Embryonic xanthophore fate in the zebrafish, *Danio rerio*

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Abstract

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Pigment patterns are one of the most diverse and visually appealing traits of any animal, playing important behavioral and ecological roles in mate choice, shoaling and predator avoidance. In the zebrafish, *Danio rerio*, these patterns result from the organization of neural crest derived pigment cells, offering an opportunity to study mechanisms of fate specification and pattern formation. While recent studies in *D. rerio* examine interactions between black melanophores, yellow xanthophores, and iridescent iridophores during adult pattern formation, little is known about the development of the individual pigment cell lineages, particularly yellow xanthophores. Here, I found that embryonic xanthophores de-differentiate at the start of pigment pattern metamorphosis, yet persist into the adult where some redifferentiate as xanthophores and others

remain in an undifferentiated state. The undifferentiated cells that remain are competent to differentiate as xanthophores, but not melanophores, suggesting these cells are fate restricted to the xanthophore lineage. My results identify at least one origin of adult xanthophores in the zebrafish thus contributing to the overall picture of pigment cell development, and more broadly, lending novel insight to neural crest lineage diversification.

Introduction

Teleost fishes exhibit incredible diversity of patterns and coloration that play important roles in mate choice, shoaling, and predator avoidance [1-4]. In the zebrafish, *Danio rerio*, patterns result from the differential numbers and arrangement of three pigment cell types: black melanophores, yellow xanthophores, and iridescent iridophores. These cells contain different pigments including melanin, pteridines, and reflecting platelets, respectively, that produce their visible coloration [5-7]. Pigment cells represent one of the many derivatives of the neural crest, a transient cell population that also contributes to peripheral nerves and glia, craniofacial bone and cartilage, and other cell and tissue types. This developmental potential makes the neural crest an ideal system for studying mechanisms of fate specification and pattern formation, particularly in the conspicuous pigment cell lineages [8-9].

In zebrafish, embryonic melanophores develop directly from the neural crest and contribute to an early larval pigment pattern. Subsequently, pigment pattern metamorphosis occurs during the larval-to-adult transformation, and establishes the definitive adult pigment pattern comprising dark stripes of melanophores bordering light interstripes of xanthophores and iridophores [10-11]. Iridophores are the first adult pigment cells to develop, and do so at the prospective interstripe region; these iridophores provide positional cues to the other pigment cells, resulting in the differentiation of xanthophores within the interstripe and the localization of adult melanophores dorsal and ventral to this region [12-15]. As the larval to adult pigment pattern transition occurs, many of the embyronic/early larval melanophores die [16-18].

Though many recent studies of zebrafish have examined interactions among pigment cell classes during adult pattern formation [12-14,19], we still know relatively little about the

specification and differentiation of the individual adult pigment cell lineages. In contrast to embryonic/early larval melanophores, which develop directly from neural crest cells, at least some of the adult melanophores and iridophores arise from post-embryonic precursor cells associated with peripheral nerves and ganglia. These cells migrate to the skin during adult pattern formation and largely replace the directly neural-crest derived melanophores and iridophores of the embryo and early larva. By contrast, the precursors to adult xanthophores have yet to be identified [20-21]. Previous studies indicate that specification of xanthophore fate requires the transcription factor Pax3 whereas the terminal differentiation of xanthophores requires Pax7 [22]. Additionally, Colony stimulating factor-1 receptor (Csf1r), a receptor tyrosine kinase, is required for xanthophore migration, survival and differentiation, and *csf1r* mutants are deficient for embryonic and adult xanthophores [12, 23]. These studies identify requirements for xanthophore development, but did not investigate the origins of the adult xanthophore lineage.

Given that peripheral nerve-associated precursors were found to generate adult melanophores and iridophores, but not xanthophores, the origin of adult xanthophores has been mysterious. I hypothesized that adult xanthophores derive from the directly neural crest-derived xanthophores of the embryo and early larva, rather than post-embryonic latent precursors. To test this hypothesis I followed labeled embryonic xanthophores from the embryo through pigment pattern metamorphosis. Although embryonic xanthophores had already differentiated, as defined by the presence of yellow pigmentation and autofluorescence visible under UV illumination, I found that a subset of these cells lose their pigmentation prior to metamorphosis, re-enter the cell cyle, and remain unpigmented and non-autoflourescent for periods of days to

weeks. Some of these “dedifferentiated” cells survive through metamorphosis and redifferentiate as adult xanthophores, whereas others persist into adulthood in the de-differentiated state. To assess their developmental potential, I challenged these persisting, de-differentiated cells to redifferentiate as either xanthophores or melanophores, after laser-ablating nearby adult melanophores or xanthophores; these analyses showed that persisting, de-differentiated cells remain competent to redifferentiate to replace xanthophores, but not melanophores, suggesting they remain fate-restricted despite the absence of overt xanthophore characteristics. These results are the first demonstration of adult xanthophore origins and fate-restriction within this lineage, and thereby extend our understanding of adult pigment cell fate specification in zebrafish.

