Developmental Biology 332 (2009) 408–417

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Genomes & Developmental Control

Foxd3 controls melanophore specification in the zebrafish neural crest by regulation of Mitf

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ARTICLE INFO

Article history:
Received for publication 26 March 2009
Revised 4 June 2009
Accepted 8 June 2009
Available online 13 June 2009

Keywords:
Foxd3
Mitf
mitfa
Melanoblasts
Melanophores
Neural crest

ABSTRACT

We describe a mechanistic model whereby Foxd3, a forkhead transcription factor, prevents neural crest-derived precursors from acquiring a melanophore fate. Foxd3 regulates this fate choice by repressing the *mitfa* promoter in a subset of neural crest cells. *mitfa* is only expressed in a Foxd3-negative subset of neural crest cells, and *foxd3* mutants show an increase in the spatial domain of *mitfa* expression, thereby suggesting that Foxd3 limits the *mitfa* domain. Furthermore, *foxd3:gfp* transgenic zebrafish reveal *foxd3* expression in xanthophore precursors and iridophores, but not in terminally differentiated melanophores. Luciferase experiments and embryo mRNA injections indicate Foxd3 acts directly on the *mitfa* promoter to negatively regulate *mitfa* expression. Taken together, our data suggests the presence of Foxd3 in a subset of precursors leads to *mitfa* repression and suppression of melanophore fate. MITF, the human *mitfa* ortholog, has recently been described as an oncogene and implicated in various forms of melanoma. Understanding the mechanisms that regulate *mitfa* and melanophore development could prove informative in the treatment and prevention of these human diseases.

Introduction

The elucidation of the genetic mechanisms that transition a multipotent precursor towards a committed fate remains an important focus of developmental biology. The neural crest-derived pigment cells of the zebrafish, *Danio rerio*, are a remarkably tractable system for studying cell fate in vertebrate taxa. Pigment cells of the skin are derived from the neural crest, a transient embryonic population of cells that migrate through the animal to generate a diverse set of cell types (reviewed by Cooper and Raible, 2009). Zebrafish produce three varieties of neural crest-derived pigment cells: the black melanophore, the silver iridophore and the yellow xanthophore. These pigment cells migrate across the zebrafish and create coloration patterns that affect the behavior and ecology of the organism. Melanophores (or melanocytes) are broadly represented among species from each vertebrate class, while xanthophores and iridophores are absent in the homoeothermic vertebrates, birds and mammals (Braasch et al., 2006; Fujii, 2000). One hundred years of mouse genetic research have elucidated many aspects of melanophore biology, including the molecular pathways regulating melanoblast development, the biochemistry of melanin production and the dynamics of melanosome dispersal (Hoekstra, 2006; Kelsh, 2004; Lin and Fisher, 2007; Parichy, 2006). However, less is known about the mechanisms that act to dictate melanophore fate specification. In this study we describe an early event in pigment cell development in zebrafish, *Danio rerio*, which prevents a pigment precursor from attaining a melanophore fate.

Positive regulators of melanophore development are well defined. In many animals the basic helix–loop–helix/leucine zipper transcription factor Mitf acts as a master regulatory gene for black pigment cell differentiation (Levy et al., 2006). Mitf is amongst the earliest genes expressed in melanoblast precursors, and is necessary and sufficient for their development. Zebrafish mutant for the Mitf ortholog *mitfa* exhibit a complete absence of body melanophores (Lister et al., 2001). Previous work has placed Mitf upstream of multiple genes necessary for melanin production and terminally differentiated melanophores including *dopachrome tautomerase*, *tyrosinase*, *tyrosinase-related protein-1*, *c-kit* and *bcl2* (reviewed by Steingrimsson et al., 2004). Ectopic expression of *Mitf* is sufficient to confer melanophore characteristics (Lister et al., 1999; Planque et al., 2004; Tachibana et al., 1996), demonstrating that it is necessary and sufficient for pigment cell specification.

Several positive regulators of zebrafish *mitfa* have been identified. The transcription factor Sox10 directly drives melanophore cell fate via the *mitfa* promoter (Elworthy et al., 2003), a regulatory relation-

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doi:10.1016/j.ydbio.2009.06.010

ship conserved in other animals (Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). Transcription of *mitfa* is also positively regulated by Wnt signals (Dorsky et al., 2000; Larue and Delmas, 2006; Saito et al., 2003). However, both Sox10 and Wnt signals play positive roles in multiple neural crest lineages (reviewed by Raible, 2006) suggesting negative regulators of *Mitf* are important for cell fate specification.

Foxd3, a winged-helix transcription factor, is a good candidate for a negative regulator of melanophore development. Originally identified as a gene expressed in murine embryonic stem cells (Clevidence et al., 1993; Hanna et al., 2002; Sutton et al., 1996), Foxd3 was later found to exhibit highly conserved pre-migratory neural crest expression throughout vertebrates (Hromas et al., 1999; Labosky and Kaestner, 1998; Odenthal and Nusslein-Volhard, 1998; Pohl and Knochel, 2001; Sasai et al., 2001). More recently, Foxd3 has been shown to play a functional role in the specification of various downstream neural crest derivatives (Ignatius et al., 2008; Lister et al., 2006; Montero-Balaguer et al., 2006; Stewart et al., 2006; Teng et al., 2008). While Foxd3 has been described as a transcriptional activator in muscle (Lee et al., 2006), it likely acts as a transcriptional repressor in the neural crest (Pohl and Knochel, 2001; Sasai et al., 2001; Yaklichkin et al., 2007). Foxd3 represses the formation of melanocytes in chick embryos (Kos et al., 2001), suggesting it is a key regulator of melanogenesis. Recently, Ignatius et al. (2008) have shown that zebrafish *foxd3* genetically interacts with histone deacetylase and that Foxd3 protein can bind the *mitfa* promoter *in vitro*, but no binding sites were identified or functional tests reported. Here we examine the functional consequence of this interaction.

We propose a model in which Foxd3 acts upon neural crest cells, via the *mitfa* promoter, to prevent melanophore fate. *foxd3* mutants reveal an increase in the spatial domain of *mitfa* suggesting Foxd3 restricts *mitfa* expression. In a wild-type background, *mitfa* is only expressed in a subset of neural crest cells that are negative for Foxd3 protein, again suggesting Foxd3 limits the *mitfa* domain. Furthermore, our *foxd3:gfp* transgenic zebrafish reveals *foxd3* expression in xanthophore precursors and iridophores but not terminally differentiated melanophores. Luciferase experiments quantify Foxd3's ability to repress the *mitfa* promoter in a winged-helix domain dependent manner. Lastly, embryo mRNA injections recapitulate the luciferase results in the context of a live zebrafish. Taken together, these results support a mechanism of cell specification whereby the absence of Foxd3 protein in pigment precursor cells allows *mitfa* activation and, subsequently, melanophore cell fate. Reciprocally, the presence of Foxd3 protein in other neural crest cells maintains *mitfa* repression and, thereby, suppresses melanophore fate.

