Interplay between Foxd3 and Mitf regulates cell fate plasticity in the zebrafish neural crest

Kevin Curran a,e, James A. Lister b, Gary R. Kunkel c, Andrew Prendergast d,e, David M. Parichy a, David W. Raible a,c,*

a Department of Biology, University of Washington, Seattle, WA 98195, USA
b Department of Human Genetics, Virginia Commonwealth University, Richmond, VA 23284, USA
c Department of Biochemistry/Biophysics, Texas A&M, College Station, TX 77843, USA
d Department of Neurobiology and Behavior, University of Washington, Seattle, WA 98195-7270, USA
e Department of Biological Structure, University of Washington, Seattle, WA 98195-7420, USA

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A B S T R A C T

Pigment cells of the zebrafish, Danio rerio, offer an exceptionally tractable system for studying the genetic and cellular bases of cell fate decisions. In the zebrafish, neural crest cells generate three types of pigment cells during embryogenesis: yellow xanthophores, iridescent iridophores and black melanophores. In this study, we present evidence for a model whereby melanophores and iridophores descend from a common precursor whose fate is regulated by an interplay between the transcription factors Mitf and Foxd3. Loss of mitfa, a key regulator of melanophore development, resulted in supernumerary ectopic iridophores while loss of foxd3, a mitfa repressor, resulted in fewer iridophores. Double mutants showed a restoration of iridophores, suggesting that one of Foxd3’s roles is to suppress mitfa to promote iridophore development. Foxd3 co-localized with pnp4a, a novel marker of early iridophore development, and was necessary for its expression. A considerable overlap was found between iridoblast and melanoblast markers but not xanthoblast markers, which resolved as cells began to differentiate. Cell lineage analyses using the photoconvertible marker, EosFP, revealed that both melanophores and iridophores develop from a mitfa+ precursor. Taken together, our data reveal a Foxd3/mitfa transcriptional switch that governs whether a bipotential pigment precursor will attain either an iridophore or a melanophore fate.

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Introduction

Mechanisms underlying cell fate decisions and the acquisition of specific characteristics required for cells to perform their differentiated functions are of central importance in developmental biology. The neural crest, a multipotent cell population that migrates from the dorsal neural tube and develops into multiple cell types including neurons, glia, craniofacial cartilage and pigment cells, has been a popular model system to study cell fate acquisition. Current models suggest a sequential process of fate restriction, with a combination of intrinsic regulators, such as transcription factors, and extrinsic cell signals in influencing differentiation decisions. One outstanding question is the relative plasticity of these cell fate decisions. While there is considerable evidence that post-migratory, neural crest-derived stem cells retain multipotency when challenged in vitro (Kim et al., 2003; Morrison et al., 2000; Morrison et al., 1999), back transplantation studies suggest that the cell fate choices of these stem cells have become restricted within the embryo (White et al., 2001). However, the relative plasticity of neural crest cells in vivo, which have begun to express lineage-restricted markers, is not well understood.

The development of the black-pigmented melanocytes, referred to as melanophores in fishes and other aquatic vertebrates, is among the best understood cell fate decisions in neural crest development (Cooper and Raible, 2009). Mitf (microphthalmia-associated transcription factor) is a central player in the specification of melanocytes and is amongst the earliest genes expressed in this lineage (Hodgkinson et al., 1993; Opdecamp et al., 1997). Genetic studies demonstrate that this bHLH–leucine zipper transcription factor is necessary for the differentiation of melanocytes and melanophores in all vertebrate taxa (Hodgkinson et al., 1998; Lister et al., 2001; Mochii et al., 1998; Tassabehji et al., 1994). Mitf directly regulates the expression of multiple genes necessary for melanophore development, including dopachrome tautomerase (dct), tyrosinase, tyrosinase-related protein-1, c-kit and bcl2 (Steingrimsson et al., 2004); furthermore, ectopic misexpression of Mitf is sufficient to confer a melanoblast phenotype (Planque et al., 2004; Tachibana et al., 1996). The Mitf gene is directly regulated by the neural crest transcription factors Sox10 and Pax3 (Bondurand et al., 2000; Elworthy et al., 2003; Lacosta et al., 2005; Lee et al., 2000; Potterf et al., 2000; Watanabe et al., 2000; 2001)

* Corresponding author. University of Washington, Department of Biological Structure, Box 357420, Seattle, WA 98195-7420, USA. Fax: +1 206 543 1524.
E-mail address: draible@uwashington.edu (D.W. Raible).

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al., 1998), and is extrinsically activated by the Wnt and cyclic AMP signaling pathways (Busca and Ballotti, 2000; Dorsky et al., 2000; Takeda et al., 2000; Widlund et al., 2002) suggesting a model in which melanocyte cell fate specification is promoted by these factors through activation of Mitf. Expression of Mitf is repressed by the forkhead transcription factor, Foxd3, suggesting that its negative regulation is also important for cell fate specification (Curran et al., 2009; Ignatius et al., 2008; Thomas and Erickson, 2009). Indeed, a recent report provides evidence that Foxd3 expression in the avian neural/gial lineage prevents gial precursors from differentiating as melanocytes (Thomas and Erickson, 2009).

Aquatic vertebrates have additional neural crest-derived pigment cells besides melanophores, including yellow xanthophores and iridescent iridophores. Iridophores are present in amphibians, fish, reptiles and certain invertebrate taxa such as cephalopods (Bagnara et al., 1968; Braasch et al., 2006; Demski, 1992; Kelsh, 2004; Mills and Patterson, 2009; Morrison, 1995). Select wavelengths of light are reflected from stacks of organelle-bound crystallized guanine platelets and are perceived by the viewer as bursts of iridescence (Bagnara et al., 2007; Ziegler, 2003). Electron microscopy studies have identified single cells that contain pigment organelles from each of the three pigment cell types, suggesting that the different pigment cells may be derived from a common precursor (Bagnara et al., 1979). However, single cell lineage analyses in zebrafish have not supported a common precursor, or clonal relationship, amongst the three pigment cell types (Dutton et al., 2001; Raible and Eisen, 1994).