Results

Embryonic xanthophores disappear during the larval to adult transformation

To better understand the dynamics of xanthophore development, I imaged pre- and post-metamorphic wild-type zebrafish larvae. By 4 days post fertilization (dpf), larvae exhibit numerous differentiated xanthophores across the flank (Figure 1A) [24]. Embryonic xanthophores disappear by 6.0 mm standard length (SL; pre-metamorphic) when the anterior lobe of the swim bladder inflates (Figure 1A’) [11]. Differentiated xanthophores (of the adult) then reappear across the flank around 8.0 mm SL, as the pelvic bud develops (Figure 1A”). Given the absence of a demonstrated, post-embryonic origin of adult xanthophores, I speculated that the disappearance of embryonic xanthophores might reflect the de-differentiation, rather than death of these cells, and that this same population might later contribute to the complement

of adult xanthophores. If embyronic and adult xanthophores indeed constitute a single lineage, I predicted that ablation of the embryonic xanthophores should lead to xanthophore deficiences in the adult. To test this notion, we used a xanthophore-specific promoter of *aldehyde oxidase 3* (*ao3*) [23] to drive expression of bacterial nitroreductase (NTR), which converts metronidazole (Mtz) into toxic metabolites, thereby killing cells that express the transgene without bystander effects [25]. We injected embryos with *ao3:NTR* at the one-cell stage and treated larvae expressing NTR mosaically with Mtz at 4 dpf when xanthophores first develop (Figure 2A, A'). Ablation of embryonic xanthophores resulted in xanthophore-free regions within the interstripe at post-metamorphic stages (Figure 2B, B', 2C, C'). This defect in the adult xanthophore population suggested that embryonic xanthophores may later develop as adult xanthophores.

Population analysis indicates that embryonic/early larval xanthophores persist in the adult

To understand the fate of embryonic/early larval xanthophores at the population level, I assessed the fate of these cells utilizing a nuclear-localized, photoconvertible fluorophore, EosFP [20]. EosFP expression was driven mosaically using the *ao3* promoter in injected fish (F0; Figure 3). All EosFP+ xanthophores (≥5 cells per larva) were photoconverted from green to red between 4–6 dpf (Figure 3A, B). To assess the fates of labeled cells, I then imaged photoconverted fish at middle metamorphic stages, when xanthophores begin appearing during adult pattern formation. Labeled EosFP+ cells persisted through pigment pattern metamorphosis and in 55% of the individuals (n=64 fish) at least one of the red EosFP+ cells developed as a xanthophore (Figure 3C–E). EosFP+ cells that did not develop xanthophore pigmentation were found within the light interstripe but also within the dark melanophore stripes.

Clonal analysis reveals that larval xanthophores persist, lose pigmentation, divide, and redifferentiate during pigment pattern metamorphosis

The above analyses described cell behaviors at a population level yet I wished to understand individual cell behaviors as well, and specifically whether or not individual cells might re-enter the cell cycle. To this end, I targeted individual EosFP+ xanthophores for photoconversion between 4–6 dpf, thereby labeling single cells, or in some instances small numbers of cells per larva (Figure 4A, A'). Fish with labeled cells were then imaged repeatedly every 5–7 days to observe cell behaviors during and after adult pigment pattern development. Labeled embryonic xanthophores persisted through adult pigment pattern metamorphosis, but did not exhibit yellow pigmentation at early/pre-metamorphic stages (Figure 4B). Some post-metamorphic cells redifferentiated into xanthophores evident by the yellow pigmentation (Figure 4C) and autofluorescence (Figure 4D). Moreover, persisting de-differentiated cells retained some proliferative capacity, dividing between 1–3 times, and 41% of the total labeled cells redifferentiated into xanthophores with the remaining cells persisting in the de-differentiated state (Table 1).

Undifferentiated, persisting embryonic xanthophores remain committed to the xanthophore lineage

csf1r mutants show a reduction in adult melanophores in addition to lack of xanthophores suggesting the potential for a common melanophore-xanthophore precursor [23]. The widespread distribution of undifferentiated, photoconverted EosFP+ nuclei throughout the stripe

and interstripe regions was consistent with the possibility that some of these de-differentiated cells might have entered a bipotent or multipotent state, in which they could contribute to both xanthophore and melanophore lineages. Alternatively, these de-differentiated embryonic xanthophores might be restricted to a xanthophore fate. To distingiush between these possibilities, I reasoned that the potential of these cells could be revealed in a regenerative context, were I to challenge the cells to replenish either xanthophores or melanophores following the laser ablation of these nearby differentiated cell types. In xanthophore-ablated regions (lacking previously redifferentiated EosFP+ xanthophores) new xanthophores repopulated the ablated area within 10–14 days and these newly differentiated xanthophores included previously de-differentiated EosFP+ cells (Figure 5). By contrast, no EosFP+ melanophores were found to develop in the vicinity of persisting, de-differentiated EosFP+ cells following the laser-ablation of melanophores (Figure 6). These data indicate that embryonic xanthophores that de-differentiate and persist into the adult are able to redifferentiate into xanthophores, but not melanophores.

