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FOXD3 regulates the lineage switch between neural crest-derived glial cells and pigment cells by repressing MITF through a non-canonical mechanism

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The first neural crest cells to emigrate from the neural tube are specified as neurons and glial cells and are subsequently followed by melanocytes of the skin. We wished to understand how this fate switch is controlled. The transcriptional repressor FOXD3 is expressed exclusively in the neural/glial precursors and MITF is expressed only in melanoblasts. Moreover, FOXD3 represses melanogenesis. Here we show that avian MITF expression begins very early during melanoblast migration and that loss of MITF in melanoblasts causes them to transdifferentiate to a glial phenotype. Ectopic expression of FOXD3 represses MITF in cultured neural crest cells and in B16-F10 melanoma cells. We also show that FOXD3 does not bind directly to the *MITF* promoter, but instead interacts with the transcriptional activator PAX3 to prevent the binding of PAX3 to the *MITF* promoter. Overexpression of PAX3 is sufficient to rescue MITF expression from FOXD3-mediated repression. We conclude that FOXD3 controls the lineage choice between neural/glial and pigment cells by repressing *MITF* during the early phase of neural crest migration.

KEY WORDS: FOXD3, MITF, Pigment cell development, Transcriptional regulation, PAX3, Neural crest, Chick, Quail

INTRODUCTION

The neural crest (NC) is a population of cells that emigrates from the neural tube early during vertebrate development. These cells migrate throughout the embryo, where they differentiate into numerous cell types. NC cells of the avian trunk emigrate from the neural tube in two waves that are distinct in their time of emigration, pathway of migration and fate. Cells in the first migratory wave migrate ventrally through the somites and differentiate as neurons and glia, whereas cells in the second wave are specified as melanocytes and migrate dorsolaterally between the dermatome and the overlying ectoderm. NC cells are fate-specified at the time they begin migrating, and this prior specification determines their pathway choice and migratory abilities (Erickson and Goins, 1995; Henion and Weston, 1997; Reedy et al., 1998). There appears to be a lineage switch that occurs in the premigratory NC between the two waves of migration, such that they are specified as neural and glial precursors before the switch and melanoblasts after. The molecular basis for this switch is unknown.

Specification is a process by which an undifferentiated cell begins to express markers and to demonstrate behavior unique to one of its derivatives. A specified cell is defined as one from which all progeny will, under normal circumstances, differentiate into a particular cell type. For example, melanoblasts (NC cells specified to become melanocytes) express markers that distinguish them from other NC cells, migrate in a distinct migratory pathway, and will differentiate into melanocytes under normal circumstances. Although melanoblasts can be identified readily by marker expression or migratory behavior, the molecular events that govern the specification of melanoblasts are poorly understood. Signaling by Wnt and BMP molecules seem to govern the lineage switch, with WNT3A driving cells to a melanogenic fate and BMP4 antagonizing melanogenesis (Jin et al., 2001). However, the molecular events that alter gene expression to effect melanoblast specification are unknown.

Microphthalmia-associated transcription factor (MITF) is the earliest expressed marker of melanoblasts identified to date and is essential for melanogenesis. It transactivates the expression of many of the genes required for melanin production and melanocyte differentiation and survival (Levy et al., 2006). In mouse embryos, MITF expression is first observed in migrating melanoblasts just dorsal to the neural tube. The importance of MITF in differentiation of the melanocytic lineage has been demonstrated by genetic mutations in several organisms: null mutations in mice, rats, quail and zebrafish all result in a complete loss of melanocytes (Hodgkinson et al., 1993; Hodgkinson et al., 1998; Mochii et al., 1998; Lister et al., 1999). Conversely, ectopic expression of MITF in several different cell types confers melanocyte-specific properties: NIH3T3 cells adopt a melanocyte-like morphology and express melanocyte-specific marker genes (Tachibana et al., 1996); avian neuroretinal cells transdifferentiate into retinal pigmented epithelium and NC-like melanocytes (Planque et al., 1999; Planque et al., 2004); and when MITF is misexpressed in medaka embryonic stem-like cells, they differentiate into melanocytes (Bejar et al., 2003).

The transcription factors PAX3 and SOX10 cooperate to induce expression of *MITF* in nascent melanoblasts (Watanabe et al., 1998; Bondurand et al., 2000; Potterf et al., 2000). In addition, WNT3A signaling upregulates the expression of *MITF*, and growth in the presence of WNT3A induces melanoblast differentiation in cultured NC cells (Takeda et al., 2000; Jin et al., 2001). However, SOX10 and PAX3 are both expressed and WNT3A is present in the neural tube long before melanoblast migration and *MITF* expression (Bondurand et al., 1998; Kuhlbrodt et al., 1998; Cheng et al., 2000; Jin et al., 2001; Cheung and Briscoe, 2003; McKeown et al., 2005; Otto et al., 2006). Since several molecules known to induce *MITF* expression are present in premigratory NC cells well before *MITF* expression begins, we wondered what prevented *MITF* from being

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expressed in the neural tube and in migrating neural and glial precursors. Answering this question will define the basis for the lineage switch. We hypothesized that a transcriptional repressor prevents the expression of *MITF* in early-migrating NC cells and that this absence of MITF is necessary to allow neural and glial specification in the NC.

The forkhead box transcriptional repressor FOXD3 (Sutton et al., 1996; Freyaldenhoven et al., 1997b; Pohl and Knochel, 2001; Sasai et al., 2001; Yaklichkin et al., 2007) is expressed in the dorsal neural tube during the first wave of NC migration and in migrating neural and glial precursors (Kos et al., 2001). It is downregulated by the time melanoblasts begin migrating. We previously demonstrated that FOXD3 represses melanogenesis in the NC. Experimental downregulation of FOXD3 results in an increase in the number of differentiating melanocytes in quail NC cultures, and in premature dorsolateral migration of chick NC cells (Kos et al., 2001). Conversely, misexpression of FOXD3 in melanoblasts results in a failure of NC cells to enter the dorsolateral pathway (Kos et al., 2001). Based on the necessity of MITF for melanoblast specification, the presence of other MITF-inducing molecules in the early NC, and the fact that FOXD3 represses melanogenesis, we hypothesized that FOXD3 controls the lineage choice between neural/glial and pigment cells by repressing *MITF* during the early phase of NC migration.