Materials and methods

Animal husbandry and establishment of transgenic lines

The Tg(*mitfa:gfp*)^{w47} transgenic line was generated by injecting one-cell embryos with an agarose gel-purified Sal I/Not I restriction fragment of the plasmid pNP-BEGFP, in which the CMV promoter from the vector pCS2+BE-GFPLT was replaced with a 931-bp fragment of the *mitfa* promoter and 5′ UTR via the SalI and HindIII sites. A single germline transgenic founder was identified from 28 adult fish. Adult fish of the *AB strain *foxd3*^{zdf1} (*sym1*; Stewart et al., 2006), the transgenic line Tg(*mitfa:gfp*)^{w47} and the transgenic line Tg(*foxd3:gfp*)^{zf15} (Gilmour et al., 2002) reporter strains were maintained on a 14 h/10 h light/dark cycle at 28.5 °C. Embryos for all experiments were obtained through natural crosses and staged according to Kimmel et al. (1995). In some experiments, phenylthiocarbamide (PTU; Sigma) was added to embryo medium at a final concentration of 0.2 mM to inhibit melanin synthesis.

Cell culture, transfections, immunostaining

B16 and NIH-3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% fetal bovine serum (Gibco) supplemented with 1× penicillin/streptomycin and incubated at 37 °C in an atmosphere of 5% CO_2. Fresh culture medium was provided every 2 days and the cells were sub-cultured before reaching 70% confluency. The melb-a mouse melanoblast cells (Sviderskaya et al., 1995) were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum, stem cell factor (10 ng/ml) and FGF2 (40 pM) supplemented with 1× penicillin/streptomycin and incubated at 37 °C in an atmosphere of 10% CO_2. The pCS2-myc-tag Foxd3 plasmid used for the melb-a cell overexpression assay was described previously (ZDB-GENE-980526-143; Lister et al., 2006). The DNA-binding mutant form of pCS2-myc-tag Foxd3 (Foxd3 BM) was created using the Quikchange mutagenesis protocol (Stratagene) with the following oligonucleotides: forward, 5′-cca ttc gcc ata act ttt cgc tca acg act gct-3′; reverse, 5′-agc agt cgt tga gcg aaa agt tat ggc gaa tgg-3′. The mutation generated a single amino acid substitution in the winged-helix domain: leucine to phenylalanine (L147F). The pCS2-*snail1b* plasmid was made by cloning a full-length open reading frame (ZDB-GENE-980526-514; Thisse et al., 1995) into pCS2 via HindIII and BamHI sites. For the NIH-3T3 luciferase assay, the coding sequence of zebrafish *sox10* (ZDB-GENE-011207-1; Dutton et al., 2001) was amplified and subcloned into the vector pCS2-FLAG with three amino terminal FLAG epitope tags. The *mitfa* proximal promoter (GenBank accession # AF211890; Dorsky et al., 2000) was mutated using the Quikchange mutagenesis protocol (Stratagene). Changed base pairs are in lower case. Site 1 (nucleotides 579–588): 5′ ATGCTGAtggCAggggATGTTT 3′. Site 2 (nucleotides 750–761): 5′-CGTTTGGGTAgggAAggagATATGA 3′. All constructs were confirmed by sequencing. About 1×10^5 cells were seeded into each well of a 24-well plate (Falcon 3047) 24 h prior to transfection. Cells were transfected by the lipofectamine method (Invitrogen) according to the manufacturer's instructions. Transfection mixtures contained 1 μl of lipofectamine and 0.8–1 μg of plasmid constructs per well in 24-well plates. Reagents for the Galacto-Light chemiluminescent reporter assay were from Tropix (Bedford, MA, USA) and D-Luciferin was from Analytical Luminescence Laboratories (San Diego, CA, USA). Luciferase output was standardized with beta-galactosidase activity. All transfections and luciferase assays were performed independently and in quadruplicate.

In situ hybridizations and immunohistochemistry

Digoxigenin-labeled riboprobe for *mitfa* (ZDB-GENE-990910-11; Lister et al., 2006) and *dct* (ZDB-GENE-000508-1; Kelsh, 2000) has been characterized previously. In situ hybridization was performed as described previously (Lister et al., 1999), using NBT/BCIP as a chromagenic substrate. Fluorescent in situ hybridization was performed as described previously (Julich et al., 2005) using anti-Dig POD for both *dct* and *mitfa*, Alexa-Fluor tyramide substrate 568 (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-myc (Abcam), 1:1000; mouse monoclonal anti-Flag (Sigma), 1:1000; mouse monoclonal anti-Pax3/7 (DP312) (Minchin and Hughes, 2008), 1:500; rabbit polyclonal anti-MITF (Novus Biological), rabbit polyclonal anti-Sox10 (Park et al., 2005), 1:1000; 1:500; anti-mouse (Alexa 488) and anti-rabbit (Alexa 568) secondary antibodies (Invitrogen), 1:750; mouse monoclonal anti-Green Fluorescent Protein (Invitrogen) and rabbit polyclonal anti-Green Fluorescent Protein (Invitrogen), 1:500 in conjunction with anti-mouse (Alexa 488), 1:1000 and anti-rabbit (Alexa 568), 1:1000 to boost signal. Brightfield images were obtained on a Nikon dissecting microscope with a Spot RT Slider digital camera (Diagnostic Instru-

ments). Fluorescent confocal images were obtained on a LSM 5 Pascal confocal microscope (Zeiss). Whole images were processed for color balancing and brightness/contrast using Photoshop CS4 (Adobe) and formatted with Illustrator CS4 (Adobe).

Embryo injections

Full-length and capped *foxd3* (100 pg/embryo), *foxd3* DNA-binding mutant (100 pg/embryo) and *sox10* mRNA (18 pg/embryo) were generated using mMessage mMachine (Ambion). Embryos for injection were obtained from natural matings, injected at the one-cell stage and imaged at shield stage (6–8 h post fertilization; hpf) using a fluorescent Nikon dissecting microscope with Spot camera.

Results

mitfa:gfp transgenic line reports mitfa positive melanoblasts

To observe zebrafish melanoblasts *in vivo* we generated a GFP reporter transgenic line using 931 bp of the *mitfa* promoter and 5′ UTR upstream of the *mitfa* start site. At 20 hpf *mitfa:gfp* is expressed in lateral stripes along the dorsal aspect of the hindbrain and trunk region (Fig. 1A). This pattern is indicative of migratory neural crest cells arising from the closing neural tube. By 26 hpf *mitfa:gfp* positive cells are present dorsally at the posterior end of the zebrafish and ventrally throughout the head and anterior trunk region (Fig. 1B). To confirm the fidelity of our *mitfa:gfp* transgenic, we co-labeled transgenic embryos with anti-GFP and a *mitfa* riboprobe and counted cells along the dorsal–lateral stripes. At 24 hpf, 98.4% of *mitfa:gfp* cells ($n = 311$) are also positive for *mitfa* transcript (Figs. 1C, E). To confirm that cells positive for *mitfa:gfp* are melanoblasts and fated to become melanophores, we examined transgenic embryos for co-expression of GFP and *dopochrome tautomerase* (*dct*) mRNA (Figs. 1D, F). *dct*, also known as *tyrosinase-related protein-2*, is a melanogenic enzyme downstream of *mitfa* that resides in the melanosome membrane within melanoblasts (Kelsh et al., 2000; Lister et al., 1999). Therefore, at 24 hpf, one would expect a proportion of *mitfa* positive cells to have up-regulated *dct* expression. We found 55% of *mitfa:gfp* positive cells ($n = 193$) are *dct* positive. In conclusion, our *mitfa:gfp* transgenic accurately marks *mitfa* positive melanoblasts at these stages.

mitfa expression is expanded in foxd3 mutants

If Foxd3 acts as a transcriptional repressor, one would expect to observe higher overall *mitfa* expression or ectopic *mitfa* expressing cells in *foxd3* mutant zebrafish. To explore this regulatory relationship,