In this study, we present a model of pigment cell fate whereby melanophores and iridophores descend from a common precursor cell. Our genetic analysis of iridophore development suggests that mitfa, the zebrafish Mitf orthologue, and Foxd3 both regulate iridophore development. Iridophores are strongly reduced with loss of foxd3 activity, whereas excess iridophores are found with loss of mitfa activity. These phenotypes suggest a model in which melanophores and iridophores derive from a common precursor whose fate is

![Fig. 1. Expression pattern of iridophores and pnp4a throughout embryonic development.](image-url)
regulated by a Foxd3/mitfa transcriptional switch. Epistasis analyses presented here support the hypothesis that Foxd3 both promotes iridophore development and blocks melanophore development by repressing mitfa. We then test if well-characterized markers for other pigment lineages overlap with a new marker of early iridoblast development, the purine nucleoside phosphorylase gene \( pnp4a \). We find a significant overlap between markers of melanoblasts and iridoblasts, but not xanthoblasts. Finally, we test the lineage relationships of these cells directly using transgenic lines and the photo-activatable protein EosFP. These analyses show that a substantial fraction of \( mitfa \)-expressing cells will subsequently differentiate as iridophores without cell division. These results indicate that cell fate choices remain plastic even after \( mitfa \) expression in zebrafish and support a model in which melanophores and iridophores develop from a common precursor cell.

**Materials and methods**

**Animal husbandry and establishment of transgenic lines**

A \( sox10:nls-eos \) plasmid was generated by PCR amplification from n1--eos using a primer set containing the SV40 nuclear localization sequence and attB1/2 recombination sites. The resulting \( nls-eos \) cassette was recombined into pDONR221 using BP cloning (Invitrogen) then combined pME-\( nls-eos \) with \( csp1 \) and the ToL2 kit components: p3E-poly4 and pDestToL2pA2 (Kwan et al., 2007) to yield \( sox10:nls-eos \). The \( foxd3^{shd}^{sh}/mitfa^{sh} \) double mutant was generated by crossing homozygous mutant \( foxd3^{shd}/mitfa^{sh} \) animals for the double mutant was generated by crossing homozygous mutant \( foxd3^{shd}/mitfa^{sh} \) and \( foxd3^{shd}/mitfa^{sh} \) offspring were raised to identify \( mitfa^{sh} \) and \( mitfa^{sh} \) with FigTree v1.2.3 (http://tree.bio.ed.ac.uk/software/figtree/). The following sequences were used for alignment: Zebrafish \( pnp4a \) (NP_001002102.1; ZDB-GENE-040625-83), Zebrafish \( pnp4b \) (NP_091206; ZDB-GENE-040426-1887), Zebrafish \( pnp5a \) (NP_998476; ZDB-GENE-040426-2553), Zebrafish \( pnp5b \) (NP_001004628; ZDB-GENE-040912-54), Zebrafish \( pnp6 \) (NP_091218; ZDB-GENE-040426-1800), Human PNP (NP_000261.2), Mouse Pnp1 (AAC37635), Mouse Pnp2 (NP_001116843), predicted Mouse Pnp3 (XP_001474586), Dro sophila Pnp (NP_647727), Yeast Pnp (NP_013310), Escherichia coli PNP (NP_416902), Mouse Mtap (NP_013117), Dro sophila Mtap (NP_013117), Zebrafish Mtap (NP_956848), Mouse Mtap (NP_077753), and Human Mtap (CAG46471).

**In situ hybridizations and immunohistochemistry**

Digoxigenin-labeled riboprobes for the genes \( pnp4a \) (ZDB-GENE-040625-83; This se et al., 2004), dct (ZDB-GENE-000508-1; Kelsh et al., 2000b), csp1 (fns, ZDB-GENE-001205-1; Parichy et al., 2000), and aox3 (ZDB-GENE-001205-2; Parichy et al., 2000) have been characterized previously. In situ hybridization was performed as described previously (Lister et al., 1999), using NBT/BCIP as a chromogenic substrate. Fluorescent in situ hybridization was performed as described previously (Julich et al., 2005) using anti-Dig POD for \( pnp4a \) and dct, anti-Fluor POD for \( pnp4a \), dct, csf1r and aox3, Alexa-Fluor tyramide substrates 568 and 488 (Invitrogen) and Roche blocking reagent and buffer. The following antibodies were used for immunohistochemistry at the indicated dilutions: rabbit polyclonal anti-Foxd3 (Lister et al., 2006), 1:500; mouse monoclonal anti-Pax3/7 (DP312; Davis et al., 2005), 1:500; mouse monoclonal anti-Green Fluorescent Protein (Invitrogen), 1:1000; anti-mouse (Alexa 488) and anti-rabbit (Alexa 568) secondary antibodies (Molecular Probes) were used at 1:750. Brightfield images were obtained on a Nikon dissecting microscope with a Spot RT Slider digital camera (Diagnostic Instruments). Fluorescent confocal images were obtained on a LSM 5 Pascal confocal microscope (Zeiss). Images were processed for color.

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**Fig. 2.** Foxd3 is necessary for \( pnp4a \) expression. (A,B) Wild-type fish co-stained with \( pnp4a \) and Foxd3, 24 hpf, anterior trunk. Green: \( pnp4a \) mRNA, red: Foxd3 antibody. (A) Punctate, cytoplasmic \( pnp4a \) mRNA signal surrounds Foxd3 positive nuclei, 63×. (B) Field reveals a \( pnp4a^{+}/Foxd3^{+} \) cell (arrow) adjacent to three \( pnp4a^{−}/Foxd3^{+} \) cells (‘), 40×. (C,D) Flat mounted head and trunk stained with \( pnp4a \) riboprobe, 22 hpf, dorsal view, anterior left, 10×. (C) wild-type (D) \( foxd3^{−}/− \) mutant (sym1). Scale bars: (A) 10 µm; (B) 20 µm; (C,D) 70 µm.