Discussion

The results of this study illuminate the fate of embryonic xanthophores, demonstrating that these cells de-differentiate (as defined by loss of xanthophore-specific visible pigmentation and autofluorescence) during early pigment pattern metamorphosis, yet these cells persist into the adult with some redifferentiating as xanthophores in the interstripe region during pigment pattern metamorphosis. The remaining undifferentiated cells are competent to differentiate as xanthophores, but lack the potential to generate melanophores, as evidenced by laser ablation/ regeneration experiments. Whereas the development of the melanophore lineage is fairly well

understood, and interactions between the different pigment cell lineages are important for proper pigment pattern development, this study is the first to examine the development of the xanthophore lineage and report findings of adult xanthophore origins.

My finding that cells of the embryonic xanthophore lineage persist from embryo to adult elucidates at least one origin of adult xanthophores, and this origin is distinct from that of latent precursor-derived adult melanophores [16, 21]. Interestingly, less than half of the persisting embryonic xanthophores re-express characteristics of differentated xanthophores in the adult (i.e., yellow pigmentation and autofluorescence). The remaining de-differentiated cells of this lineage persist throughout the flank, residing in both the interstripe and stripe. Despite their varied locations, the ability of these cells to replenish xanthophores but not melanophores after ablation of neighboring pigment cells suggests that these cells are fate-restricted, at least in this context.

My findings demonstrate a de-differentitaion of embryonic xanthophores at the start of adult pigment pattern metamorphosis. Embryonic xanthophores express yellow pigmentation and are autofluorescent during larval stages, but as the fish approaches metamorphosis these differentiated cells revert back to a less differentiated state and are present, but not yellow. Xanthophore coloration results from pteridines and carotenoids present within pigment organelles inside the cell [26]. Carotenoids are dietarily derived organic pigments whereas pteridines are synthesized through biochemical pathways within the xanthophores [27]. While both components contribute to the coloration of these cells, synthesized pterdines seem to play a more critcal role because even in the absence of carotenoids in the diet of adult fish, xanthophores appear yellow. Although the molecular mechanisms of de-differentiation in

xanthophores remain unknown, the absence of yellow coloration during the larval to adult transition might result from a downregulation of the pteridine synthesis pathway.

In conclusion, these results further our understanding of pigment cell fate specification in zebrafish by dissecting the development of the poorly studied xanthophore lineage during the larval to adult transition. Furthermore, this report highlights dedifferentiation in pigment cells. Together, these analysis contribute to the overall picture of pigment cell development and provide novel insights into basic mechanisms of neural crest lineage diversification.

Methods

Fish rearing conditions and transgenes

Fish stocks were reared in standard conditions at 28.5°C 14L:10D. Individuals were housed in beakers for the duration of the experiments while fish with all fluorescent cells converted were grown on a standard zebrafish flow system. BAC recombineering for *tol2*-mediated transgenesis was used to generate the *ao3:NTR* transgene for embryonic/early larval xanthophore ablations [28]. Gateway cloning of entry plasmids into pDest vectors containing *Tol2* repeats was used to generate the *ao3:Eos* transgene [29, 30]. To express nuclear-localized *EosFP* in xanthophores, a 3.6kb fragment upstream of *ao3* and including the first intron was cloned. Standard methods for microinjection of plasmids and *Tol2* mRNA were used. For individual cell photoconversions of EosFP from green to red, illumination through a DAPI filter was used for 30 seconds to convert individual cells, or for 5 minutes to convert all EosFP+ cells within the fish, using a 20X objective and a constricted aperture. All photoconversions were performed between 4-6 dpf.

Imaging

Fish were viewed and repeatedly imaged on a Zeiss Observer inverted compound epifluorescence microscope and images were taken using AxioVision software using Axiocam HR or MR3 cameras. Image stacks were processed using Zeiss Axiovision Extended Focus modules. Laser ablations were performed using a Micropoint Laser Ablation system mounted to a Leica DM5500B upright compound epifluorescence microscope.

