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

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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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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-