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**pnp4a phylogenetic tree**

Alignment of PNP amino acid sequences was performed with ClustalX 2.0.10 (www.clustal.org). Phylogenetic trees were drawn with FigTree v1.2.3 (http://tree.bio.ed.ac.uk/software/figtree/). The following sequences were used for alignment: Zebrafish \( pnp4a \) (NP_001002102.1; ZDB-GENE-040625-83), Zebrafish \( pnp4b \) (NP_091206; ZDB-GENE-040426-1887), Zebrafish \( pnp5a \) (NP_998476; ZDB-GENE-040426-2553), Zebrafish \( pnp5b \) (NP_001004628; ZDB-GENE-040912-54), Zebrafish \( pnp6 \) (NP_091218; ZDB-GENE-040426-1800), Human PNP (NP_000261.2), Mouse Pnp1 (AAC37635), Mouse Pnp2 (NP_001116843), predicted Mouse Pnp3 (XP_001474586), Dro sophila Pnp (NP_647727), Yeast Pnp (NP_013310), Escherichia coli PNP (NP_416902), Mouse Mtap (NP_013117), Dro sophila Mtap (NP_013117), Zebrafish Mtap (NP_956848), Mouse Mtap (NP_077753), and Human Mtap (CAG46471).
balancing and brightness/contrast using Photoshop CS4 (Adobe) and formatted with Illustrator CS4 (Adobe).

**EosFP photoconversion and cell lineage tracing**

Photoconversion experiments were performed on individual cells expressing the sox10:nls-eos and mitfa:gfp transgenes. sox10:nls-eos plasmid was injected into one-cell Tg(mitfa:gfp)W547 embryos. The resulting embryos express GFP signal throughout the cytoplasm of mitфа positive cells and transiently express photoconvertible green Eos in the nuclei of a subset of sox10 cells. At 24 hpf, embryos were de-chlorinated, anesthetized with MESAB and individually mounted for examination on a Zeiss Axiosplan2 compound scope. Using a 20× objective with a constricted diaphragm, a single double-positive sox10:nls-eos/mitfa:gfp cell per zebrafish was briefly exposed (3–5 s) to ultraviolet light (405 nm). The resulting cell displayed a photoconverted red sox10:nls-eos nucleus and maintained the green mitfa: gfp cytoplasm. Photoconverted zebrafish were returned to embryo media in a 28.5 °C incubator. At 48 and 72 hpf, photoconverted zebrafish were analyzed with brightfield light and incident light to identify cell fate.

**Iridophore cell counts and morpholino oligonucleotide injection**

Tail iridophores (those appearing caudal to the cloaca) were counted at approximately 51–54 hpf on a dissecting microscope with epi-illumination from a fiber optic light source. foxd3 and mitfa antisense morpholino oligonucleotides have been previously described: foxd3 (Lister et al., 2006) and mitfa (Nasevicius and Ekker, 2000).

**Results**

**pnp4a is a novel iridoblast marker**

The first iridophores terminally differentiate by 42 hpf, as revealed by iridescence of their organelle-bound reflecting platelets. These initial iridophores sparsely populate the dorsal stripe and the surface of the eye (Fig. 1A). By 72 hpf, differentiated iridophores more densely populate the dorsal stripe, ventral stripe, ventral yolk stripe and the eye (Fig. 1B). To characterize earlier events during iridophore development we needed to identify an early marker of iridophore precursors. By microarray screening, we found that transcript for the purine nucleoside phosphorylase gene, pnp4a, is enriched in skin with attached pigment cells in wild-type zebrafish as compared to the closely related iridophore-deficient species, D. albolineatus (D.M. Parichy, unpublished). pnp4a is one of five highly conserved pnp genes found in zebrafish, compared to three genes expressed in mouse and one in human (see Fig. S1 in the supplementary material).

We determined the spatiotemporal pattern of pnp4a gene expression by whole-mount mRNA in situ hybridization (Figs. 1C–J, LM). We found that expression of pnp4a begins at 20 hpf, approximately 22h prior to iridophore terminal differentiation. pnp4a is expressed solely in a subset of neural crest cells (Figs. 1C–E) and does not appear in other tissue types in the developing larval zebrafish (Figs. 1F,G). pnp4a first appears at 20 hpf in the head (Fig. 1C). A dorsal view (22 hpf) reveals staining restricted to the neural crest dorsolateral stripes (Fig. 1D). At 24 hpf, pnp4a+ cells are observed along characteristic neural crest migratory pathways (Figs. 1E,M). At 42 hpf, pnp4a positive cells are restricted to a spatial pattern that is characteristic of differentiated iridophores: the dorsal stripe, ventral stripe, ventral yolk stripe and eye, as seen with NBT/BCIP in situ hybridization (Fig. 1F) and fluorescent in situ hybridization (Fig. 1G). High magnification images reveal pnp4a positive cells in the trunk (Fig. 1H), tail (Fig. 1I), and the surface of the eye and dorsal yolk ball (Fig. 1J). To confirm that terminally differentiated iridophores express pnp4a, we imaged the dorsal stripe iridophore pattern of individual WT embryos at 52 hpf (Fig. 1K), processed embryos individually for pnp4a mRNA expression and imaged the resulting pattern of pnp4a staining (Fig. 1L; note that native iridescence of iridophores is lost following histological processing). The positions of pnp4a+ cells and terminally differentiated iridophores correspond precisely, thereby illustrating the specificity of pnp4a to iridophores.

If pnp4a is an enzyme present in iridoblasts, one would expect a pnp4a reduction in an iridophore mutant. Leukocyte tyrosine kinase (ltk) is critical for early iridophore development (Lopes et al., 2008). We assayed pnp4a expression in homozygous ltk mutant embryos by in situ hybridization. Heterozygous ltk mutant parents were crossed...
and 26% (6 of 23) of offspring exhibited a dramatic reduction of pnp4a signal (Figs. 1M,N). Similar reductions in pnp4a signal were observed at 30 hpf in offspring from heterozygous ltk mutant parents: 25% (7 of 28; data not shown). Taken together, these results confirm that pnp4a is a specific cell marker for iridoblasts.