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Figures

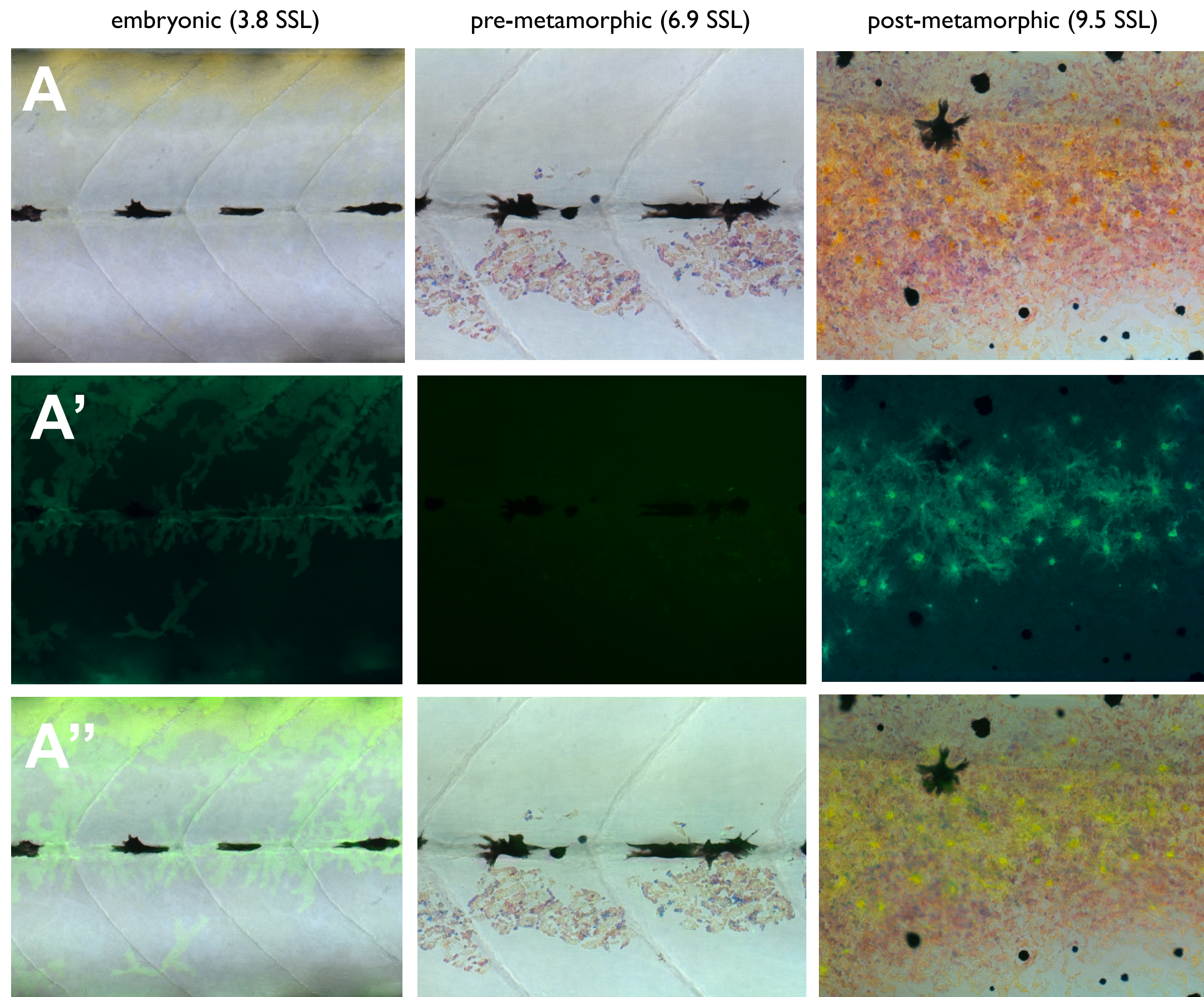

Figure 1. Xanthophore development in wild-type zebrafish.

Embryonic xanthophores develop across the flank by 4 days post fertilization (3.8 SSL) during which time these cells are visibly yellow and exhibit autofluorescent properties in the GFP channel (A). During early/pre-metamorphic stages (6.9 SSL), yellow xanthophores have disappeared and no autofluorescence is detected, but iridophores are developing across the flank (A'). Post metamorphosis (9.5 SSL), many yellow, autoflorescent xanthophores are visible across the flank of the fish(A''). All images were obtained using the 20X objective.

