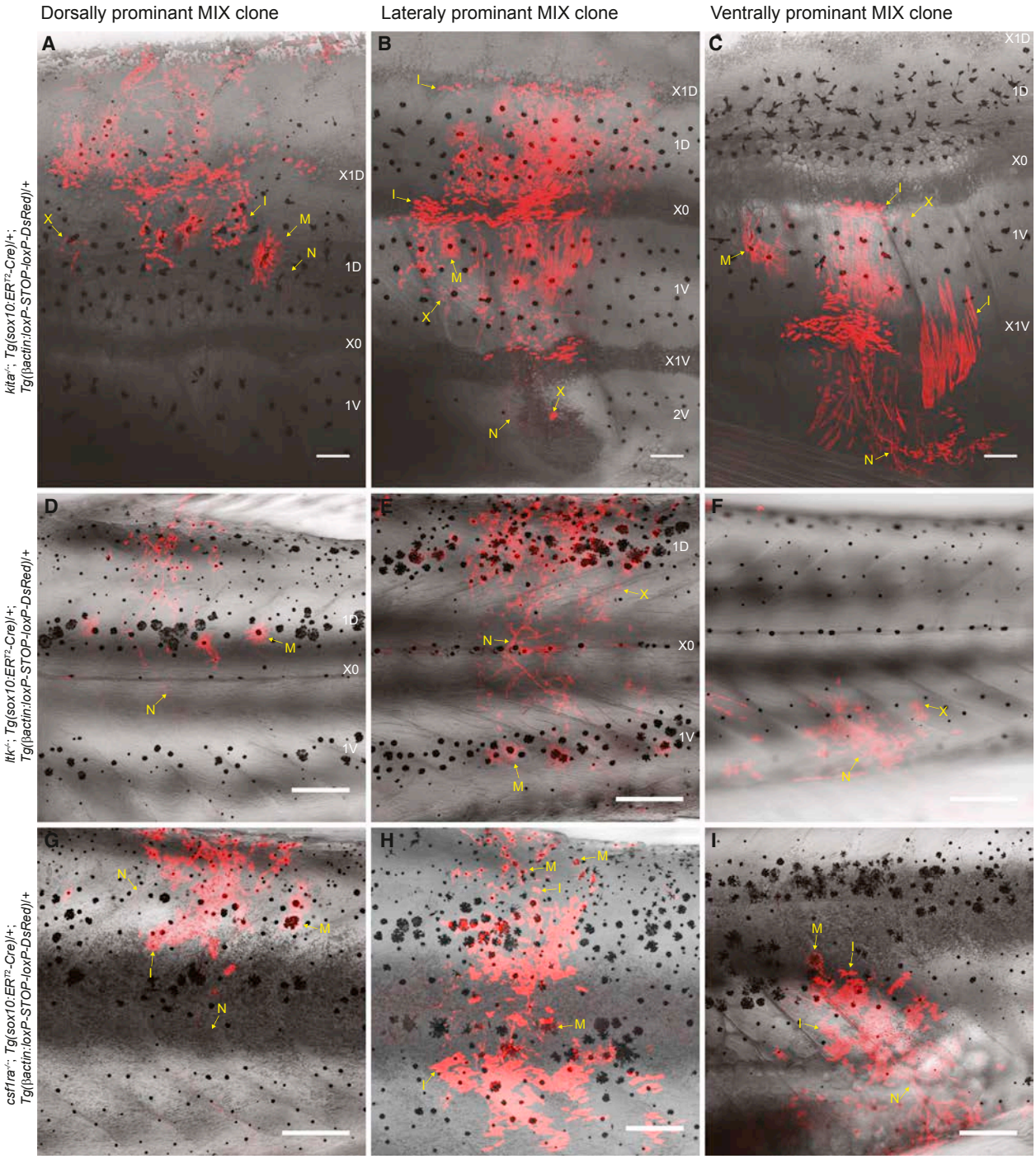

Figure 7. Dorsoventral Organization of Clones in Pigmentation Mutants

(A–I) Clones in the background of (A–C) *sparse/kita* lacking a subset of melanophores (n = 18 clones), (D–F) *shady/ltk* lacking iridophores (n = 20 clones), and (G–I) *pfeffer/csf1ra* lacking xanthophores (n = 20 clones). Scale bars, 250 μm. See also Figure S7.

the absence of any two given pigment cell types, the remaining third cell type tends to cover the entire skin (Fadeev et al., 2015; Frohnhöfer et al., 2013; Irion et al., 2014a). Thus, cross-communication between pigment cells, their precursors, and the environment are important in determining the pigment cell fate.