**Foxd3 is necessary for pnp4a expression**

The transcriptional repressor Foxd3 is necessary for iridophore differentiation (Lister et al., 2006). While Foxd3 expression was not previously found in overtly differentiated iridophores, we showed that GFP expression persists in iridophores in a Tg(foxd3:gfP) transgenic line (Curran et al., 2009), suggesting that this protein may be expressed at earlier stages in the iridophore lineage. To further explore the Foxd3/iridophore relationship, we co-stained migratory neural crest at 24 hpf with Foxd3 antibody and pnp4a probe. We found that 63% of pnp4a+ cells (n = 78) co-expressed Foxd3 protein (Fig. 2A). Reciprocally, 24% of Foxd3+ cells (n = 123) co-expressed pnp4a transcript (Fig. 2B). This would be expected as Foxd3 is expressed in multiple neural crest derivatives, including glia, enteric neurons and dorsal root ganglion neurons (Gilmour et al., 2002; Lister et al., 2006). To test if Foxd3 is necessary for pnp4a expression, we next assayed pnp4a staining in homozygous mutant foxd3 embryos at 22–24 hpf by in situ hybridization (Figs. 2C,D). Heterozygous parents were crossed and 23% (6 of 26) of progeny exhibited dramatically reduced iridophores and loss of pnp4a signal were observed at 30 hpf in double mutant embryos (Figs. 3D,E). As expected, the loss of melanophore phenotype observed in mitfa−/− was not rescued in double mutants, as mitfa is necessary for melanophore specification and differentiation (Lister et al., 1999). Additionally, other neural crest derivatives, including enteric neurons, dorsal root ganglia, jaw cartilage and glial cells remained reduced in the double mutant (data not shown). Therefore, mitfa is epistatic to foxd3 with respect to the iridophore phenotype. These results demonstrate that Foxd3 repression of mitfa is a necessary step in iridophore development and suggest the hypothesis that melanophores and iridophores derive from a common precursor.

To address the question of whether eye iridophores are regulated by Foxd3 in a similar manner to trunk iridophores, we performed iridophore cell counts on the eyes of 48 hpf zebrafish under various conditions (see Fig. S2 in the supplementary material). In agreement with trunk iridophores, we observed ectopic eye iridophores in mitfa−/− mutants. However, in contrast to trunk iridophores, we did not observe a reduction in eye iridophores in either the foxd3 MO or after loss of function of mitfa and foxd3. These results contrast with initial expression of pnp4a in the eye at early stages (Fig. 2). However we see later expression of pnp4a in foxd3 mutants wherever iridophores are found (data not shown). We conclude that Foxd3 does exert transcriptional control on the timing and intensity of pnp4a eye expression; however Foxd3 is dispensable for eye iridophore differentiation.

mitfa-expressing neural crest cells re-acquire Foxd3 expression

Foxd3 expression serves as a robust marker for pre-migratory neural crest (Hromas et al., 1999; Labosky and Kaelstrom, 1998; Odenthal and Nusslein-Volhard, 1998; Pohl and Knochel, 2001; Sasai et al., 2001), however, as development proceeds Foxd3 is downregulated in the head and anterior trunk then later reappears in specific neural crest derivatives, such as glia associated with the lateral line (Kelsh et al., 2000a; Gilmour et al., 2002; Lister et al., 2006). We find that Foxd3 exhibits similar biphasic expression in mitfa+ chromatoblasts. Previously, we demonstrated that the vast majority (greater than 90%) of 18 hpf neural crest cells, which begin to express mitfa, are Foxd3 negative (Curran, 2009). To test if Foxd3 is reactivated in a subset of mitfa+ cells, we used an identical assay to examine Foxd3 expression in mitfa:gfP transgenic animals at 24 hpf. We have previously shown that all mitfa:gfP+ cells express endogenous mitfa transcript (Curran et al., 2009). Foxd3 displayed nuclear expression in a substantial proportion of mitfa+ neural crest cells (Figs. 4A–C). Cell counts reveal that 48% of mitfa+ cells (n = 748) are Foxd3 positive at 24 hpf (Fig. 4D). Thus, despite the down-regulation of Foxd3 in the vast majority of mitfa+ cells at 18 hpf,

Fig. 4. mitfa positive neural crest cells re-acquire Foxd3 expression. (A–C) Confocal images taken from lateral aspect of anterior trunk, 40×. (A) Foxd3, (B) mitfa:gfP, (C) merged: red channel: Foxd3, green channel: mitfa:gfP. (D) Cell counts of mitfa:gfP positive cells that are either Foxd3 positive or negative. Counts derived from 40× confocal images at 24 hpf, numbers given as percent of total. 52% of mitfa:gfP+ cells are Foxd3+ (432/848). 48% of mitfa:gfP+ cells are Foxd3+ (316/748). Scale bar = 30 µm.
nearly half of mitfa+ cells have begun to re-express Foxd3 6 h later. These results suggest the hypothesis that re-expression of Foxd3 in a subset of mitfa+ cells promotes their differentiation as iridophores.

Iridoblast marker co-localizes with melanoblast markers

To assess the cell lineage relationship between melanoblasts and iridoblasts we quantified the degree of overlap between the iridoblast marker, pnp4a, and melanoblast genes, mitfa and dct. All cell counts were collected from confocal images from the lateral aspect of the anterior tail region of fixed zebrafish. Cells co-stained to reveal both mitfa and pnp4a mRNA reveal a considerable overlap at 24 hpf (Fig. 5A), this co-localization progressively diminishes as development proceeds (Figs. 5B,C). To further quantify mitfa expression, we took advantage of mitfa:gfp transgenic animals. At 24 hpf, 42% of pnp4a+ cells stain for mitfa:gfp and 57% of mitfa+ cells stain for pnp4a mRNA (Figs. 5D–F; Table 1). A similar degree of overlap between mitfa:gfp and pnp4a mRNA persists until 28 hpf (Fig. 5P; Table 1). At 50 hpf the population of co-localized cells drops considerably; only 3% of pnp4a+ cells stain for mitfa and 8% of mitfa+ cells stain for pnp4a.