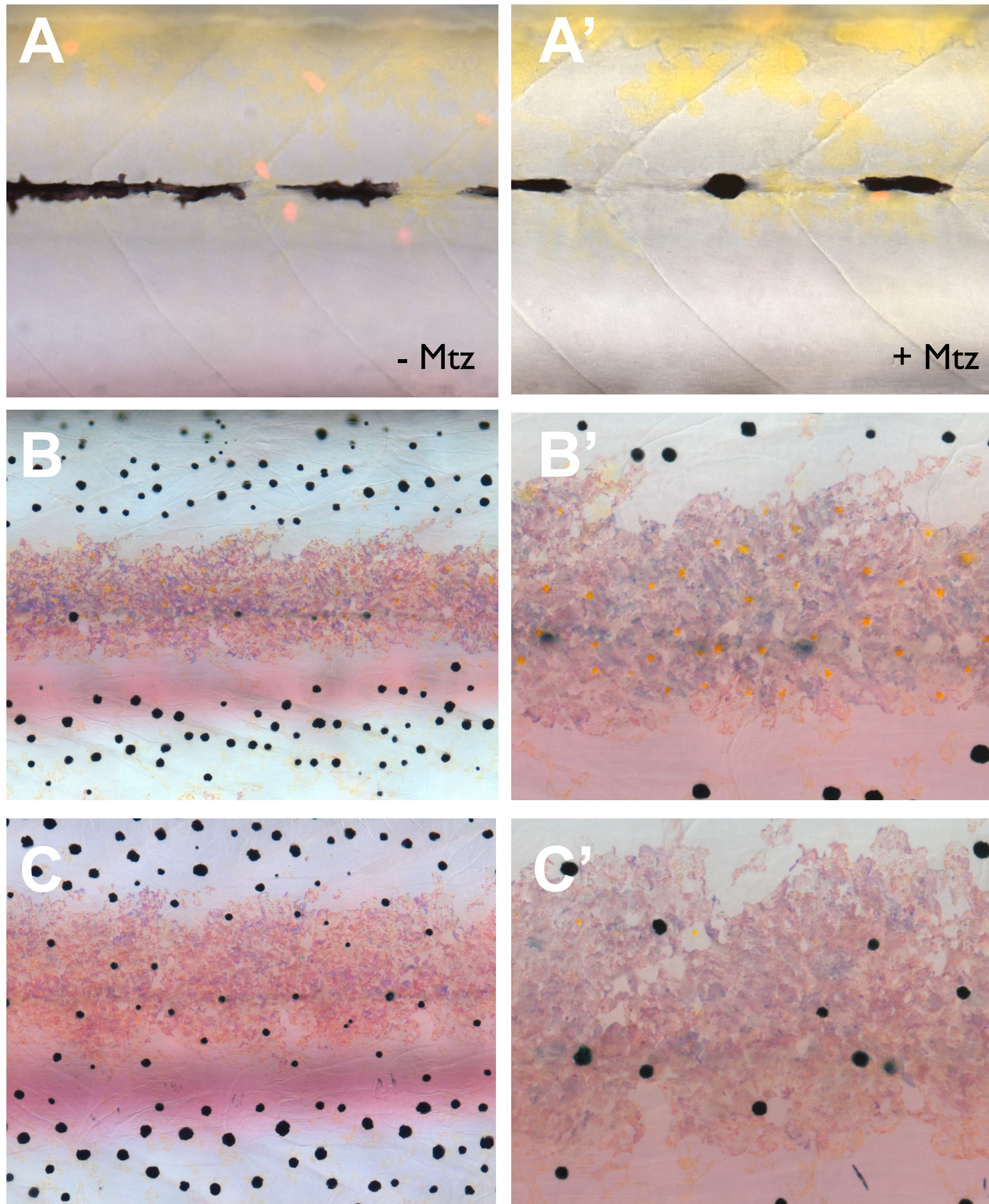

Figure 2. Embryonic xanthophore ablation produced defects in adult xanthophore development.

(A, A') Wild type siblings injected with *ao3*:Eos:NTR plasmid and treated without (A) or with (A') metronidazole (Mtz) at 4 days post fertilization (approx. 3.5 SSL). EosFP+ cells were photoconverted immediately following Mtz treatments. The loss of red EosFP+ cells in Mtz treated fish indicates death of these cells through autonomous toxic metabolite production. Non-drug treated fish do not contain defects in adult xanthophore development within the interstripe (B, 10X; B', 20X) while drug-treated fish exhibit xanthophore-free regions within the interstripe (C, 10X; C', 20X).