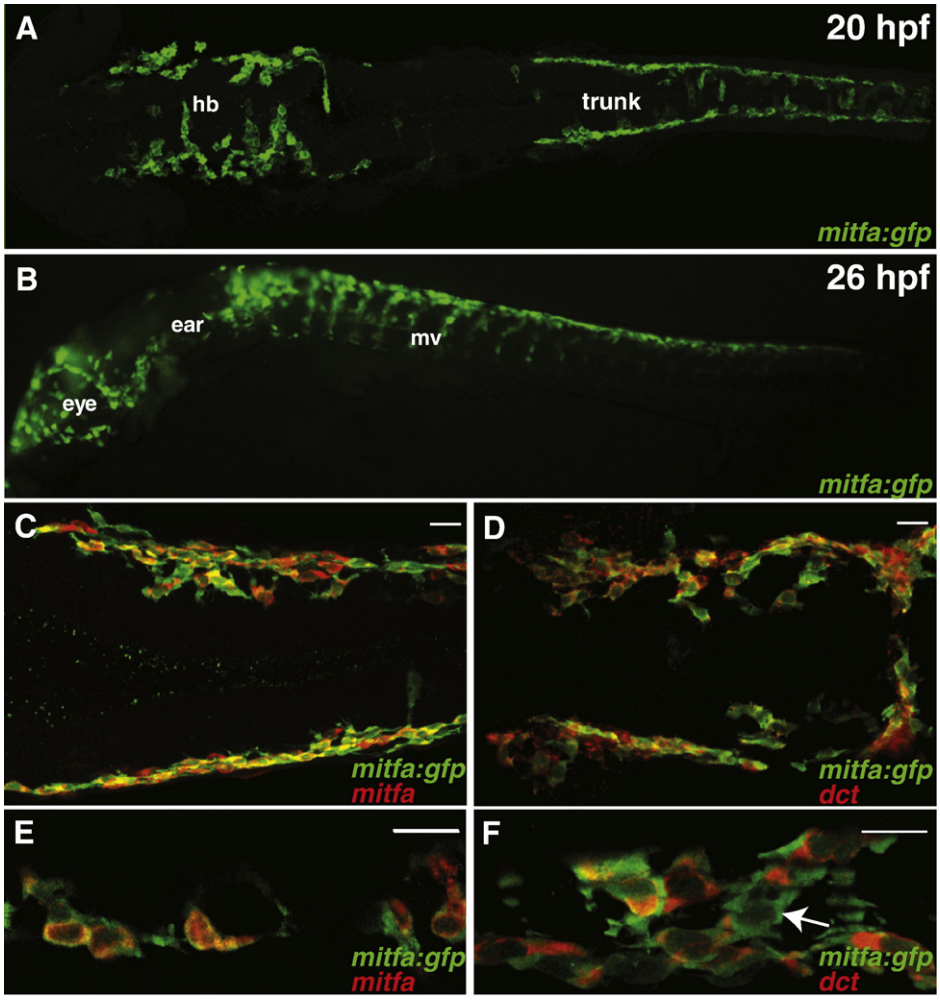


Fig. 1. *mitfa:gf*p transgenic reports *mitfa* positive melanoblasts. (A) Live GFP expression from 20 hpf *mitfa:gfp* transgenic zebrafish, streams of migratory neural crest cells arise from lateral stripes, dorsal view, anterior left, 10×. hb = hindbrain. (B) Live GFP expression from 26 hpf *mitfa:gfp* transgenic zebrafish, *mitfa* positive neural crest cells migrate ventrally (mv), lateral view, anterior left, 10×. (C, E) *mitfa:gfp* transgenic recapitulates *mitfa* expression, dorsal view, anterior left, 24 hpf. Green: GFP expression, red: *mitfa* mRNA. (C) 20× scale bar = 20 μm. (E) 40× scale bar = 10 μm. 98.4% of *mitfa:gfp* cells ($n = 311$) are *mitfa* positive. (D, F) *mitfa:gfp* transgenic labels early melanoblasts, dorsal view, anterior left, 24 hpf. Green: GFP expression, Red: *dct*. (D) 20×. Scale bar = 20 μm. (F) 40×. Scale bar = 10 μm. Arrow points to *mitfa:gfp* positive cell that has yet to activate *dct* expression. 55% of *mitfa:gfp* positive cells ($n = 193$) are *dct* positive.

we assessed possible spatial and temporal expansions of *mitfa* expression in *foxd3* mutant embryos using in situ hybridization. The *foxd3*^{zdf1} mutation is a nucleotide deletion that disrupts the forkhead DNA-binding domain of the *foxd3* gene (Stewart et al., 2006). At 40 hpf, we observe a spatial expansion of *mitfa* in the head region and anterior trunk (Figs. 2A, D). At later time points, 48 and 60 hpf, the increase in *mitfa* expression is noticeable, although less pronounced (Figs. 2B, C, E, F). Similar results were recently reported by Ignatius et al. (2008), who found *mitfa* signal in melanoblasts to be more robust in *foxd3* morpholino-injected zebrafish than in uninjected controls.

Foxd3 and mitfa exhibit a mutually exclusive relationship in migratory neural crest cells

Foxd3 expression is a robust marker of pre-migratory neural crest but is down-regulated in a subset of cells as development proceeds, possibly via an autoregulatory negative feedback mechanism (Lister et al., 2006; Stewart et al., 2006). If Foxd3 acts to repress *mitfa* expression, we would predict their distribution to be mutually exclusive. To assess the relationship between *mitfa* and Foxd3 expression, we used Tg(*mitfa:gfp*) zebrafish and anti-Foxd3 antibody on a field of neural crest cells along the dorsal aspect of the anterior trunk region of zebrafish embryos (Figs. 3A, B). At 24 hpf, we find that expression of Foxd3 protein does not substantially overlap with *mitfa:gfp*. Analysis of *mitfa* transcript by in situ hybridization confirms this relationship (Fig. 3C). To quantify the mutually exclusive relationship between *mitfa* and Foxd3 expression, we looked at the earliest stages of GFP transgene initiation to determine whether there was any overlap in expression, and found that approximately 90% of GFP positive cells were Foxd3 negative (Fig. 3D). In contrast, Sox10, a known *mitfa* activator (Dutton et al., 2001) shows extensive overlap (Fig. 3E). Cell counts reveal that 91.8% of *mitfa:gfp* positive cells ($n=250$) are Sox10 positive at this stage.

Foxd3 expression is absent in melanophores, but present in iridophore and xanthophore precursors

Using the previously characterized Tg(*foxd3:gfp*) zebrafish (Gilmour et al., 2002), we looked for *foxd3* expression in the three zebrafish pigment cell types: xanthophores, iridophores and melanophores. The thick yellow pigmentation in xanthophores obscures cellular boundaries and makes accurate cell counts difficult. To better identify xanthophores at 27 hpf, we used the Pax3/7 antibody, recently determined to be exclusively expressed in the xanthophore lineage at this stage (Minchin and Hughes, 2008). We found 94% of Pax3/7 xanthoblasts ($n=165$) are *foxd3:gfp* positive (Figs. 4A, B). Terminally differentiated iridophores illuminate under incident light, allowing accurate cell counts. At 72 hpf, we observed 88.3% of differentiated iridophores ($n=342$) to be *foxd3:gfp* positive (Figs. 4C, D). Terminally differentiated melanophores lie adjacent to iridophores along the dorsal stripe at 72 hpf (Fig. 4D). These melanophores display no overlapping *foxd3:gfp* expression, however, it is possible the melanin accumulation within melanophores masks GFP expression. To address this concern, we treated fish with the melanin synthesis blocker PTU at 24 hpf, then analyzed *foxd3:gfp* expression in partially melanized zebrafish at 72 hpf (Figs. 4E, F). We observed *foxd3:gfp* expression in only 5% of melanophores ($n=314$). We conclude that *foxd3* expression is maintained in xanthophores and iridophores, however, *foxd3* expression is down-regulated in melanophores.