Fig. 5. Iridoblast marker co-localizes with melanoblast markers. (A–O) Confocal images collected from lateral aspect of anterior tail region of fixed zebrafish, 20×. (A–C) Cells co-stained with mitfa riboprobe (red) and pnp4a riboprobe (green) reveal a considerable overlap at 24 hpf (A) and a diminishing overlap as development proceeds (B,C). mitfa:gfp transgenic reveals that mitfa+ cells overlap with pnp4a expression at 24 hpf (D–F) and resolve at 50 hpf (G–I). (F) Color merged: green: GFP expression, red: pnp4a mRNA (inset 40×). Wild-type embryos reveal that dct+ cells overlap with pnp4a mRNA at 24 hpf (J–L) then resolve at 26 hpf (M–O). (M) dct (K,N) pnp4a. (L,O) Color merged: green: dct mRNA, red: pnp4a mRNA (inset 40×). (P,Q) Percent of overlap between chromatoblast markers (see Table 1). (P) Green line = % of mitfa:gfp+ cells that are mitfa:gfp+/pnp4a+. Red line = % of pnp4a+ cells that are mitfa:gfp+/pnp4a+. (Q) Green line = % of dct+ cells that are dct+/pnp4a+. Red line = % of pnp4a+ cells that are dct+/pnp4a+. Scale bars: (A–C) 40 µm; (D–O) 60 µm; (F, K, inset) 30 µm.
(Figs. 5G–LP; Table 1). To further test if melanoblasts and iridoblasts share developmental genes, we examined overlap with a second melanoblast marker, dct (Kelsch et al., 2000b). A similar pattern of overlap was observed with pnp4a and dct, however, dct and pnp4a resolve somewhat earlier than mitfa and pnp4a. At 24 hpf, 55% of pnp4a+ cells stain positive for dct mRNA and 62% of dct+ cells stain positive for pnp4a (Figs. 5J–L; Table 1). By 26 hpf, 2 h later, these cell markers are largely resolved; only 7% of pnp4a+ cells stain for dct and 4% of dct+ cells are pnp4a+. These temporal differences in dct and mitfa:gfp expression may reflect increased stability of GFP protein relative to transcript. In conclusion, we observed that approximately 50% of melanoblasts and iridoblasts share developmental genes at 24 hpf, while the markers resolve into their respective cell types at 50 hpf.

Neither iridoblast nor melanoblast markers co-localize with xanthoblast markers

To assess the cell lineage relationship between xanthoblasts and the other chromatoblasts, iridoblasts and melanoblasts, we quantified the degree of overlap between the xanthoblast markers colony stimulating factor-1 receptor (csf1r, formerly fms) and aldehyde oxidase 3 (aox3, formerly xanthine dehydrogenase) with the other cell markers, pnp4a, and the melanoblast markers, dct and mitfa. All cell counts were collected from confocal images from the lateral aspect of the anterior tail region of fixed zebrafish. csf1r encodes a receptor tyrosine kinase and is essential for early larval xanthophore development, whereas aox3 catalyses the synthesis of the xanthophore pteridine pigment, xanthopterin (Parichy et al., 2000). At 24 hpf, 2% of pnp4a+ cells stain positive for csf1r and 5% of csf1r+ cells stain positive for pnp4a (Figs. 6A–CM; Table 2). By 28 hpf, there is no overlap between csf1r and pnp4a (Fig. 6M; Table 2), aox3 and pnp4a display a similar pattern of overlap. At 24 hpf, only 1% of pnp4a+ cells stain for aox3 and 2% of aox3+ cells stain for pnp4a (Figs. 6D–F,N; Table 2), while at 28 hpf there is no overlap between markers (Fig. 6N; Table 2). In conclusion, we observed no significant overlap of the xanthoblast development genes, csf1r and aox3, with the iridoblast gene, pnp4a. The Pax3/7 sub-family of genes encodes paired box transcription factors. They are critical for xanthophore development but express broadly early in the neural crest (Minchin and Hughes, 2008). In contrast to csf1r and aox3, Pax3/7 antibody staining shows a significant overlap with pnp4a until 50 hpf (see Figs. 52 [A–FJ] and Table S1 in the supplementary material). Pax3/7 also co-localizes strongly with mitfa:gfp positive cells between 24 and 32 hpf (see Figs. 53 [G–LK] and Table S1 in the supplementary material).

The percentage of overlap between melanoblast markers and xanthoblast markers remains below 1% at each time point. At 24 hpf, only 0.6% of dct+ cells stain for csf1r and 0.4% of csf1r+ cells stain for dct (Figs. 6G–I; Table 3). The percentage of overlap between dct and csf1r falls to 0% at 28 hpf (Table 3). At 24 hpf, 0.7% of dct positive cells stain for aox3 and 0.5% of aox3+ cells stain for dct (Figs. 6J–L; Table 3). The percentage of overlap between dct and aox3 also arrives at 0% at 28 hpf (Table 3). In conclusion, we observed no significant overlap of the xanthoblast development genes, csf1r and aox3, with the melanoblast development gene, dct, between 24 and 35 hpf.

### Table 1

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|                  | 50                                   | 11                               | 264                             |

Mеланофоны и иридофоны происходят от общего предшественника mitfa +

If a subset of pigment cell precursors express both iridoblast and melanoblast genes at 24 hpf, it is possible that these precursors maintain the potential to acquire either an iridophore or a melanophore fate. To explore this cell lineage relationship, we employed the photoconvertible gene reporter, EosFP. EosFP emits green fluorescence (516 nm) that changes to red (581 nm) upon near-UV irradiation (≈405 nm) due to a photo-induced modification involving a break in the peptide backbone adjacent to the chromophore (Wiedenmann et al., 2004). Sox10, a high mobility group transcription factor, directly activates the zebrafish mitfa proximal promoter and is necessary to specify all zebrafish non-ectomesenchymal neural crest derivatives (Elworthy et al., 2003; Lee et al., 2000; Verastegui et al., 2000). Injecting sox10:mitfa:eos into mitfa:gfp transgenic fish allows tracking of mitfa + neural crest cells. We photoconverted individual cells that expressed both nuclear sox10:mitfa:eos and cytoplasmic mitfa:gfp at 24 hpf (Fig. 7A). The resulting photoconverted cell displayed a red sox10:mitfa:eos nuclear signal and a green cytoplasmic mitfa:gfp signal (Fig. 7B). This distinct color marking allowed individual cells from 24 hpf mitfa:gfp zebrafish to be tracked over a 48 hour time period, thereby permitting us to assess their fates.