In the present study, we show that MITF is required during the earliest stage of melanoblast specification and that loss of MITF in these cells causes them to transdifferentiate into glia. We also show that FOXD3 represses expression of MITF in nascent melanoblasts and in melanoma cells by inhibiting transcription from the *MITF-M* (the melanocyte-specific isoform of *MITF*) promoter. Finally, we show that FOXD3 does not repress transcription of *MITF* by directly binding the *MITF-M* promoter, but instead prevents the binding of PAX3.

MATERIALS AND METHODS

Whole-mount immunostaining

For whole-mount immunolabeling of MITF, White Leghorn chick embryos were fixed in 4% paraformaldehyde (PFA) and incubated for several hours in PBTT (PBS supplemented with 0.1% Tween 20 and 0.5% Triton X-100). They were then incubated in ImageIT FX (Invitrogen) for 2-3 hours. The embryos were then incubated with an anti-MITF antibody (C5+D5; 1:50, Zymed) overnight in PBS containing 3% BSA, followed by overnight incubation with Alexa Fluor 488-conjugated anti-mouse secondary antibody (1:1000, Invitrogen). Embryos were then embedded in paraffin, sectioned and stained with HNK-1 supernatant and Cy5-conjugated goat anti-mouse secondary antibody (1:2000, Jackson).

Immunostaining of cultured cells

Neural tubes from the last-formed somite to somite 8 were dissected from stage 12-13 Japanese quail (*Coturnix japonica*) embryos according to established protocols (Kos et al., 2001). In some experiments, the neural tubes were removed and replated after 24 hours and cultured for an additional 48-72 hours. In other experiments, clusters of melanoblasts that form on the neural tube (Loring et al., 1981) were removed after 48 hours and subcultured. Cells were transfected using Fugene HD (Roche).

For staining with glial marker antibody 7B3 and anti-MITF on cultured cells, NC cells were first fixed in 4% PFA. All subsequent steps were carried out in blocking solution (2% BSA, 0.01 M phosphate buffer pH 7.3, 0.5 M NaCl, 0.1% Na azide, 0.3% Triton X-100). After overnight incubation in 7B3 and anti-MITF (1:100), cells were incubated in blocking solution containing 4% goat serum with Cy5-conjugated goat anti-mouse IgM (μ chain-specific; 1:200, Jackson) and Cy3-conjugated goat anti-mouse IgG (γ chain-specific; 1:500, Jackson) for 30 minutes. hMGFP-positive and -negative cells were counted after imaging.

For staining with anti-MITF alone, NC cells were cultured and transfected as above, or B16-F10 cells (ATCC) were cultured in Opti-MEM I (Invitrogen) supplemented with 2% horse serum and 100 units/ml penicillin/streptomycin (Invitrogen) and transfected with Fugene HD. Cells were fixed with 4% PFA and permeabilized with PBTT. They were then blocked with 3% BSA in PBS, followed by incubation with anti-MITF (1:100) for 1 hour. After washing, the cells were incubated in Alexa Fluor 555-conjugated goat anti-mouse IgG (1:1000, Invitrogen) for 30 minutes.

MITF shRNA

The MITF shRNA and scrambled control constructs were made in psiStrike-hMGFP (Promega), which expresses hMGFP independently under the control of a CMV promoter to mark transfected cells. Oligonucleotides used were MITF-shRNA-F and MITF-shRNA-R for the MITF shRNA, and MITF-scram-F and MITF-scram-R for the scrambled control (see Table 1) Oligonucleotides were hybridized and ligated into the vector according to the manufacturer's protocol. Cell proliferation was monitored by cotransfecting a construct expressing RFP-ligase, which is localized to sites of DNA replication during S phase and visible as puncta in the nucleus (Easwaran et al., 2005). Apoptosis was assayed using Magic Red Caspases 3&7 reagent (Neuromics).

Table 1. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)
MITF-shRNA-F	ACCGAACGAAGAAGAAGATTTAAAGTTCTCTTAAATCTTCTTCTTCGTTCTTTTTC
MITF-shRNA-R	TGCAGAAAAAGAACGAAGAAGAAGATTTAAGAGAACTTTAAATCTTCTTCTTCGTT
MITF-scram-F	ACCGAGATATAGGACTAAAAGAAAGTTCTCTTCTTTTAGTCCTATATCTCTTTTTC
MITF-scram-R	TGCAGAAAAAGAGATATAGGACTAAAAGAAGAGAACTTTCTTTTAGTCCTATATCT
FOXD3-stop-F	TGACTAGTTGACACGG
FOXD3-stop-R	GATCCCGTGTCAACTAGTCA
pMITF-310F	TTTCCTTACTACTTTCCTTAAAAACTTTTAACC
pMITF-200R	GACCTTTTAAGTGATGGGCTG
EMSA-F	TATTAGTATGACTGGAACGAAAGATGAATTGCAAATTAGCCTTGAGCAAA
EMSA-R	TTTGCTCAAGGCTAATTTGCAATTCATCTTTCGTTCCAGTCATACTAATA
MITF-515F	AGTGGCTCAGCACCTTGAAATCCT
MITF+90R	CTTATACGGTTGTGAGAGAAGTGACT
FOX1-mutF	TTTAACCTTAGTGCTTCCAGCTGCCACCATTGTCTATTAGTA
FOX1-mutR	CTAATAGACAATGGTGGCAGCTGGAAGCACTAAGGTTAAAAG
FOX2-mutF	TAAAAGGTCCTTTTGCCAGCTGCAAAAAGCATGACGT
FOX2-mutR	CGTCATGCTTTTTGCAGCTGGCAAAAGGACCTTTTAAG
FOX3-mutF	AGAGCCCTTGTGATGCCAGCTGCACTCTACATGCGTGG
FOX3-mutR	ACGCATGTAGAGTGCAGCTGGCATCACAAGGGCTCTTC

FOXD3 constructs

pFOXD3 was constructed by inserting the coding region of *FOXD3* into the bicistronic vector pMES (Swartz et al., 2001), which expresses EGFP using an IRES. pFOXD3-VP16 was constructed by replacing the C-terminal domain of *FOXD3* from the 3′-most *Kas*I site with the transactivation domain of HSV protein VP16. pFOXD3-ΔC was made by digesting pFOXD3 with *Pvu*II and *Bam*HI and inserting a synthetic oligonucleotide made by hybridizing oligonucleotides FOXD3-stop-F and FOXD3-stop-R (Table 1).