Foxd3 represses endogenous MITF in cell culture

To test whether Foxd3 expression is sufficient to repress endogenous Mitf expression we transfected pCS2+ plasmid expressing zebrafish *foxd3* under the CMV promoter into melb-a mouse melanoblast cells. Melb-a cells constitutively express Mitf (Lei et al., 2002). We then assayed expression of endogenous Mitf expression with an anti-MITF antibody. Expression of Foxd3 strongly repressed Mitf expression (Fig. 5A). In contrast, *foxd3* with a leucine to phenylalanine substitution in the DNA-binding, winged-helix domain (L167F), a mutation of a highly conserved amino acid shown to block DNA binding of Fox proteins in other systems (Hacker et al., 1995), was incapable of repressing endogenous Mitf, implying Foxd3 must bind directly to DNA to repress Mitf (Fig. 5B). To quantify the relationship between Foxd3 and endogenous Mitf expression, cell counts were performed (Fig. 5C). 96.5% of cells expressing Foxd3 repressed Mitf expression ($n=58$). 2.2% of cells expressing Foxd3 binding mutant repressed Mitf expression ($n=47$). We conclude that zebrafish *foxd3* prevents endogenous Mitf in cell culture.

Foxd3 represses the mitfa promoter in mouse fibroblasts and mouse melanoma cells

To determine whether Foxd3 could directly regulate the *mitfa* promoter, we used a pGL3-SV40 luciferase reporter plasmid compri-

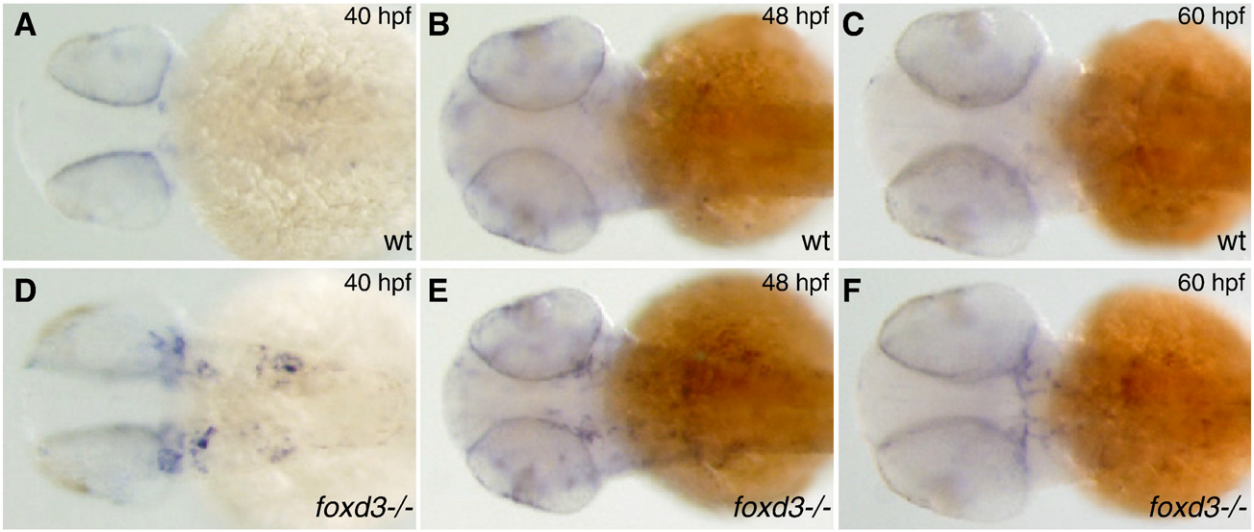


Fig. 2. *mitfa* expression expanded in Foxd3 mutant. *mitfa* mRNA expanded in head and anterior trunk of Foxd3 mutant. Dorsal view, anterior left, 5×. (A–C) *mitfa* expression in wild-type zebrafish (D–F) *mitfa* expression in *foxd3*−/− (*sym1*). (A, D) 40 hpf (B, E) 48 hpf (C, F) 60 hpf.