Brightfield light overlaid with red fluorescence allowed photoconverted cells that accumulated melanin to be scored as melanophores (Figs. 7D–F). In a similar fashion, incident light overlaid with red fluorescence allowed photoconverted cells that iridiscend to be scored as iridophores (Figs. 7G–I). Photoconverted cells were successfully tracked (n = 144) and identified as attaining either a melanophore or iridophore fate (see Table 4 for all values). 104 photoconverted cells acquired a melanophore fate (72% of scored cells); 40 photoconverted cells acquired an iridophore fate (28% of scored cells) (Fig. 7C; Table 4). We conclude that both melanophores and iridophores are derived from a mitfa + neural crest precursor. Notably, none of the labeled cells divided, suggesting that mitfa:gfp/sox10:mitfa:eos cells were post-mitotic at 24 hpf. To confirm that mitfa:gfp + cells are not mitotically active, we co-stained with anti-phosphohistone H3, which detects mitotic cells in M-phase. 579 (99%) of mitfa:gfp cells were phosphohistone H3 negative; 6 (1%) of mitfa:gfp cells were phosphohistone H3 positive (n = 585) (see Fig. S4 in the supplementary material). These results demonstrate that post-mitotic, chromatoblast cell fate decisions remain plastic.

### Discussion

The results of this study identify a partially fate-restricted neural crest precursor, marked by mitfa expression, with the capacity to produce both melanophores and iridophores in zebrafish embryos. We propose that there are two avenues for neural crest cells to acquire the melanophore or iridophore fate (Fig. 8). A cell can either develop directly into one of the two chromatophores or it may pass through a bi-potent stage before acquiring its ultimate fate. Our results suggest that cells expressing mitfa are still plastic: some cells will continue to express this gene and become melanophores while other cells will eventually repress mitfa and form iridophores. We refer to these mitfa + precursors as bi-potent, since they retain the potential to produce either melanophores or iridophores. As indicated by both photolabeling and phosphohistone H3 staining, each cell is post-
mitotic, and therefore gives rise to either an iridophore or a melanophore, but not both. We hypothesize that the cell fate decision is decoupled from cell division and that cells remain plastic after their final mitosis. Foxd3 acts as a molecular switch upon this precursor population by repressing melanophore fate and promoting iridophore fate, in part by repressing mitfa expression. We note that there is no evidence for any biochemical difference between a chromatophore that passes through a double-positive phase and one that develops directly; both sets of precursors express pnp4a.

**pnp4a as a novel iridophore marker**

Our study of early iridophore development was facilitated by the identification of pnp4a as a specific marker, originally identified as enriched in the iridophore inter-stripe region of adult zebrafish dermal tissue. pnp4a is a highly conserved member of the purine nucleoside phosphorylase family with nearly 50% similarity to the *E. coli* protein (see Fig. S1 in the supplementary material). The biochemical function of pnp4a has not been tested in zebrafish, however, functional experiments with PNPase orthologues have been performed in both mouse and *E. coli* (Della Ragione et al., 1996; Seeger et al., 1995). PNPase is an enzyme involved in purine synthesis, which metabolizes guanosine into guanine, a nitrogenous base, and ribose phosphate, a sugar (Bzowska et al., 2000). Therefore, it would follow that pnp4a would be of paramount importance for zebrafish iridescent pigmentation, which results from light reflecting off accumulated guanine crystals. Taken together, pnp4a seems to act as an early expressing enzyme in iridophore differentiation, analogous to the
manner with which dct is used in melanin biosynthesis in early melanoblasts and melanocyte stem cells. Itk, a leucocyte tyrosine kinase known to be critical for early iridophore development (Lopes et al., 2008) is necessary for pnp4a expression. The very low level of Itk expression precluded further co-localization analysis with pnp4a and other pigment cell markers.

A bi-potent melanophore/iridophore precursor in the chromatoblast lineage

EosFP photoactivation experiments demonstrate that mitfa:gfp+ cells are capable of forming either melanophores or iridophores, allowing us to infer that expression of mitfa is not sufficient to drive cells towards the melanophore fate. Our results also suggest, however, that not all mitfa:gfp+/pnp4a+ cells become iridophores. Comparing cell ratios between marker co-localization assays and cell-labeling experiments reveals significantly more double-positive mitfa:gfp+/pnp4a+ cells than the number of mitfa+ cells that become iridophores (p<0.001 by Chi-square analysis; see Table S2 in the supplementary material). These findings suggest a model in which mitfa+/pnp4a+ cells are precursors for both melanophores and iridophores. However, we do not believe that all melanophores and iridophores are derived from this cell type. There are many more melanophores than double-positive pnp4a+/mitfa+ cells, suggesting an additional melanophore source. Moreover, while there is an iridophore increase with loss of mitfa, the number of ectopic iridophores is much lower than the number of melanophores lost (data not shown). Similarly, there is not a complete loss of iridophores in foxd3 mutants and the number of iridophores is not completely restored in foxd3/mitfa double mutants, suggesting some iridophores are regulated by independent mechanisms. As our data shows that all differentiated iridophores express pnp4a, these iridoblasts may be represented by the mitfa:gfp+/pnp4a+ population.