Production of FOXD3-GST

The *Eco*RV/*Not*I fragment of *FOXD3* was cloned into pGEX4T-2 (Amersham). FOXD3-GST was expressed in *Escherichia coli* BL21 grown in 2×YT medium to OD_{600} 0.8. IPTG was then added to 0.3 μM, and the cells were grown at room temperature for an additional 2 hours. After centrifugation the cells were lysed using FastBreak (Promega). The cleared lysate was incubated with glutathione-sepharose 4B (Amersham) and purified FOXD3-GST was eluted with reduced glutathione.

Electromobility shift assay (EMSA)

B16-F10 nuclear extract was obtained by incubating trypsinized washed cells in membrane lysis buffer (10 mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT) on ice for 10 minutes. Igepal CA-630 was added to 1% (v/v) final concentration and the cells were vortexed and centrifuged to pellet nuclei. The nuclei were lysed in nuclei lysis buffer (20 mM Hepes pH 8.0, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 25% glycerol) and the chromatin was sheared by passage through a 26-gauge needle.

A long EMSA probe was produced by PCR using biotin-labeled primers pMITF-310F and pMITF-200R (Table 1). The short EMSA probe containing only the PAX3 site was made by biotin-labeling oligos EMSA-F and EMSA-R (Table 1) with the Biotin 3′ End DNA Labeling Kit (Pierce) according to the manufacturer's protocol. EMSA reactions consisted of 1 μg B16 nuclear extract and 10 fmol biotinylated probe in 10 μl EMSA binding buffer (Guo et al., 2002). In some reactions, unlabeled probe and/or purified FOXD3-GST and/or 200 ng anti-PAX3/7 (C-20; Santa Cruz Biotechnology) were added. The probe was added after a 15-minute incubation on ice, followed by an additional 15 minutes on ice. The reactions were run on a 5% non-denaturing polyacrylamide gel, transferred to a nylon membrane and visualized with the Chemiluminescent Nucleic Acid Detection Module (Pierce).

Cloning of genomic *MITF* and promoter analysis

The *MITF* gene was cloned from a chick lambda genomic library (Stratagene) The sequence fragments were assembled into the complete genomic sequence (GenBank accession number FJ196874). pMITF-Rluc was made by amplifying the chick *MITF* promoter from a genomic subclone by PCR using primers MITF-515F and MITF+90R (Table 1) and cloning the product into pGL4.70 (Promega). pMITF-EGFP was made by replacing the luciferase gene with EGFP. pMITF-EGFP was tested by electroporation into the neural tube of chick embryos at stage 15-16 according to established protocols (Kos et al., 2001; Harris et al., 2008). Potential FOXD3 binding sites [predicted by the MATCH (Kel et al., 2003) transcription factor binding site search program] were mutated by site-directed mutagenesis using FOX-mut primers (Table 1).

B16-F10 cells were transfected as described above using Fugene HD. Plasmids used were pMITF-Rluc and its derivatives, pFOXD3, pFOXD3-VP16 and pFOXD3-ΔC. Other constructs used, but not described above, include CMV-PAX3 (IMAGE clone 6518115), empty pGL4.70, CMV-BF-Rluc [which contains eight copies of the brain factor binding site and a minimal CMV promoter kindly provided by Peter Vogt (Scripps Institute, La Jolla, CA, USA), inserted into pGL4.70] and CMV-Rluc (which contains the CMV promoter inserted into pGL4.70). pCS2-βGal was included as an internal control. *Renilla* luciferase activity was measured 24 hours after transfection using the *Renilla* Luciferase Assay System (Promega) according to the manufacturer's protocol on a Sirius luminometer (Berthold). *Renilla* luciferase data were normalized to β-galactosidase activity measured using ONPG substrate.

GST pulldown

To perform the GST pulldown, 10 μg B16-F10 nuclear extract was incubated with either 5 μg FOXD3-GST or 5 μg GST and 14 μl glutathione-sepharose in EMSA buffer for 3 hours at 4°C. After washing, the sample was eluted by boiling in Laemmli buffer. The samples were analyzed by western blotting using anti-PAX3/7 (C-20) and anti-SOX10 (N-20) antibodies (1:2000, Santa Cruz Biotechnology). The western blot was visualized using an HRP-conjugated donkey anti-goat secondary antibody (1:10,000, Santa Cruz Biotechnology) and chemiluminescence.

RESULTS

MITF expression pattern

Previous results from our laboratory established that in avian NC cells, FOXD3 is expressed only in neural and glial precursors and represses melanoblast specification (Kos et al., 2001). Additionally, MITF is absolutely required for the differentiation of melanocytes (see Introduction). These two observations, taken together, led us to propose that FOXD3 inhibits melanoblast specification by repressing expression of *MITF*. We first determined the MITF expression pattern in chick NC cells in order to compare it with the expression pattern of FOXD3. MITF was first observed sporadically in NC cells as early as Hamburger-Hamilton (HH) stage 15 (Fig. 1A), although such cells were rare. MITF-positive cells accumulated in the migration staging area (MSA), the extracellular wedge between the dorsal neural tube and the medial somite (Weston, 1991), through stage 19 (Fig. 1B,C). At stage 20 (Fig. 1D), the MITF-positive cells in the MSA began to clear and MITF-positive cells could be seen in the dorsolateral migratory pathway. MITF was observed at later stages in cells in the dorsolateral migratory pathway and in the ectoderm (Fig. 1E,F). This is consistent with the timing of dorsolateral migration as observed when melanoblasts are labeled with Smyth line serum or HNK-1 (Erickson et al., 1992; Reedy et al., 1998).