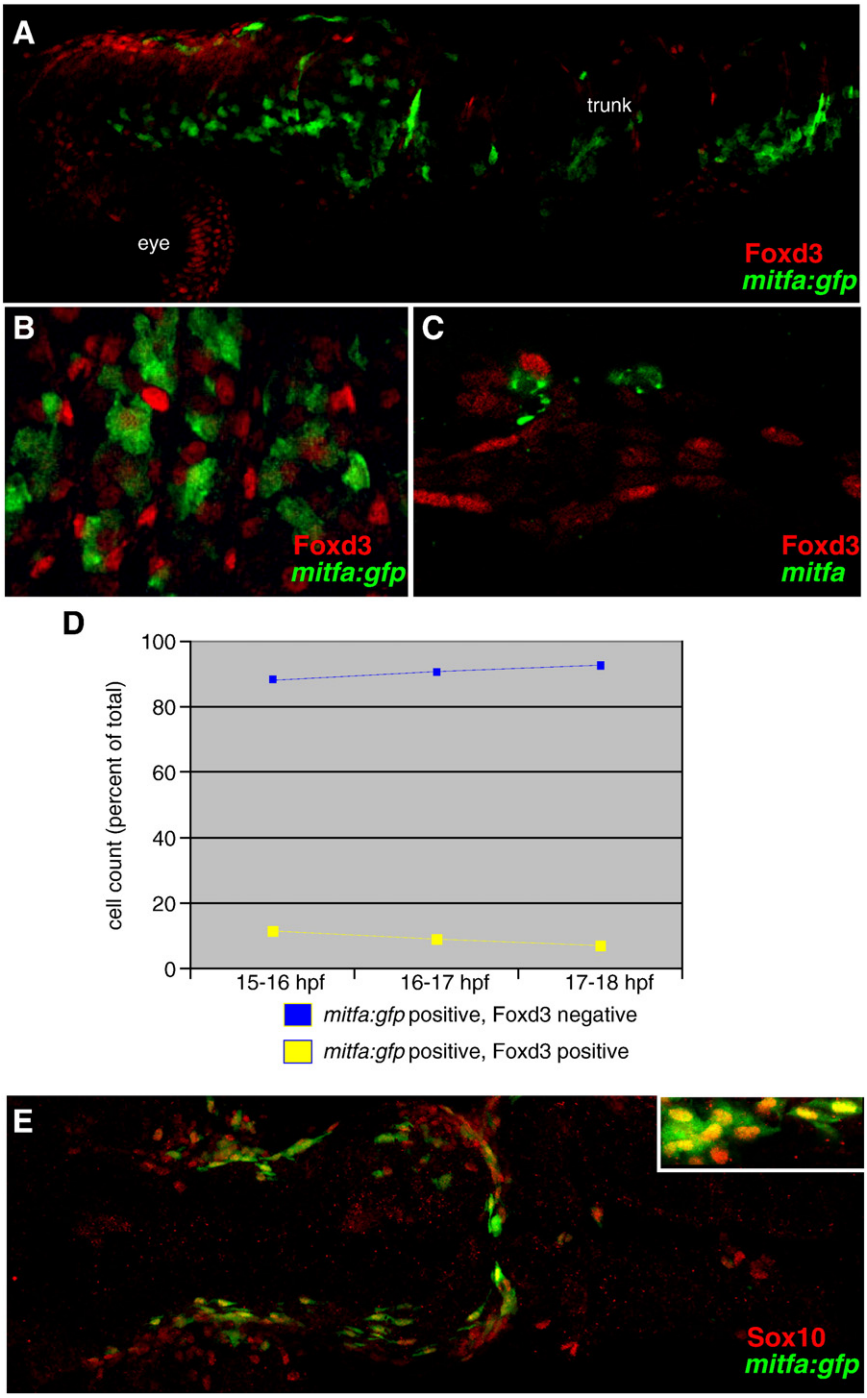


Fig. 3. Foxd3 and *mitfa* expressed in separate populations of neural crest cells. (A) *mitfa:gfp* positive neural crest cells are mutually exclusive with Foxd3 positive cells, 18 hpf, lateral view, anterior left, 10×. (B) *mitfa:gfp* up-regulates in Foxd3 negative neural crest cells, 18 hpf, dorsal view, anterior trunk region, 20×. (A, B) Red: Foxd3 Ab. Green: GFP expression in *mitfa:gfp* transgenic line (C) *mitfa* up-regulates in Foxd3 negative neural crest cells, 24 hpf lateral view, anterior trunk region. Red: Foxd3 Ab. Green: *mitfa* mRNA. 20×. (D) Cell counts of *mitfa:gfp* positive cells derived from 40× confocal images of migratory neural crest cells at three time points: 15–16 hpf, 16–17 hpf and 17–18 hpf. Blue line = percent of total *mitfa:gfp* positive cells counted which are Foxd3 negative. Yellow line = percent of total *mitfa:gfp* positive cells counted which are Foxd3 positive. Stage 15–16 hpf, 88% of *mitfa:gfp* positive cells are Foxd3 negative (583/664); 12% of *mitfa:gfp* positive cells are Foxd3 positive (81/664). Stage 16–17 hpf, 89% of *mitfa:gfp* positive cells are Foxd3 negative (497/560); 11% of *mitfa:gfp* positive cells are Foxd3 positive (63/495). Stage 17–18 hpf, 91% of *mitfa:gfp* positive cells are Foxd3 negative (448/495); 9% of *mitfa:gfp* positive cells are Foxd3 positive (47/495). (E) *mitfa:gfp* positive neural crest cells overlap with Sox10 expression, 91.8% of 250 *mitfa:gfp* positive cells are Sox10 positive, 20 hpf, dorsal view, anterior left, 10× (inset 20×).

sing 931 bp of the zebrafish *mitfa* proximal promoter (Dorsky et al., 2000) to perform luciferase assays in NIH-3T3 mouse fibroblasts and B16 mouse melanoma cell lines. In B16 melanoma cells, the *mitfa* promoter has high basal activity. Co-transfection of the *foxd3* plasmid caused a 19-fold decrease in the activity of *mitfa:luciferase* as compared to basal *mitfa:luciferase* alone (Fig. 6A). A similar co-transfection using the DNA-binding mutant version of *foxd3* resulted in a return to basal *mitfa:luciferase* expression levels (Fig. 6A). Acting as a negative control, *snail1b*, a transcriptional repressor with no known *mitfa* activity, was transfected into the assay. *snail1b* failed to repress *mitfa:luciferase* (Fig. 6A). Foxd3 transfection had no effect on luciferase expression under control of an SV40 promoter (data not shown), suggesting that *mitfa* control elements were necessary for repression. Co-transfection with the transcriptional repressor *snail1b* (LaBonne

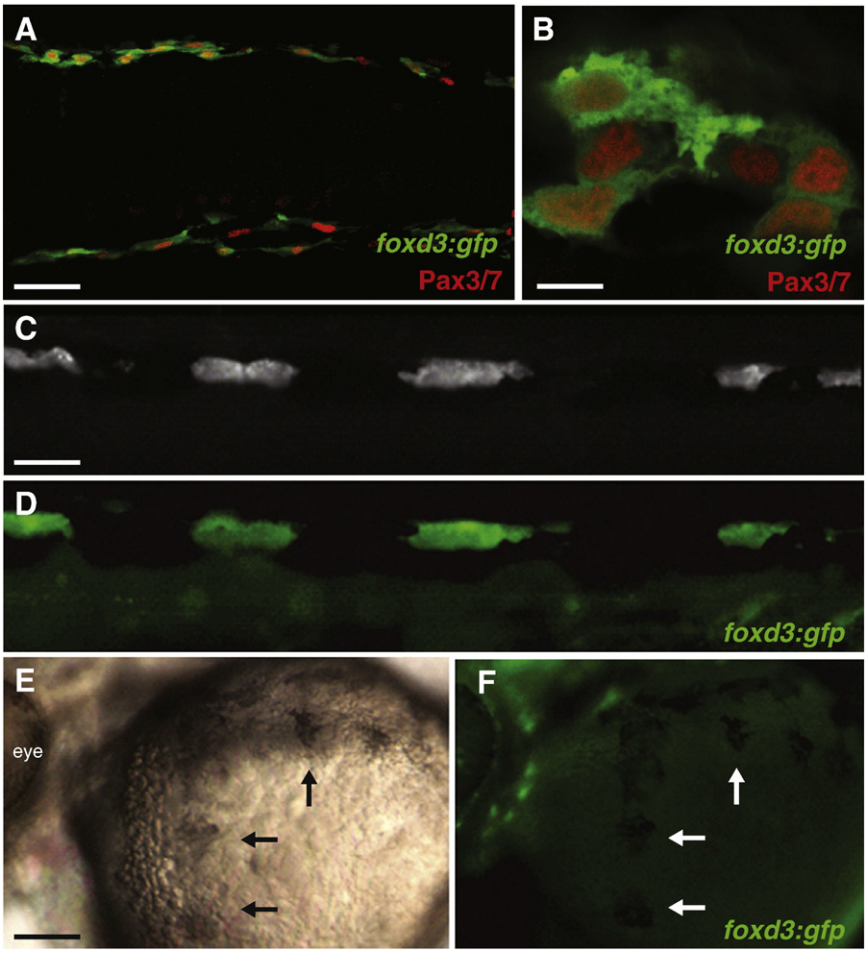
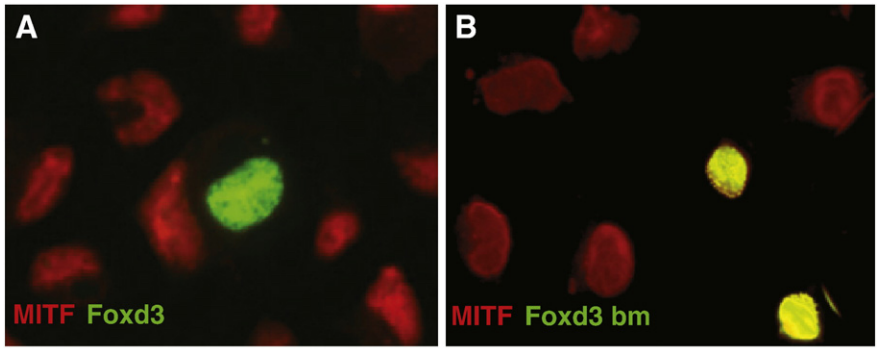