A close relationship between iridophores and melanophores has been proposed previously. Bagnara et al. (1979) first suggested the idea that pigment cells share a common origin after observing single chromatophores housing multiple pigment organelles. Iridophores from the iris of the dove contained partially melanized reflecting platelets; likewise, single chromatophores from the tapetum lucidum of the teleost fish, Dasyatis sabina, revealed melanosomes and reflecting platelets bound within the same intracellular membrane. This chromatophore mosaicism suggested that pigment cells, specifically iridophores and melanophores, share a common precursor capable of activating multiple pigment synthesis pathways. Ide and Bagnara (1980) found that proliferating bullfrog melanophores in clonal culture lose melanosomes and form reflecting platelets (iridosomes) when cultured in a medium containing high guanosine content, suggesting that developing melanoblasts maintain the capacity to trans-differentiate to an iridophore fate. Previous work in zebrafish has also demonstrated a close developmental relationship between iridophores and melanophores, parade (pde), a pigment phenotype mutant, accumulates ectopic, mosaic chromatophores that contain organelles characteristic of both iridophores (iridosomes) and melanophores (melanosomes) (Kelsh et al., 1996). More recently, Lopes et al. (2008) presented a progressive fate restriction model, which describes a sox10+/ltk+ pigment precursor prepared to adopt either an iridophore or melanophore fate. Since Itk positive cells accumulate in the posterior trunk and tail region of sox10 mutants, the authors hypothesize that the iridoblasts are trapped in a multipotent pigment precursor state. Our data builds on these reports to support a chromatophore lineage model based on an iridoblast/melanoblast precursor that is separate from xanthophore precursors.

Our temporal analysis of genetic markers revealed a substantial overlap of iridoblast and melanoblast markers. In contrast, there is less than 1% overlap between either of the xanthoblast markers, csf1r and aox3, and markers of either iridoblasts or melanoblasts. This result suggests that the xanthoblast lineage is distinct and, in agreement with previous clonal analysis (Dutton et al., 2001; Raible and Eisen, 1994), does not provide convincing evidence for a tri-potent chromatophore precursor capable of producing all three chromatoblast cells. However, it remains possible that the observed 0.4% overlap between csf1r+ and dct+ cells and the 0.5% overlap between aox3+ and dct+ cells represent a small population of tri-potent chromatoblast precursors. Expression of Pax3 has previously been shown to be required for xanthophore development but not for melanophores or iridophores. Pax3/7 antibody labels xanthoblasts (Minchin and Hughes, 2008), however, in our hands this antibody additionally labels the majority of mitfa+ and pnp4a+ cells, demonstrating that it is not specific to xanthoblasts.

It remains possible that other cell types, including glia and xanthophores, could descend from mitfa+ precursors. Thomas and Erickson (2009) reported that MITF repression causes cells to acquire glial characteristics. Furthermore, a recent report using chick embryos shows that myelinating krox20+ Schwann cells retain the competence to differentiate into melanocytes (Adameyko et al., 2009). These results illustrate a close lineage relationship between glial cells and melanoblasts. Minchin and Hughes (2008) found that Pax3 knock-down led to a loss of xanthophores accompanied by a small increase in melanophores, supporting the possibility of a common chromatoblast precursor between melanophores and xanthophores. We observed that photoconverted cells neither adopt xanthophore or glial morphology nor migrate in a manner characteristic of either cell type. To remain spatially unbiased, we photoconverted cells from all expression domains (dorsal trunk, brain, yolk ball and lateral trunk). However, by only photoconverting cells at a single time point (24 hpf).

| Table 2 |
|------------------|-----------------|-------------------|-------------------|-------------------|
| Iridoblast and xanthoblast co-localization analysis. | | | | |
| Time points (hpf) | Double-positive cells (pnp4a+/csf1r+) | pnp4a+ only | csf1r+ only | % of pnp4a+ that are pnp4a+/csf1r+ | % of csf1r+ that are pnp4a+/csf1r+ |
| 24 | 1 | 147 | 57 | 2 | 5 |
| 26 | 5 | 120 | 66 | 4 | 7 |
| 28 | 0 | 234 | 210 | 0 | 0 |
| 35 | 0 | 145 | 153 | 0 | 0 |

| Time points (hpf) | Double-positive cells (pnp4a+/aox3+) | pnp4a+ only | aox3+ only | % of pnp4a+ that are pnp4a+/aox3+ | % of aox3+ that are pnp4a+/aox3+ |
| 24 | 4 | 396 | 196 | 1 | 2 |
| 26 | 6 | 114 | 61 | 5 | 9 |
| 28 | 0 | 189 | 215 | 0 | 0 |
| 35 | 0 | 109 | 163 | 0 | 0 |

| Table 3 |
|------------------|-----------------|-------------------|-------------------|-------------------|
| Melanoblast and xanthoblast co-localization analysis. | | | | |
| Time points (hpf) | Double-positive cells (dct+/csf1r+) | dct+ only | csf1r+ only | % of dct+ that are dct+/csf1r+ | % of csf1r+ that are dct+/csf1r+ |
| 24 | 1 | 166 | 249 | 0.6 | 0.4 |
| 26 | 0 | 132 | 123 | 0 | 0 |
| 28 | 0 | 89 | 78 | 0 | 0 |
| 35 | 0 | 65 | 68 | 0 | 0 |

| Time points (hpf) | Double-positive cells (dct+/aox3+) | dct+ only | aox3+ only | % of dct+ that are dct+/aox3+ | % of aox3+ that are dct+/aox3+ |
| 24 | 1 | 142 | 199 | 0.7 | 0.5 |
| 26 | 0 | 132 | 99 | 0 | 0 |
| 28 | 0 | 56 | 78 | 0 | 0 |
| 35 | 0 | 83 | 56 | 0 | 0 |
we introduced a temporal bias that may have limited the diversity of descendents; partially restricted glial/melanophore or xanthophore/melanophore precursors could theoretically exist before the onset of mitfa expression.