In the chick, FOXD3 is never observed in the dorsolateral migratory pathway, and expression is lost from the neural tube by the time melanoblasts begin migrating (Kos et al., 2001). In the mouse, FOXD3 is expressed in the neural tube and in cells in the dorsolateral migratory pathway, but MITF and FOXD3 are never observed in the same cell (Dottori et al., 2001).

Loss of MITF in NC cells causes them to transdifferentiate into glial cells

Although *MITF* mutants have revealed that MITF is important early in the differentiation of melanocytes, the consequence of the loss of MITF was not clear. Are melanoblasts specified in the absence of MITF but quickly die, or are melanoblast never specified? We hypothesized that the loss of MITF expression in early melanoblasts will cause them to transdifferentiate into glial cells for the following reasons: (1) clonal cultures of NC cells have identified a bipotent precursor capable of giving rise to glia and melanocytes, but no melanocyte-neuron precursor has been identified (Dupin and Le Douarin, 2003); (2) melanocytes or glia isolated from quail embryos will dedifferentiate and give rise to a mixed culture of melanocytes and glia upon treatment with endothelin 3 (Dupin et al., 2000); (3) melanocytic nevus cells can adopt a Schwann cell-like character and express markers for early Schwann cell differentiation (Reed et al., 1999). In addition, MITF upregulates expression of *Tbx2* in melanoblasts, a member of a developmentally important gene family known to maintain cell and tissue identity, suggesting a mechanism for the loss of melanocytes observed in MITF-deficient mice (Carreira et al., 2000).

We made a short hairpin RNA (shRNA) construct to knock down MITF expression incorporated in a vector that also expresses hMGFP to identify transfected cells. We tested the shRNA for function by transfecting the construct into B16-F10 mouse melanoma cells and G361 human melanoma cells (the sequence targets a region of *MITF* that is identical in the chick, human and

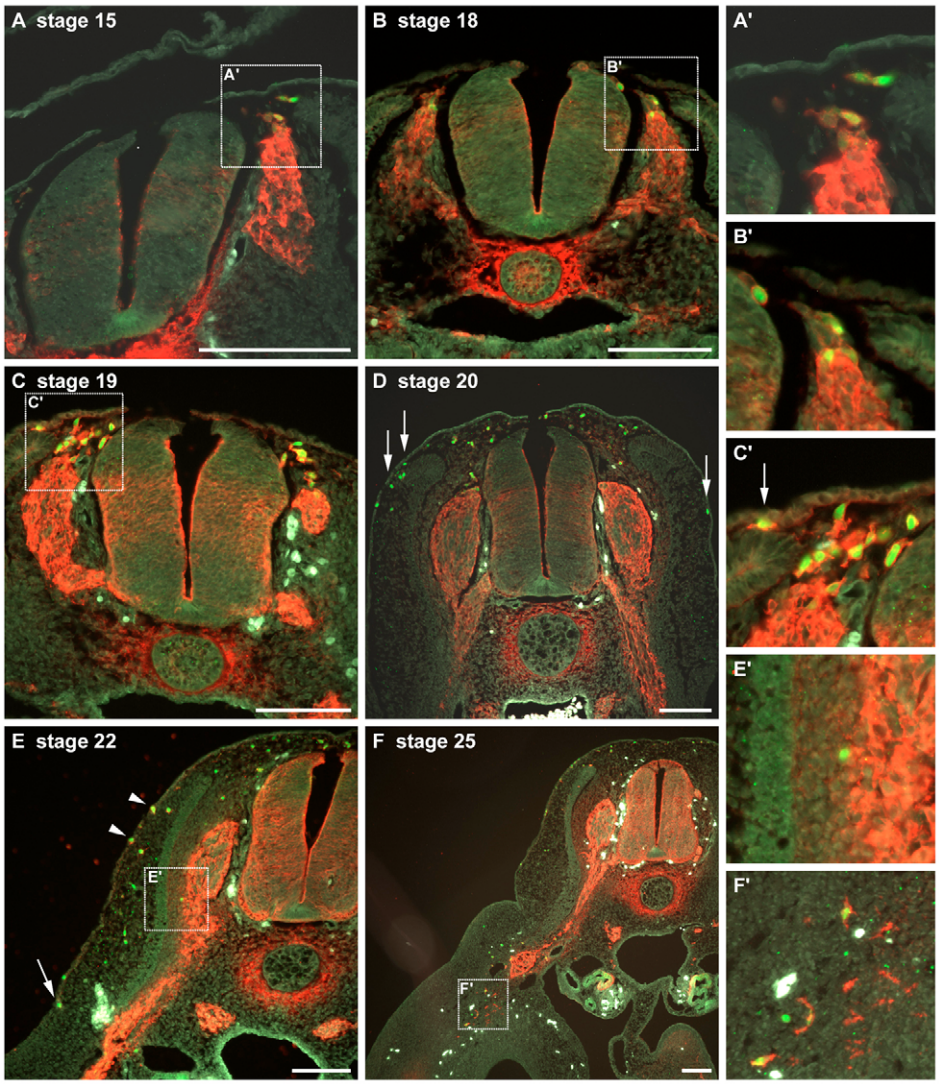

Fig. 1. MITF is expressed early in the migration of chick melanoblasts. (**A-F′**) Chick embryos were labeled with anti-MITF (green), sectioned and then labeled with HNK-1 (neural crest marker, red). The white cells are autofluorescent red blood cells. Images of stages 18-25 (B-F) are at the axial level of the forelimb. The boxed areas in A-C,E,F are shown at higher magnification in A′-C′,E′,F′, respectively. MITF expression is observed as early as stage 15 in the trunk (A) and MITF-expressing cells increase in number and accumulate in the migration staging area (MSA) until stage 19 (B,C). A few melanoblasts have entered the dorsolateral pathway at stage 19 (C′, arrow). By stage 20, the accumulation of MITF-positive cells in the MSA has ceased and cells are observed in the dorsolateral migratory pathway (D, arrows). MITF-positive cells reach the lateral edge of the somite by stage 22 (E, arrow) and some have invaded the ectoderm (arrowheads). MITF-positive cells are found in the mesenchyme of the limb bud at stage 25 (F). A small number of MITF-positive cells are observed in the ventral migratory pathway (E′) at all stages. Scale bars: 10 μm.

mouse genes). hMGFP-positive cells did not stain with an antibody against MITF, whereas the rest of the cells in the culture were MITF positive (see Fig. S1 in the supplementary material).