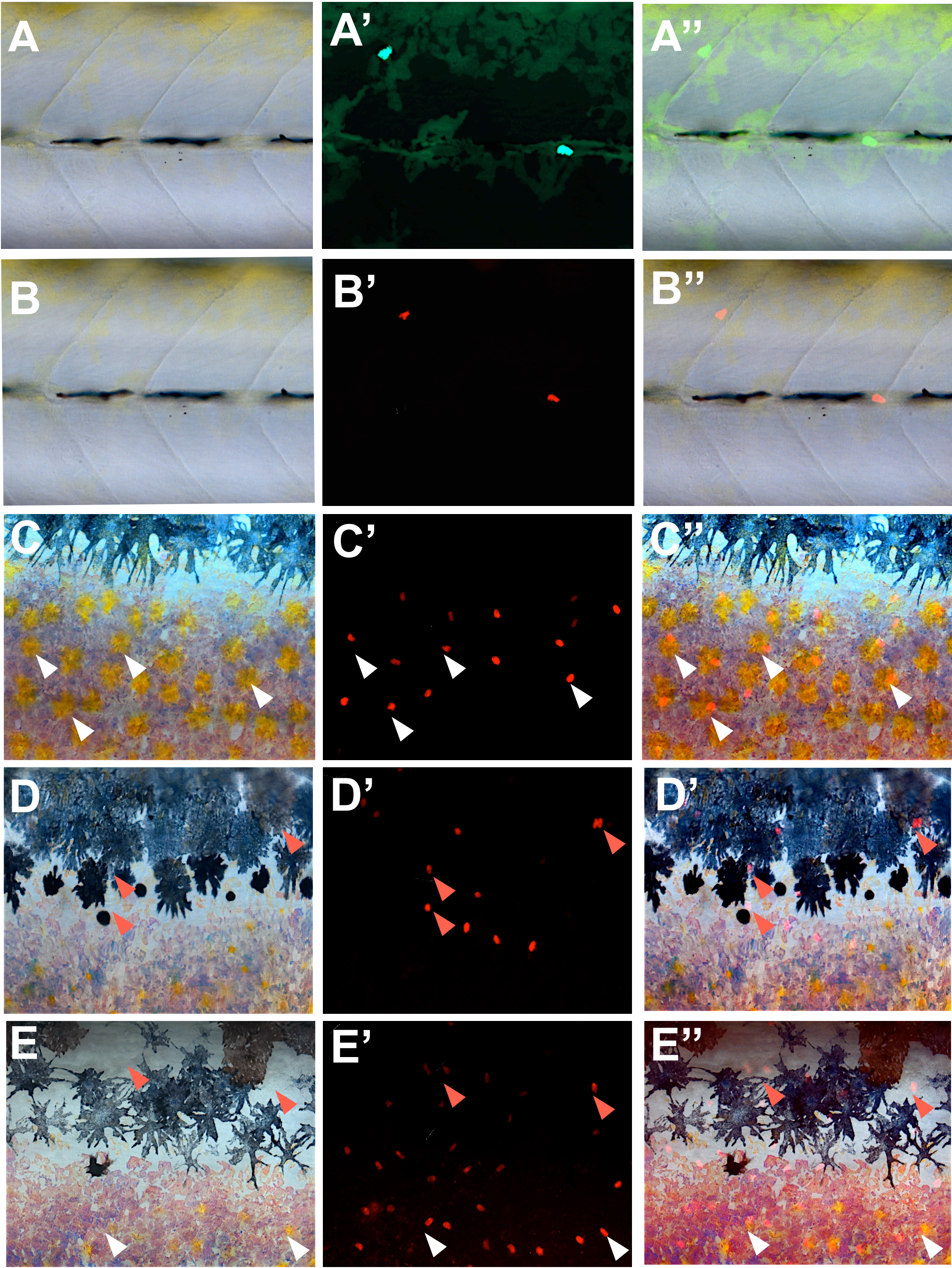

Figure 3. Embryonic xanthophores persist into the adult and some redifferentiate as xanthophores.

Representative examples of fish pre- (A) and post- (B) photoconversion of EosFP+ cells. Individuals with all EosFP+ cells photoconverted from green to red were grown to post-metamorphic stages. At these stages, EosFP+ cells became xanthophores (e.g., white arrowheads) within the interstripe (C) or remained undifferentiated (e.g., red arrowheads) in the stripe (D). In some individuals, some EosFP+ cells were found both the interstripe and stripe (E).

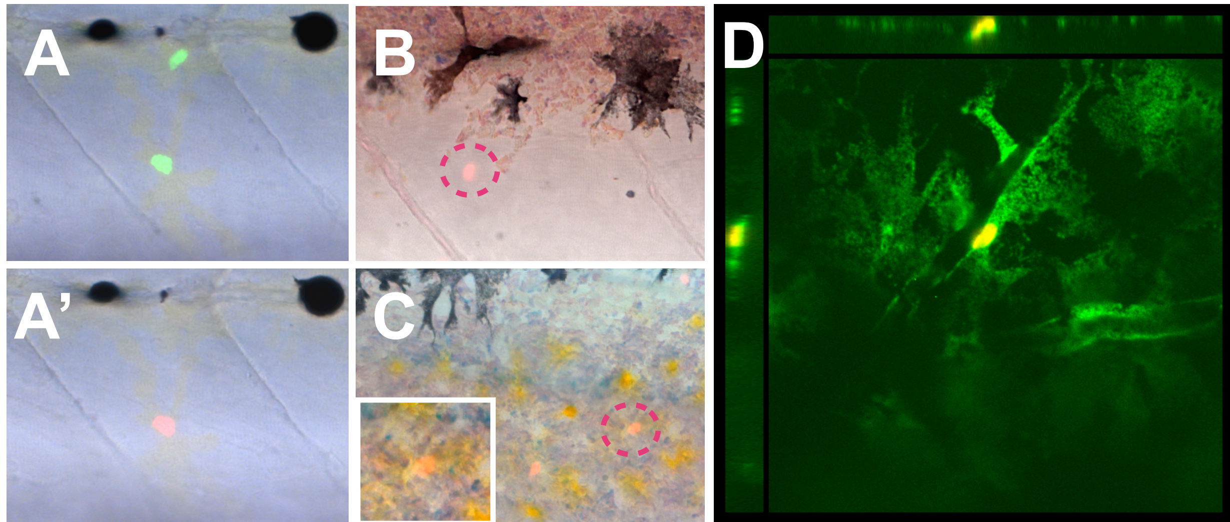

Figure 4. Embryonic xanthophores persist in an undifferentiated state until metamorphosis when some redifferentiate as xanthophores.