Foxd3 as a molecular switch between melanophores and iridophores

We propose that Foxd3 promotes pnp4a expression in cells that are initially positive for mitfa. We demonstrate that Foxd3 is necessary for pnp4a expression at 24 hpf and, using in situ fluorescence imaging, we reveal that Foxd3 positive cells express pnp4a transcript. Foxd3 often serves as a transcriptional repressor (Curran et al., 2009; Pohl and Knochel, 2001; Stewart et al., 2006; Yaklichkin et al., 2007), however, Foxd3 transcriptional activation is not unprecedented. Lee et al. (2006) observed that Foxd3 directly bind the myf5 promoter so as to maintain myf5 expression in somites and adaxial cells, thus driving somitogenesis. It currently remains unclear whether Foxd3 directly binds the pnp4a promoter to activate its expression.

Foxd3 has previously demonstrated the capacity to bind and repress the zebrafish mitfa promoter, thereby preventing melanophore fate (Curran et al., 2009; Ignatius et al., 2008) or to indirectly

Table 4
Photoconversion lineage tracing.

<table>
<thead>
<tr>
<th>EosFP photoconversion data</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
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<td>Photoconverted cells</td>
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<td>73</td>
<td>70</td>
<td>231</td>
</tr>
<tr>
<td>Photoconverted cells that could be tracked</td>
<td>40</td>
<td>51</td>
<td>53</td>
<td>144</td>
</tr>
<tr>
<td>Melanophore descendants</td>
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<td>36</td>
<td>33</td>
<td>103</td>
</tr>
<tr>
<td>Iridophore descendants</td>
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<td>15</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td>% of photoconverted cells that become melanophores</td>
<td>85</td>
<td>71</td>
<td>62</td>
<td>72</td>
</tr>
<tr>
<td>% of photoconverted cells that become iridophores</td>
<td>15</td>
<td>29</td>
<td>38</td>
<td>28</td>
</tr>
</tbody>
</table>

Fig. 7. Melanoblasts and iridoblasts share a mitfa+/bi-potent precursor. (A,B) Confocal image of a double-positive sox10:nls-eos/mitfa:gfp cell surrounded by mitfa:gfp cells, lateral view, anterior trunk, 24 hpf, 40×. (A) Unconverted, pre-UV exposure. (B) Photoconverted, post-UV exposure. (C) Bar graph: 72% of identified photoconverted cells acquire a melanophore fate; 28% of identified photoconverted cells acquire an iridophore fate (n = 1-44) Bars = s.d. (for all values see Table 4). (D–F) Photoconverted sox10:nls-eos/mitfa:gfp cell acquires melanophore fate, lateral view, anterior trunk, 48 hpf, 40×. (D) Brightfield. (E) Red channel. (F) Merged brightfield/red channel. (G–I) Photoconverted sox10:nls-eos/mitfa:gfp cell acquires iridophore fate, lateral view, anterior trunk, 48 hpf, 40×. (G) Incident light. (H) Red channel. (I) Merged incident/red channel. Scale bars: (A,B, D–I) 30 µm.

Fig. 8. Model for chromatophore development from the neural crest. We propose a model for pigment cell lineages that includes two pathways for melanophores and iridophores to differentiate. Pigment cells may develop directly from neural crest cells or transit through a bi-potent pigment precursor stage. Between 18 and 24 hpf, neural crest cells begin to express lineage-specific markers. Xanthoblasts require csf1r and commit to the pteridine pigment synthesis pathway, as indicated by aox3 expression. Iridoblasts require ltk and continue along the purine synthesis pathway as indicated by pnp4a expression. Melanoblasts require mitfa and continue along the melanin synthesis pathway as indicated by dct expression. In addition, some mitfa+ cells will retain the capacity to produce either melanophores or iridophores, a process regulated by expression of Foxd3. Foxd3 is initially expressed in all neural crest cells at 18 hpf, then downregulated. Foxd3 reappears in approximately half of mitfa+ bi-potent precursors at 24 hpf, resulting in repression of mitfa, activation of pnp4a and promotion to an iridophore fate. A reciprocal population will remain Foxd3 negative, continue mitfa expression and follow the melanophore path.
inhibit avian MITF transcription by binding activators (Thomas and Erickson, 2009). Accordingly, we have previously shown that mitfa expression is initially exclusive with Foxd3. At 18 hpf, more than 90% of mitfa-expressing neural crest cells are Foxd3 negative (Curran et al., 2009). In contrast, by 24 hpf, approximately 50% of mitfa-expressing cells stain positive for Foxd3, suggesting that a subset of mitfa+ cells have re-acquired Foxd3 activity. This transcriptional control of chromatophore fate is further illustrated with our iridophore count experiments. Loss of mitfa resulted in ectopic iridophore development, loss of foxd3 caused a loss of iridophores, while the foxd3/mitfa double mutants allowed a partial rescue, suggesting that Foxd3 promotes iridophore development, in part, by repressing mitfa. It should be noted that neither the foxd3fl/fl mutant (Stewart et al., 2006) nor the Foxd3 morphant (Lister et al., 2006) resulted in a complete loss of iridophores, and we observed some corresponding ppm4a+ cells in foxd3fl/fl mutants at 28 and 32 hpf (data not shown). Residual Foxd3 function is likely to remain after MO injection and while the foxd3fl/fl mutation was originally reported as a null, recent work has demonstrated that mutant Foxd3 protein retains some function (Chang and Kessler, 2010). Low levels of Foxd3 activity may be sufficient for the few iridophores that specify in Foxd3 mutant and morphino-injected embryos.

Our study demonstrates a remarkable flexibility in development under the onset of expression of a key differentiation regulator. The MITF transcription factor has previously been thought of as a “master regulatory” gene for melanocyte differentiation, with its expression both necessary and sufficient for acquisition of melanoblast characteristics. Our results contrast with recent reports that suggest mitfa expression is sufficient to commit a neural crest cell towards the melanophore fate (Ignatius et al., 2008; Thomas and Erickson, 2009). However direct lineage tracing has not previously been described. It has been shown previously that mammalian melanoblasts can be maintained in an undifferentiated state after the onset of Mitf expression through the continued action of Pax3 (Lang et al., 2005). We speculate that neural crest cells in this state might retain the flexibility to differentiate along multiple lineage pathways.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.04.023.

References