After confirming the efficacy of the MITF shRNA construct, we used it to knock down expression of MITF in cultured quail melanoblasts derived from melanoblast clusters. After 24 hours in culture, melanoblasts were transfected with either the MITF shRNA construct or with the scrambled control construct. After an additional 48 hours, the cells were fixed and stained with the glial marker 7B3 (Henion et al., 2000) and with anti-MITF antibodies. We also immunolabeled with the Hu antibody (a neural marker), but saw no difference in the number of Hu-positive cells. No hMGFP-positive (i.e. MITF shRNA-positive) cells were stained with the anti-MITF antibody in the shRNA-transfected cultures (Fig. 2C,E), compared with 82% of the hMGFP-negative cells in the same culture and 65-69% of the cells (whether hMGFP negative or positive) in the control-transfected cultures (Fig. 2A,E), indicating that the construct successfully eliminated expression of MITF. In the control-transfected cultures, 26-29% of the cells were 7B3 positive (Fig. 2B,E). In the shRNA-transfected cultures, 75% of the hMGFP-positive cells were 7B3 positive, compared with 16% of the hMGFP-negative cells (Fig. 2D,E). A similar experiment, in which the cells were assayed at 72 hours and the empty shRNA vector was used as a control, yielded comparable results (see Fig. S2 in the supplementary material). Although hMGFP was detectable in many pigmented cells in the control-transfected cultures, hMGFP and pigment granules were never observed in the same cell in the shRNA-transfected cultures (Fig. 2F). Because MITF functions in melanocyte survival (McGill et al., 2002), we assayed some cultures for apoptosis and cell proliferation (see Fig. S2 in the supplementary material). No differences in cell survival or proliferation were detected. Based on these data, we conclude that loss of MITF in cultured melanoblasts shifts the fate of the cells from a melanocyte fate to a glial phenotype.

FOXD3 represses expression of *MITF*

To determine whether FOXD3 can repress expression of *MITF*, we transfected pFOXD3, which simultaneously expresses FOXD3 and EGFP, into B16-F10 mouse melanoma cells. Mouse *Mitf* expression was lost in all EGFP-positive cells (Fig. 3D-F), as assayed by staining with an anti-MITF antibody. Although there was some heterogeneity in staining intensity among cells, all EGFP-negative cells were MITF positive. Transfection with empty pMES did not repress expression of *Mitf* (Fig. 3A-C).

To determine whether FOXD3 could repress expression of *MITF* in avian melanoblasts, cultured quail NC cells were transfected with pFOXD3 or with empty pMES and the cells then labeled with an

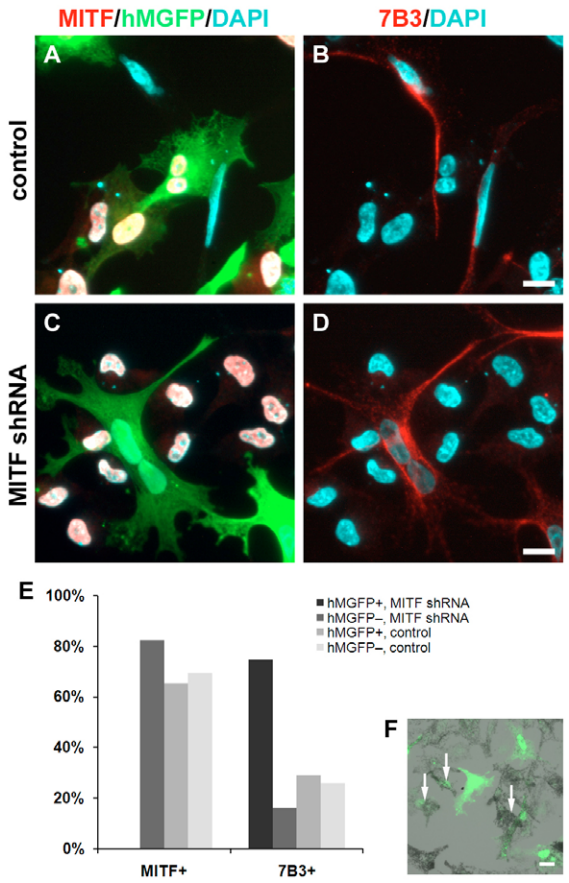

Fig. 2. Loss of MITF in quail melanoblasts causes them to express a glial marker. (**A-D**) Quail melanoblasts were transfected with either the empty shRNA vector (A,B) or the MITF shRNA construct (C,D), both of which also express hMGFP to mark transfected cells. After 48 hours, the cells were stained with anti-MITF (A,C) and 7B3 (B,D), a glial marker. (**E**) No hMGFP-positive (MITF shRNA-positive) cells in the shRNA-transfected cultures were also MITF positive. The hMGFP-positive cells in the shRNA-transfected cultures display an increased number of 7B3-positive cells compared with the non-transfected cells in the same culture (hMGFP negative) and with all the cells in the control-transfected cultures. (**F**) After 72 hours, numerous hMGFP-positive cells produce pigment in the control-transfected cultures (arrows), but no such cells are found in the shRNA-transfected cultures. $P<0.001$ by χ^2 analysis. Scale bars: 10 μm.