Fig. 4. *foxd3* expression is absent in melanophores, but present in iridophores and xanthophore precursors. (A, B) *foxd3:gfp* maintains expression in xanthoblasts, 27 hpf, dorsal view, anterior left. Green: anti-GFP, red: Pax3/7 (mouse DP312 Ab). 94% of Pax3/7 xanthoblasts ($n = 165$) are *foxd3:gfp* positive. (A) 20×, scale bar = 40 μm. (B) 40×, scale bar = 7.5 μm. (C, D) *foxd3:gfp* maintains expression in iridophores, 72 hpf, lateral view, dorsal stripe, anterior left. Scale bar = 25 μm. 88.3% of differentiated iridophores ($n = 342$) are *foxd3:gfp* positive. (C) Incident light reveals 4 iridophores. (D) Green: live *foxd3:gfp*. (E, F) *foxd3:gfp* expression absent in melanophores, 72 hpf, lateral view, yolk ball, anterior left, embryo treated with PTU. Arrows: partially melanized melanophores, scale bar = 90 μm. 5% of melanophores ($n = 314$) express *foxd3:gfp*. (E) Brightfield. (F) Green: live *foxd3:gfp*.

and Bronner-Fraser, 2000), failed to repress *mitfa:luciferase*, suggesting that repression by Foxd3 was specific (Fig. 6A).

Transfections with an ectopic activator in NIH-3T3 cells confirmed the preceding results. NIH-3T3 cells have low basal activation of *mitfa:luciferase*, as would be expected from a fibroblast cell. To compensate for low basal *mitfa* expression, *sox10*, a known *mitfa* activator (Elworthy et al., 2003) was co-transfected to enact a 5 fold increase over basal *mitfa:luciferase* expression levels (Fig. 6B). Co-transfection

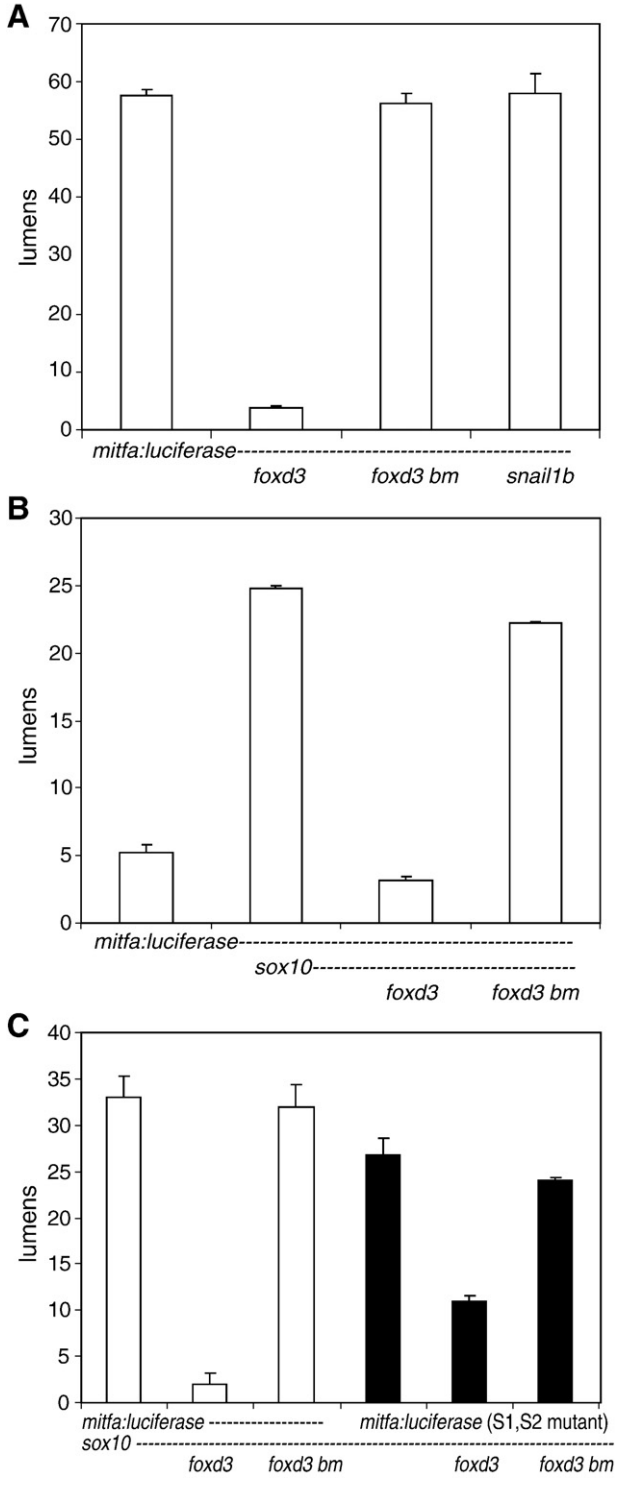


C

Foxd3 + / MITF +	2	Foxd3 bm + / MITF +	45
Foxd3 + / MITF -	56	Foxd3 bm + / MITF -	1

Fig. 5. Melb-a cell culture analysis displays Foxd3 repression of endogenous MITF expression. (A, B) Transfected melb-a (mouse melanoblast) cells, (20×). Red: anti-MITF rabbit polyclonal, green: anti-Myc mouse monoclonal. (A) Cells transfected with full-length zebrafish *foxd3-myc* sequence show nuclear exclusion of endogenous mouse MITF. (B) Cells transfected with DNA-binding mutant version of zebrafish *foxd3-myc* display co-localized staining with endogenous mouse MITF. (C) Cell counts collected from 5 separate transfection experiments. 56 melb-a cells transfected with full-length *foxd3* became MITF negative, 2 remained positive. 1 melb-a cell transfected with DNA-binding mutant version of zebrafish *foxd3* became MITF negative, 45 transfected cells remained positive.

of *foxd3* with *sox10* blocked the *mitfa* promoter activation normally observed with *sox10* alone (Fig. 6B). These data suggest Foxd3 can antagonize Sox10's activation of the *mitfa* promoter. When *foxd3* DNA-binding mutant was used, we observed no repression of *sox10* activation (Fig. 6B). Taken together, these results suggest Foxd3 represses the *mitfa* promoter by binding to DNA. Ignatius et al. (2008) recently reported binding of Foxd3 to *mitfa* promoter DNA *in vitro*, consistent with direct activation, and identified two putative Forkhead binding sites but did not test them for function. We tested the function of these presumptive Foxd3 binding sites in the *mitfa* promoter and observed a partial relief of repression after the two sites were mutated (Fig. 6C). *foxd3* transfections with NIH-3T3 cells yielded a 13 fold repression using the full-length *mitfa* promoter (Fig. 6C). In contrast, *foxd3* transfections using our mutagenized S1,S2 *mitfa* promoter yielded only a 2.5 fold repression (Fig. 6C). This suggests the S1 and S2 sites are involved with Foxd3 repression of *mitfa*, however, additional Foxd3 binding sites may also be present or additional indirect mechanisms may be at play.