Multipotent Progenitors as Stem Cells for Postembryonic Neurons and Pigment Cells

Following work in birds and mammals (Adameyko et al., 2009), several studies highlighted a close association between neuronal arbors and pigment cell progenitors, and suggested that they play an important role in pigment cell development

(Budi et al., 2011; Dooley et al., 2013a; Singh et al., 2014). At the level of the progenitors, restriction to a given cell fate may occur as the progenitors move toward the skin along the peripheral nerves. The progenitors associated with the DRG remain multipotent and are the adult stem cells that give rise to pigment cells and neurons during metamorphosis. Neurons are continuously added to the fish DRG: the larval DRG contains five to six neurons at 5 dpf, and this number increases many fold by 22 dpf (McGraw et al., 2012). The new neurons are added by proliferating, non-neuronal, *sox10*-positive cells that reside within the DRG (McGraw et al., 2012). We show that the DRG-associated cells generate neurons as well as pigment cells beyond the larval stages, indicating that these cells remain multipotent. It has been observed that the DRG-associated cells regenerate new melanophores upon depletion of existing melanophores (Dooley et al., 2013a). This indicates that some, if not all, *sox10*-positive multipotent progenitors at the DRG behave like stem cells. Consistent with this, pigment cells regenerate after several rounds of targeted ablation, or after pigment cell death due to genetic mutations as in *tyrp1A* (Krauss et al., 2014b; O'Reilly-Pol and Johnson, 2009). Between embryogenesis to early metamorphic stages, the multipotent progenitors proliferate and increase their pool size. The increase in the number of progenitors leads to a progressive reduction in the size of clones that nevertheless generate all three pigment cell types.

It was recently suggested that the emergence of peripheral nerve-associated multipotent progenitors allowed vertebrates to afford faster growth rate and bigger bodies: the nerves serve as a niche for the progenitors and facilitate their long-distance transport throughout the body (Dyachuk et al., 2014; Ivashkin et al., 2014). Taken together, our data indicate a general role of peripheral nerves in the development and transport of multipotent progenitors. Our clonal analysis and long-term imaging provide the in vivo dynamics of progenitor behavior and suggest that the progenitors for adult pigment cells reside in several locations in proximity to nerve arbors along the dorsoventral axis (schematic in Figure 6E). Consistent with this, we observe three classes of MIX clones that are anatomically associated with the three major nerve routes along the dorsal, horizontal, and ventral myosepta. The neuronal arbors might act as a niche as well as supply routes for pigment cell precursors to different locations in the skin along the dorsoventral axis. It is known that melanoblasts populate the spinal nerves during metamorphosis and travel along these nerves to reach the skin (Budi et al., 2011; Dooley et al., 2013a). It is possible that as the progenitors migrate along the spinal nerves, they become partially fate restricted depending upon the route of exit to the skin, as has been observed for the neural crest cells: in the embryo, neural crest cells that migrate along the dorsolateral route generate larval xanthophores and melanophores, whereas cells migrating along the medial route generate iridophores, melanophores, neurons, and glia (Kelsh et al., 2009). It is noteworthy that the stripe iridophores are primarily produced by progenitors that exit via the horizontal myoseptum, whereas most melanophores are produced by progenitors that use dorsal and ventral routes to reach the skin. Also, MX clones are primarily associated with the ventral routes (Figures 4D, 5D, and S5A2).

In summary, we describe the cell-fate potential of neural crest-derived progenitors between larval stages and early metamorphosis, and identify key features of the pigment cell progenitors during color pattern formation in zebrafish. Our data imply multipotency of nerve-associated progenitors, a feature that may have facilitated rapid divergence of color patterns during the evolution of *Danio* species.