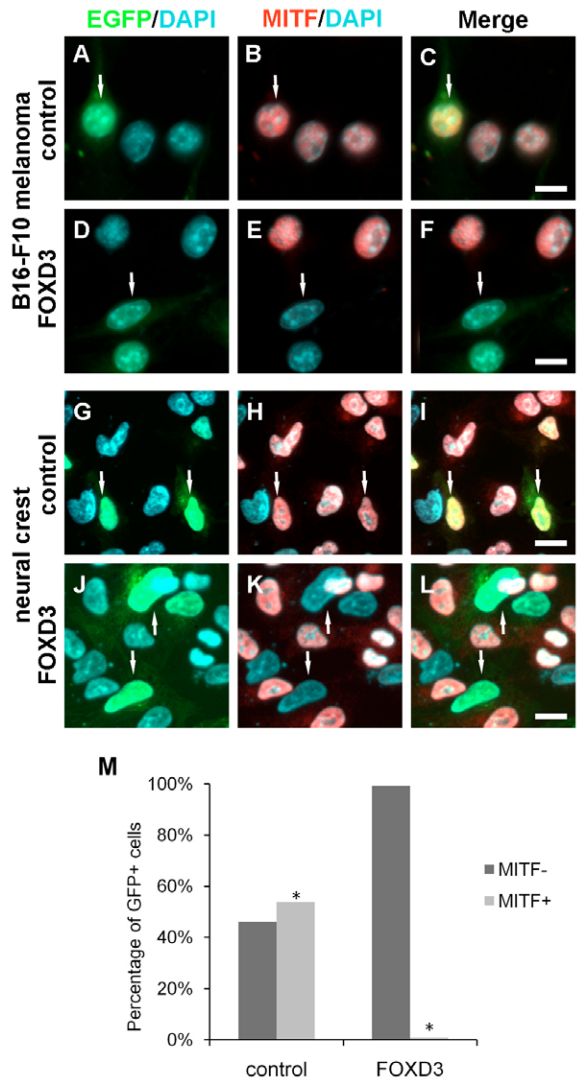

Fig. 3. FOXD3 represses MITF expression in B16-F10 mouse melanoma cells and in cultured quail neural crest cells. (**A-L**) B16-F10 cells (A-F) and quail neural crest cells (G-L) were transfected with constructs expressing either EGFP alone (A-C,G-I), or FOXD3 and EGFP (D-F,J-L). After 24 hours, the cells were fixed and labeled with an anti-MITF antibody (red). In both cell types, MITF immunoreactivity is lost when FOXD3 is expressed. (**M**) Fifty-three percent of EGFP-positive quail neural crest cells were also MITF positive in the control transfection, compared with 0.9% in the FOXD3-transfected cultures. *$P<0.001$ by χ^2 analysis. Scale bars: 10 μm.

anti-MITF antibody. In cultures transfected with the control vector, 53.9% of the EGFP-positive cells were also MITF positive (Fig. 3G-I,M), as compared with only 0.9% in the cultures transfected with pFOXD3 (Fig. 3J-M). This dramatic difference leads us to conclude that FOXD3 represses expression of *MITF* in both B16 melanoma cells and in cultured quail NC cells.

The chick proximal *MITF* promoter contains elements responsible for FOXD3-mediated repression

The mammalian *MITF* gene is expressed as several different tissue-specific isoforms that differ primarily in their use of alternate first exons, with exons 2-9 in common (Steingrimsson et al., 2004; Levy et al., 2006). *MITF-M* is expressed exclusively in NC-derived melanocytes, and its expression is driven by the promoter upstream of exon 1M, which is the 3′-most exon (Fuse et al., 1996; Steingrimsson et al., 2004). We cloned the chick *MITF* gene from a lambda phage library and identified three exon 1s (1D, 1B, 1M). This genomic structure mirrors that of its mammalian counterparts (Fig. 4A), which have several first exons of which exon 1M is the most 3′. An enhancer located 14 kb upstream in the human sequence contains several SOX10 binding sites and functions in expression from the *MITF-M* promoter (Watanabe et al., 1998). We found nothing homologous to this enhancer in the chick sequence, although at the same location we found a ~600 bp region that closely matches a region in the human gene located ~48 kb upstream of

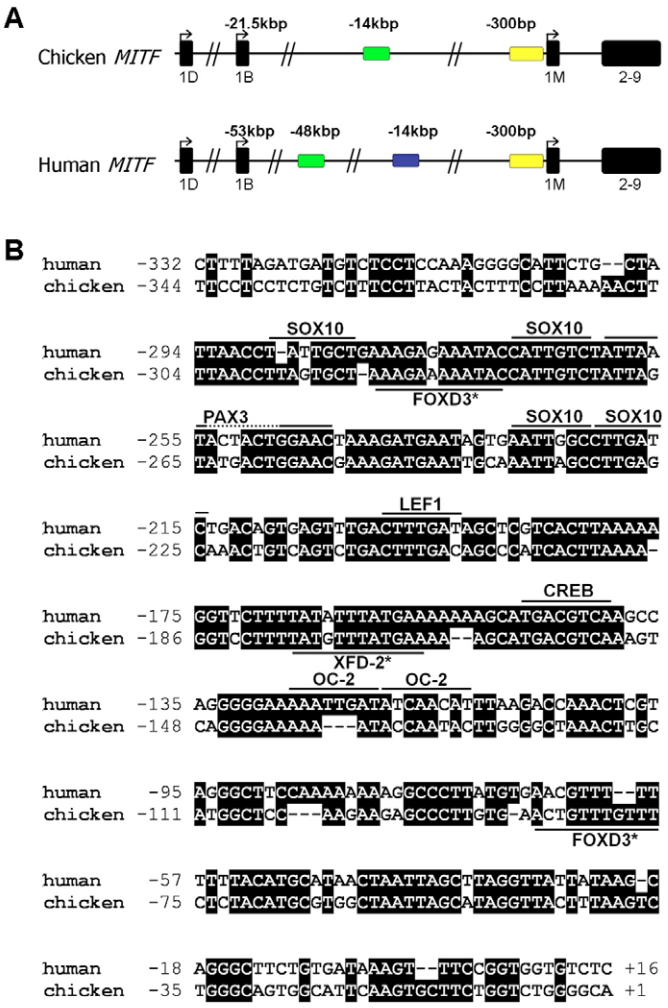

Fig. 4. Genomic structure of chick *MITF* and its promoter. The chick *MITF* gene is similar in structure to its mammalian counterparts. (**A**) Comparison with the human homolog. Exon 1M is the 3′-most first exon in both genes, with exon 1B further upstream, although the distance between them is shorter in the chick than in the human. The melanocyte distal enhancer (MDE) (Watanabe et al., 2002) in the human gene is shown in blue, and another highly conserved region of unknown function is shown in green; the conserved proximal promoter region is in yellow. (**B**) Many of the transcription factor binding sites identified in the proximal promoter of the human sequence are conserved in the chick sequence. Predicted FOXD3 sites in the chick sequence are marked with an asterisk.

exon 1M (Fig. 4A). This region does not contain predicted binding sites for any of the previously identified transcription factors important for expression from the *MITF-M* promoter and its function remains to be determined.