Foxd3 represses the mitfa promoter in zebrafish embryos

To examine *mitfa* promoter activity in live zebrafish embryos, we used a transient mRNA co-injection assay in the *mitfa:gfp* transgenic zebrafish. GFP is first observed in melanophore precursors at 15–18 hpf, reflecting endogenous *mitfa* expression (Fig. 3D). Under normal conditions, a 6 hpf shield stage embryo displays no GFP (Fig. 7B). Injection of *sox10* mRNA into one-celled stage *mitfa:gfp* embryos results in robust precocious GFP expression at 6 hpf (Fig. 7D), recapitulating *sox10*'s effect on *mitfa* expression in zebrafish (Elworthy et al., 2003). Upon co-injection of both *foxd3* mRNA and *sox10* mRNA, precocious *mitfa:gfp* expression is strikingly absent (Fig. 7F), while co-injection of DNA-binding mutant *foxd3* mRNA and *sox10* mRNA results in a return to the robust precocious GFP expression observed with injections of *sox10* alone (Fig. 7H). We conclude that, in an overexpression assay in a live zebrafish embryo, Foxd3 can prevent *mitfa:gfp* expression in a manner dependent on its DNA-binding domain.

Discussion

Our results demonstrate that Foxd3 can repress the activity of the zebrafish *mitfa* promoter and thus would inhibit melanophore differentiation. These results are consistent with previous results in avian embryos, where electroporation of Foxd3 inhibited melanocyte differentiation (Kos et al., 2001). We also find that transfection of *foxd3* into melb-a mouse melanoblast cells blocks Mitf expression. However, we have not been able to test whether Foxd3 overexpression in zebrafish embryos alters melanophore differentiation because of its earlier effects on axis specification (Lister et al., 2006; Steiner et al., 2006). We observed enhanced levels of *mitfa* signal in *foxd3* mutants, consistent with the idea that Foxd3 represses *mitfa*. However, this *mitfa* de-repression was spatially limited to the head and anterior trunk region. Consistent with these observations, there is little change in melanophore cell number in *foxd3* mutants (Stewart et al., 2006). These observations suggest that additional negative regulators of *mitfa* might exist in other regions of the zebrafish embryo, such as the

Fig. 6. Foxd3 represses the *mitfa* promoter in mouse fibroblasts and mouse melanoma cells. (A, B) Luciferase assays assess Foxd3 activity on 931-bp. region of zebrafish *mitfa* promoter (*mitfa:luciferase* construct). (A) B16 mouse melanoma cells display high levels of basal *mitfa:luciferase* activity. Basal *mitfa:luciferase* activity decreased 19-fold upon co-transfection with full-length zebrafish *foxd3*. Basal activity not significantly altered upon co-transfection with DNA-binding mutant version of zebrafish *foxd3*. Negative control: *snail1b* failed to repress basal *mitfa:luciferase* activity. (B) NIH-3T3 mouse fibroblast co-transfection with zebrafish *sox10* increases *mitfa:luciferase* activity 5 fold. Transfections with *mitfa:luciferase*, *sox10* and *foxd3* results in 7.6 fold decrease from *mitfa:luciferase* and *sox10* activation alone. Transfections with *mitfa:luciferase*, *sox10* and DNA-binding mutant version of *foxd3* result in no significant change from *mitfa:luciferase* and *sox10* activation. (C) NIH-3T3 mouse fibroblast luciferase assays reveal partial functionality of proposed Foxd3 binding sites on 931-bp. region of zebrafish *mitfa* promoter (*mitfa:luciferase* construct). Proposed Foxd3 binding sites (S1 and S2) have been scrambled in the *mitfa:luciferase* (S1,S2 mutant) construct. (C) Transfections with *mitfa:luciferase*, *sox10* and *foxd3* results in a 13 fold decrease from *mitfa:luciferase* and *sox10* activation alone. Transfections with *mitfa:luciferase*, *sox10* and DNA-binding mutant version of *foxd3* result in no significant change from *mitfa:luciferase* and *sox10* activation. Transfections with *mitfa:luciferase* (S1,S2 mutant), *sox10* and *foxd3* results in only a 2.5 fold decrease from *mitfa:luciferase* (S1,S2 mutant) and *sox10* activation alone. Transfections with *mitfa:luciferase* (S1,S2 mutant), *sox10* and DNA-binding mutant version of *foxd3* result in no significant change from *mitfa:luciferase* (S1,S2 mutant) and *sox10* activation alone. (A–C) Data are means +/− standard deviation.