Yellow embryonic xanthophores carrying the ao3:eos transgene express green EosFP by 4 days post fertilization (approx. 3.5 SSL) (A). Following photoconversion, a single cell now contains red EosFP allowing for clonal lineage analysis (A'). The single photoconverted EosFP+ cell persists in an undifferentiated state during the larval to adult pigment pattern transition (B). After pigment pattern metamorphosis, the photoconverted EosFP+ cell redifferentiates as a xanthophore (eg. red dashed circle) (C). High resolution three-dimensional spinning disc imaging of a representative redifferentiated xanthophore reveals autofluorescence (GFP channel) overlaps with the photoconverted EosFP+ nucleus (D) confirming this cell is a differentiated xanthophore.

Table 1. Summary of proliferation and final fate of embryonic xanthophores.

Clonal analysis of single or small groups of photoconverted (green to red) EosFP+ cells demonstrates that across groups cells divide between 1 and 3 times, represented by the final number of cells compared to the intial number $\pm$ SE. The final fate of all photoconverted EosFP + cells is represented as average percent xanthophores or undifferentiated cells within groups.

Number of Cells Converted	n (fish)	Final Number of Cells	Average % Xanthophores	Average % Undifferentiated
1	28	3.0 $\pm$ 2.2	11.8	88.2
2	9	5.1 $\pm$ 2.1	37.0	63.0
3	1	6	83.3	16.7
4	4	5.3 $\pm$ 2.7	33.3	66.7
5	2	9.0 $\pm$ 7.1	38.9	61.1

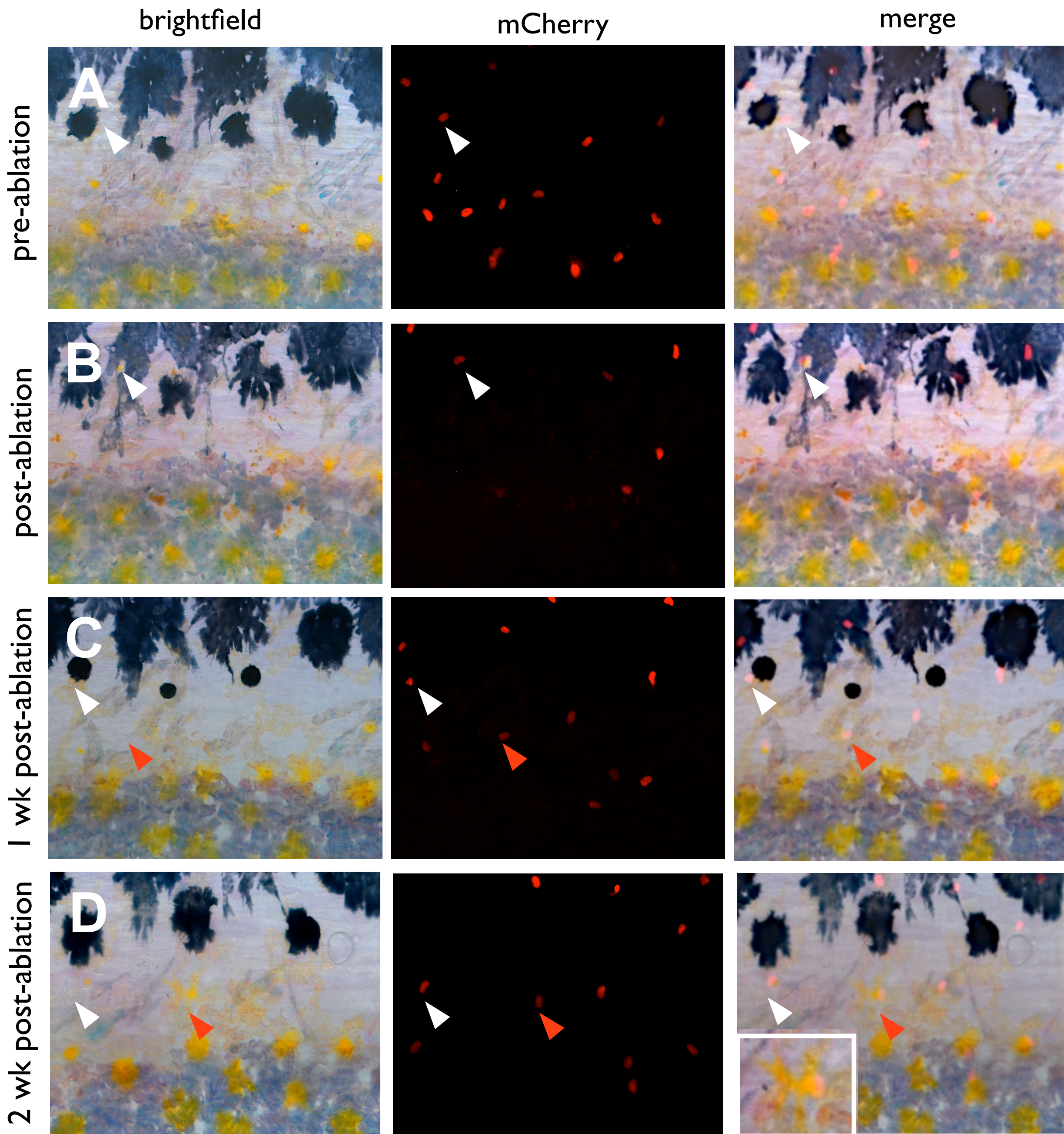

Figure 5. Undifferentiated embryonic xanthophores can regenerate as xanthophores.