The region of the *MITF-M* promoter from approximately –300 to –150 is highly conserved among mammalian and avian species. It contains the SOX10, PAX3, TCF/LEF1, OC2 (ONECUT2) and CREB binding sites that have been shown to be responsible for expression of *MITF* in NC-derived melanocytes (Steingrimsson et al., 2004; Vance and Goding, 2004; Levy et al., 2006). This region of the chick *MITF* gene contains sequences that match the transcription factor binding sites identified in the mammalian genes (Fig. 4B). To determine whether this region is sufficient for proper expression of *MITF* in the chick embryo, we constructed pMITF-EGFP, which contains the sequence from –515 to +90 around the transcription initiation site (as determined by 5′ RACE, see Fig. S3A in the supplementary material) of the *MITF-M* promoter positioned upstream of EGFP. When this construct was transfected into B16-F10 melanoma cells, we observed strong expression of EGFP. When we electroporated the same construct into the neural tube of stage 15 chick embryos, NC cells in the dorsolateral pathway, which are known to be melanoblasts, were EGFP positive. No EGFP was observed in the neural tube, but a few EGFP-positive cells were observed in the ventral pathway, consistent with the staining pattern observed with the anti-MITF antibody (see Fig. S3B,C in the supplementary material). Therefore, this region of the gene is sufficient to achieve proper expression of MITF in B16 melanoma cells and in melanocyte precursors in the chick embryo.

Next, the same promoter fragment was cloned upstream of the *Renilla* luciferase reporter gene in pGL4.70 to create pMITF-Rluc. pMITF-Rluc was transfected into B16-F10 cells and the *Renilla* luciferase activity measured. When FOXD3 was coexpressed in the assay by cotransfection with pFOXD3, *Renilla* luciferase activity was reduced ~30-fold. We conclude that this proximal region of the *MITF* promoter contains elements responsible for FOXD3-mediated repression of *MITF*.

We used multiple programs to identify potential transcription factor binding sites, but did not find any exact matches to the consensus FOXD3 binding site. However, the online program MATCH (Kel et al., 2003) identified two FOXD3 sites and one related site, XFD-2, that were reasonable matches to the consensus FOXD3 binding site (Fig. 4B). These potential FOXD3 binding sites were mutated by site-directed mutagenesis and the ability of the constructs to respond to FOXD3 was examined, as above, using the *Renilla* luciferase assay. These mutant constructs were transfected into B16-F10 cells, either with or without pFOXD3. Although the overall transcriptional activity of each construct varied somewhat compared with the native promoter, the mutant promoter constructs retained the ability to be repressed by FOXD3 (Fig. 5A). Thus, a FOXD3 binding region could not be identified by transcriptional activity.

FOXD3 does not bind the *MITF-M* promoter in vitro

We wanted to know whether or not this FOXD3-mediated repression of *MITF* is due to a direct interaction of FOXD3 with the *MITF* promoter. B16-F10 nuclear extract was incubated with a probe spanning the conserved region of the *MITF-M* promoter in an electromobility shift assay (EMSA). As expected, a pattern of shifted bands was observed representing different complexes of transcription factors bound to the probe (Fig. 5B). If FOXD3 can bind the *MITF-M* promoter, purified FOXD3-GST added to the reaction would be expected to further shift some or all of the bands owing to the increase in molecular weight. Instead, adding increasing amounts of FOXD3 resulted in the gradual disappearance of some of the bands (Fig. 5B). These data suggest that rather than binding to the DNA directly, FOXD3 is inhibiting the binding of some other factor.

PAX3 and SOX10 are necessary for expression of *MITF*. Loss of either is sufficient to reduce or eliminate expression from the *MITF-M* promoter (Watanabe et al., 1998; Bondurand et al., 2000; Potterf et al., 2000). Likewise, loss of either during development eliminates the melanocyte. We therefore hypothesized that FOXD3 might be repressing expression of MITF by inhibiting the binding of either SOX10 or PAX3. We added anti-PAX3 antibody to the reaction containing the labeled probe plus B16-F10 nuclear extract and found that the same bands that are supershifted with the antibody are lost with the addition of FOXD3. When both FOXD3 and anti-PAX3