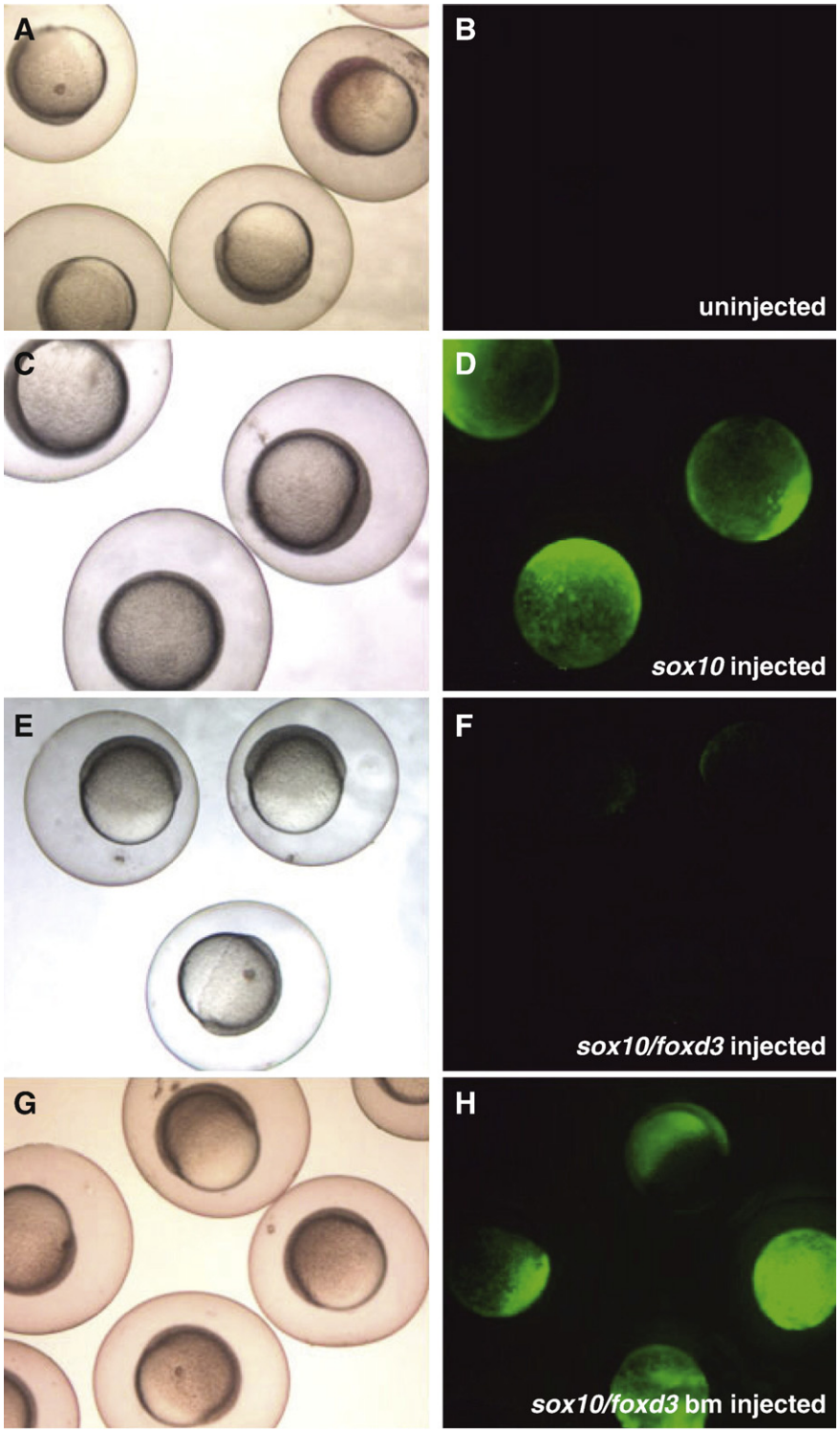


Fig. 7. Foxd3 represses the *mitfa* promoter in zebrafish embryos. (A–H) One-cell *mitfa:gfp* transgenic zebrafish embryos microinjected with mRNA and imaged live at 6–7 hpf, shield stage 5×. (A, C, E, G) Brightfield images. (B, D, F, H) Green: live GFP expression from *mitfa:gfp*. (A, B) Un-injected embryos reveal no fluorescence at shield stage (observed in 64/64 embryos). (C, D) Embryos injected with *sox10* mRNA produce robust, precocious *mitfa:gfp* expression at shield stage (observed in 62/64 embryos). (E, F) Co-injection of full-length *foxd3* with *sox10* mRNA prevents *mitfa:gfp* expression at shield stage (observed in 47/52 embryos). (G, H) Embryos co-injected with *sox10* and DNA-binding mutant version of *foxd3* mRNA display a return to robust, precocious *mitfa:gfp* expression at shield stage (observed in 43/49 embryos).

posterior trunk and tail. Additionally, specific activators are needed in addition to loss of repression to increase *mitfa* expression.

Our data are consistent with Foxd3 acting directly as a transcriptional repressor on the *mitfa* promoter. Foxd3 has been demonstrated to act as a repressor in other systems (Pohl and Knochel, 2001; Yaklichkin et al., 2007). Foxd3 might regulate *mitfa* indirectly through protein–protein interactions rather than directly by promoter binding. However, several lines of evidence support a direct interaction. We found that transfection of *foxd3* inhibits the effects of exogenously supplied *sox10* activator in our assays with NIH-3T3 cells, suggesting a direct action. Additionally, introducing the L167F mutation into the winged-helix domain of Foxd3, a mutation of a highly conserved

amino acid shown to block DNA binding of Fox proteins in other systems (Hacker et al., 1995), eliminated the effects of Foxd3 on *mitfa*. However, it is possible that Foxd3 interfered with an endogenous co-activator that was not sufficient to activate *mitfa* on its own. There remains the possibility that a properly configured winged-helix domain might be necessary for interaction with other proteins if Foxd3 were interacting independently of direct DNA binding. There is some precedent for this idea: Guo et al. (2002) utilized immunoprecipitation assays to show Oct-4 physically interacts with the DNA-binding domain of FoxD3. In this manner, Oct-4 acts as a co-repressor modulating Foxd3 repressive activity in mammalian endoderm development. It is possible similar protein co-repressors are responsible for modulating Foxd3 activity in our assays. However, we observed Foxd3 repression of *mitfa* in mouse melanoblast (melb-a cells), mouse melanoma (B16 cells), mouse fibroblast (NIH-3T3 cells) and zebrafish embryos. Therefore, if protein–protein co-repressors are involved, this indirect interaction must be present in each of these four systems.

We have not been able to demonstrate consistent interactions of Foxd3 protein with the *mitfa* promoter *in vitro*, suggesting direct interactions may be of low affinity. We note that Ignatius et al. (2008) utilized electromobility shift assays to determine that Foxd3 binds two sites from the *mitfa* promoter. When we mutated these sites and tested them in luciferase assays, we found that inhibition was alleviated from a 13 fold to a 2.5 fold repression. This partial relief of repression suggests that additional Foxd3 binding sites may be present on the *mitfa* promoter or that indirect, protein–protein interactions are at play.

The repression of *mitfa* by *foxd3* sheds light on the possible mechanism behind the genetic interaction between *foxd3* and *kit*, a receptor tyrosine kinase necessary for melanophore survival (Cooper et al., 2009). Inactivation of *foxd3* partially rescues melanophore cell death seen in *kit* mutants. Since Mitf is a positive activator of the pro-survival gene *bcl2* (McGill et al., 2002), inhibition of Mitf by Foxd3 would be expected to promote cell death. Inactivation of Foxd3 in turn would release this inhibition and have pro-survival effects that might partially overcome the loss of Kit function.

Our *foxd3:gfp* data suggests that, within pigment cells, Foxd3 repression is unique to melanophores. Xanthoblasts and iridophores maintain *foxd3*:*gfp* expression, suggesting Foxd3 does not interfere with their development and differentiation. Foxd3 appears to play a positive role in iridophore specification as iridophores are strongly reduced in both *foxd3*^{zdf1} mutant zebrafish (Stewart et al., 2006) and in *foxd3* morphant zebrafish (Lister et al., 2006). Therefore, the restricted spatial expansion of *mitfa* observed in the *foxd3*^{zdf1} mutant may represent ectopic *mitfa* expression in a pigment precursor population of neural crest cells. Interestingly, *mitfa* exerts a repressive role on iridophore development, as seen in increased cell counts of larval trunk and tail iridophores in *mitfa* mutants as compared to wild-type zebrafish (Lister et al., 1999). Fully understanding the possible mechanisms by which Foxd3 regulates transcription will be necessary to determine how Foxd3 acts to promote the development of other neural crest lineages, such as iridophores, peripheral neurons and glial cells.

While this work was under review, Thomas and Erickson (2009) described a similar inhibitory regulation of the avian *MITF* gene by FOXD3. In this study, FOXD3 regulated *MITF* indirectly through protein–protein interactions with PAX3, a positive regulator of Mitf in avian and mammalian species (Watanabe et al., 1998). In zebrafish, Pax3 does not regulate melanophores but rather xanthophores (Minchin and Hughes, 2008), and we find no evidence of regulation of the zebrafish *mitfa* promoter by Pax3 (J.A.L., unpublished). Thus while the regulatory interactions between Foxd3 and Mitf may be widely conserved, specific mechanisms may vary. Thomas and Erickson (2009) provide evidence for the regulatory interaction between Foxd3 and Mitf playing a role in regulating glial cell development, suggesting that this interaction may have more widespread effects on neural crest development than the regulation of pigment cell precursors described here.

Our work regarding Foxd3 repression of *mitfa* corroborates with previous findings from mammalian melanocyte research (reviewed by Harris and Erickson, 2007). The conservation of genetic mechanisms regulating melanogenesis across vertebrates makes the tractable zebrafish system an attractive tool to study human diseases associated with melanocytes. A recent study has correlated the occurrence of vitiligo, a chronic skin condition causing hypo-pigmentation, in one family to a single base change (G>T) which up-regulates Foxd3 promoter activity in cultured cells (Alkhateeb et al., 2005). Furthermore, MITF has recently proven to be an amplified oncogene in a significant fraction of human melanomas and to play an oncogenic role in human clear cell sarcoma (Levy et al., 2006). Understanding the molecular mechanisms that properly repress *mitfa* expression may offer insight into improved strategies for treating these life-threatening diseases.

Acknowledgments

The authors wish to thank David White for fish care, John Kanki for the *foxd3*^{zdf1} fish, Bruce Appel for the Sox10 antibody, the Biology Imaging Facility for confocal assistance and Dot Bennett for melb-a cells. Additional thanks to Cynthia Cooper for laboratory training. This work has been supported by grants from NIH to D.W.R. and J.A.L. K.C. would like to thank Washington Research Foundation, ARCS Foundation and the Developmental Biology Training Grant (NICHD T32 HD007183-2681) for their financial support.

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