When individual xanthophores are laser ablated, EosFP+ cells can redifferentiate into xanthophores to repopulate deficient areas (e.g., red arrowhead). One week following ablation, EosFP+ cells in the region migrate to ablated areas (e.g., red and white arrowhead) (C). Two weeks post-ablation EosFP+ cells begin to differentiate into new xanthophores (e.g., red arrowhead; inlet) (D).

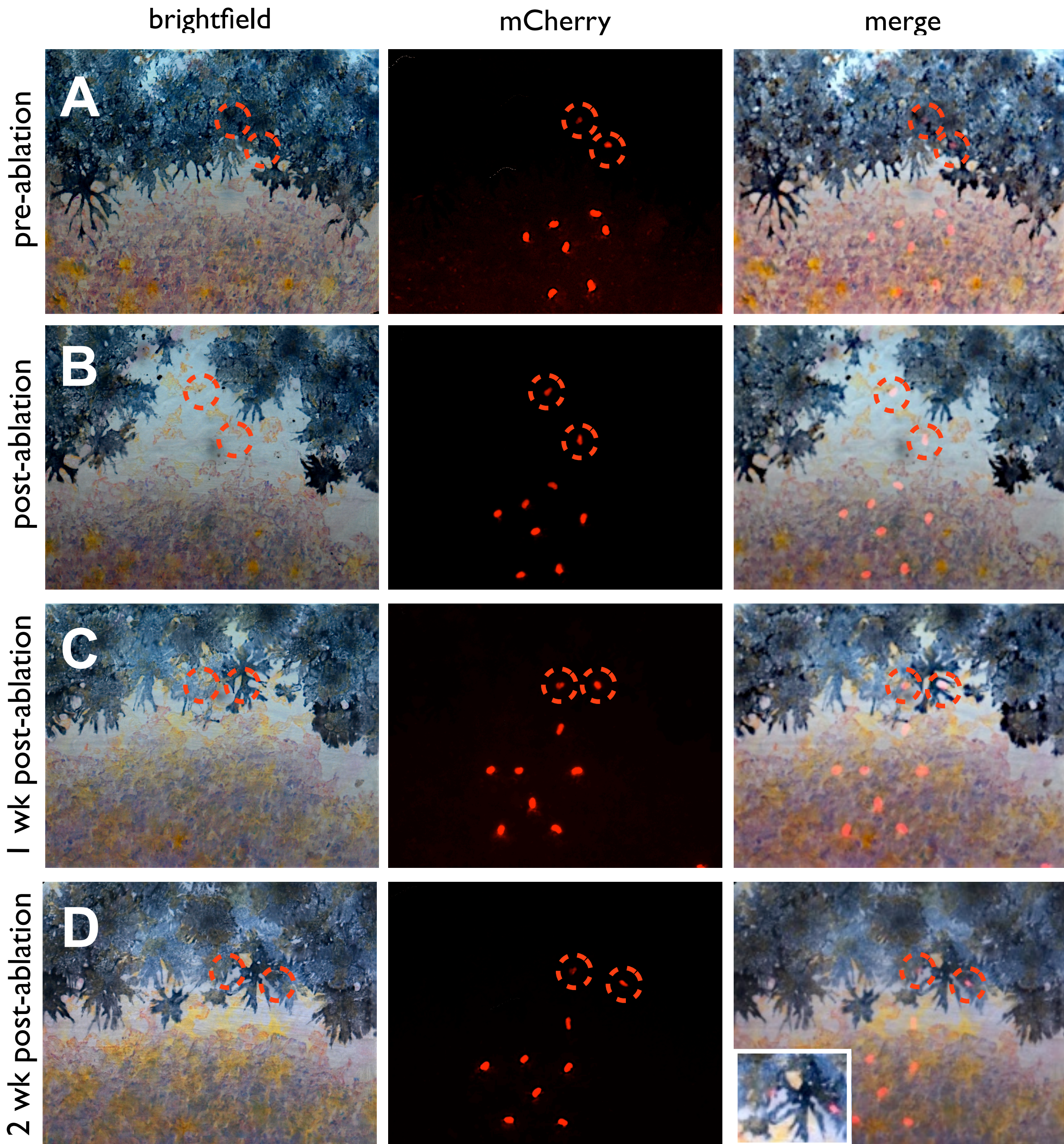

Figure 6. Undifferentiated embryonic xanthophores do not regenerate as melanophores.

When individual xanthophores are laser ablated, EosFP+ cells do not redifferentiate into melanophores to repopulate deficient areas (e.g., red dashed circle). One week following ablation, EosFP+ cells in the region migrate to ablated areas, but do not differentiate into pigment cells (C). Two weeks post-ablation, new melanophores repopulate ablated region, but none result from EosFP+ cells (e.g., red dashed circle, inlet) (D).