EXPERIMENTAL PROCEDURES

Zebrafish Lines

Wild-type (WT, Tü strain from the Tübingen zebrafish stock center), *Tg*(*sox10*:*ER^{T2}-Cre*) (Mongera et al., 2013), *Tg*(*βactin2*:*loxP-STOP-loxP-DsRed-express*) (Bertrand et al., 2010), *Tg*(*sox10*:*H2BmCherry-2A-GPIGFP*), abbreviated as *Tg*(*sox10mG*) (Richardson et al., 2016), *sparse* (Kelsh et al., 1996), *shady* (Lopes et al., 2008), and *pfeffer* (Maderspacher and Nüsslein-Volhard, 2003). All animal experiments were performed in accordance with the rules of the State of Baden-Württemberg, Germany, and approved by the Regierungspräsidium Tübingen (Aktenzeichen: 35/9185.46-5 and 35/9185.82-7).

Cre Induction

Zebrafish of appropriate stage (16 hpf, 5 dpf, 15 dpf, and 21 dpf), carrying a single copy of *Tg*(*sox10*:*ER^{T2}-Cre*) and *Tg*(*βactin2*:*loxP-STOP-loxP-DsRed-express*), were treated with 5 μM 4-hydroxytamoxifen (4-OHT; Sigma, H7904) for 1–2 hr at 28°C. Cre activation was carried out for 3 hr for animals on 21 dpf. 5 mM stock solution of 4-OHT was prepared by dissolving it in absolute ethanol, and stored at −20°C. For 4-OHT treatment, batches of ~40 larvae were transferred to plastic Petri dishes (Greiner Bio-one) containing 40 ml of embryonic medium and 4-OHT was added to a final concentration of 5 μM. For Cre activation at 16 hpf, embryos were dechorionated in Pronase prior to 4-OHT treatment. Subsequently, fish were washed three times in E2 and transferred to fresh medium. Larvae and young adult fish (2–3 months post fertilization) were screened for DsRed-labeled clones under a Zeiss LSM 5Live confocal microscope equipped with a Plan-Apochromat 10× objective (0.45 NA; air; 2 mm working distance).

Imaging

Confocal scans were acquired using a Zeiss LSM 780 NLO microscope equipped with 488-Argon laser and 561-diode laser, C-Apochromat 10× objective (0.45 NA; water immersion; 1.8 mm working distance), and LD LCI Plan-apochromat 25× objective (0.8 NA; water, glycerol, oil immersion; 0.57 mm working distance). For the repeated imaging of zebrafish, fish at appropriate developmental stage were anesthetized by adding 30 μl of 0.4% Tricaine (MS-222, Sigma) per 1 ml of embryonic medium 2. Anesthetized animals that were in early stages of metamorphosis (~6.5 mm standard length) were mounted in 0.5% low-melting agarose (NuSieve GTG Agarose, catalog number 50080, Lonza) dissolved in Tricaine-containing embryonic medium on 35 mm glass bottom dishes (MatTek Cultureware). Older animals were mounted in a minimum amount of Tricaine-containing embryonic medium such that animals do not desiccate and yet remain still. Older animals do not survive well if mounted in low-melting agarose, possibly due to blockade of the gills. On the other hand, younger individuals tend to desiccate quickly and hence it is important to mount these in low concentrations of low-melting agarose. After imaging, animals were transferred to fresh fish water or embryonic medium, and their breathing was facilitated by gently flowing water across the operculum with a small plastic pipette. On average, it takes 15–20 min per animal from anesthetization to recovery after imaging. Recovered fish were transferred to and raised in mouse cages (1–1.5 l of running fish water) with each mouse cage housing a single fish.

Generation of Melanophore Mosaics by *Albino* Knockout

Albino mutant F_0 mosaics were generated as described by Irion et al. (2014b). Adult zebrafish were anesthetized with Tricaine and treated with epinephrine hydrochloride (E4642, Sigma) prior to photograph acquisition using Canon 5D Mark II.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2016.06.020.

AUTHOR CONTRIBUTIONS

Conceptualization, A.P.S. and C.N.-V.; A.P.S. performed experiments; A.D. was responsible for additional clones induced at 16 hpf; P.M. collected *shady* and *pfeffer* mutant data; U.S. helped with crosses and genotyping; C.L. was responsible for *Tg*(*sox10mG*) line and U.I. for *albino* knockouts; Analysis, A.P.S. and C.N-V.; Writing, A.P.S. and C.N-V., with input from A.D. and U.I.

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