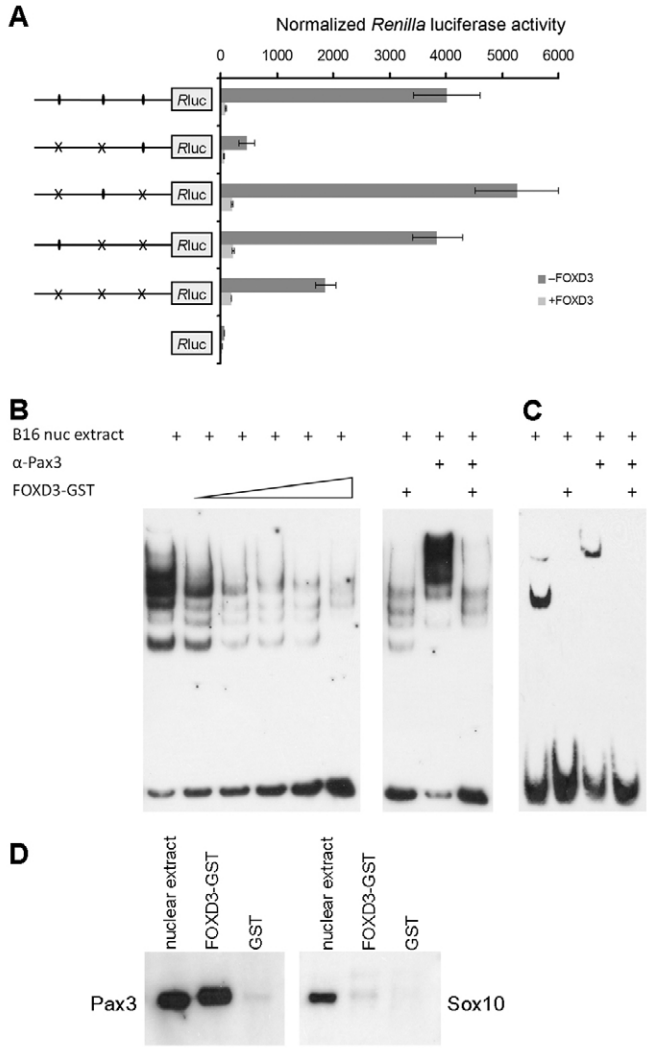

Fig. 5. FOXD3 interacts with PAX3 and prevents it from binding to the *MITF* promoter. (**A**) pMITF-Rluc or a mutant derivative (150 ng), pCS2-βGal (100 ng) and, in some cases, pFOXD3 (250 ng), were transfected into B16-F10 cells. *Renilla* luciferase activity was measured and normalized to β-galactosidase activity. The proximal promoter of the chick *MITF* gene drives expression in B16-F10 cells and that expression is repressed by coexpression of FOXD3. Mutation of the predicted FOXD3 binding sites (the core of the FOXD3 recognition sequence was replaced by *Pvu*II sites) does not alleviate repression. The data are representative of at least four separate trials. Error bars indicate the 95% confidence interval. (**B**) The ability of FOXD3 to bind the *MITF* promoter was analyzed by EMSA. A probe representing a large portion of the *MITF* promoter formed several bands of reduced mobility that correspond to different protein complexes bound to DNA when incubated with B16-F10 nuclear extract. When FOXD3-GST is added in increasing amounts, no further mobility-shifted bands are observed, although the intensity of some of the bands is reduced. An anti-PAX3 antibody added to the reaction further shifts some of the same bands that FOXD3-GST eliminates. When both FOXD3-GST and anti-PAX3 are added together, the band pattern is similar to that when FOXD3-GST alone is added. (**C**) When a shorter probe containing just the PAX3 binding site is used, a single band is observed with nuclear extract alone. Anti-PAX3 further shifts this band. FOXD3-GST added to the reaction completely eliminates the band in the presence or absence of anti-PAX3. (**D**) Western blot analysis of the products of a GST pulldown assay reveals that FOXD3-GST interacts with PAX3 but not SOX10.

were included, the anti-PAX3-supershifted bands are lost and the resulting band pattern is similar to that observed with FOXD3 alone (Fig. 5B,C).

To verify that the affected band was indeed the result of PAX3 binding to the probe, a shorter probe containing only the PAX3 binding site was used. In this case, a single shifted band was observed when B16 nuclear extract alone was used. Addition of anti-PAX3 further retarded the migration of the probe. Addition of FOXD3-GST to the reaction, either with or without anti-PAX3, eliminated the shifted band (Fig. 5B,C). Based on these data, we suggest that FOXD3 does not bind to the *MITF-M* promoter directly, but instead represses transcription by inhibiting the binding of PAX3. Because multiple bands are observed in the EMSA with the longer probe, and because FOXD3 eliminates more than one band, it is possible that FOXD3 also prevents the binding of other factors in addition to PAX3. These were not investigated further because loss of PAX3 alone is sufficient to prevent expression of *MITF* (Kamaraju et al., 2002) and because the factors responsible for these additional bands are not known.

Both FOXD3 and PAX3 are known to physically interact with other transcription factors (Magnaghi et al., 1998; Wiggan et al., 1998; Hollenbach et al., 1999; Guo et al., 2002; Hollenbach et al., 2002). After observing that FOXD3 appears to prevent the binding of PAX3 to the *MITF* promoter, we wished to determine whether FOXD3 could physically interact with PAX3 in the absence of DNA. We therefore incubated B16-F10 nuclear extract with purified FOXD3-GST and glutathione-sepharose in a GST pulldown assay. PAX3 was detectable by western blot, but SOX10 was not (Fig. 5D). We conclude that FOXD3 can physically and specifically interact with PAX3.

Based on our model, FOXD3 represses expression of *MITF* by inhibiting the binding of PAX3. We tested two predictions based on this model. The first is that mutants of FOXD3 that do not contain the transcriptional repression domain will repress *MITF* similar to wild-type FOXD3. To this end, we constructed two FOXD3 mutants (Fig. 6A) in which the C-terminal domain, which contains the transcriptional repression motif (Yaklichkin et al., 2007), was removed and replaced with the VP16 transactivation domain (pFOXD3-VP16), or was truncated (pFOXD3-ΔC). Control experiments verified that FOXD3-VP16 functions as a transcriptional activator and that FOXD3-ΔC does not alter transcriptional activity when bound to the DNA (see Fig. S4 in the supplementary material). Both mutants repressed transcription similar to wild-type FOXD3 (Fig. 6B). The second prediction is that excess PAX3 in the cell should bind all the FOXD3 and the remaining unbound PAX3 should be able to bind the *MITF* promoter and activate transcription. When we overexpressed PAX3 in B16-F10 cells that express FOXD3, the FOXD3- mediated repression of *MITF* was reduced significantly (Fig. 6C).

Although FOXD3 does not directly bind to the region of the *MITF-M* promoter tested here, we cannot rule out the possibility that there are functional FOXD3 binding sites elsewhere in the gene. However, as the constructs tested here are capable of driving expression in the correct cell-specific manner, and FOXD3 represses expression from this region of the gene, we conclude that the region tested is regulated by the interaction of FOXD3 with PAX3 rather than with the DNA.

DISCUSSION

In this report, we have investigated the regulation of the transcription factor MITF, which is required for melanoblast differentiation. First, we show that MITF is expressed shortly after melanoblasts emigrate from the neural tube, at a time consistent with an early, if not primary, role in melanoblast specification. We demonstrate that

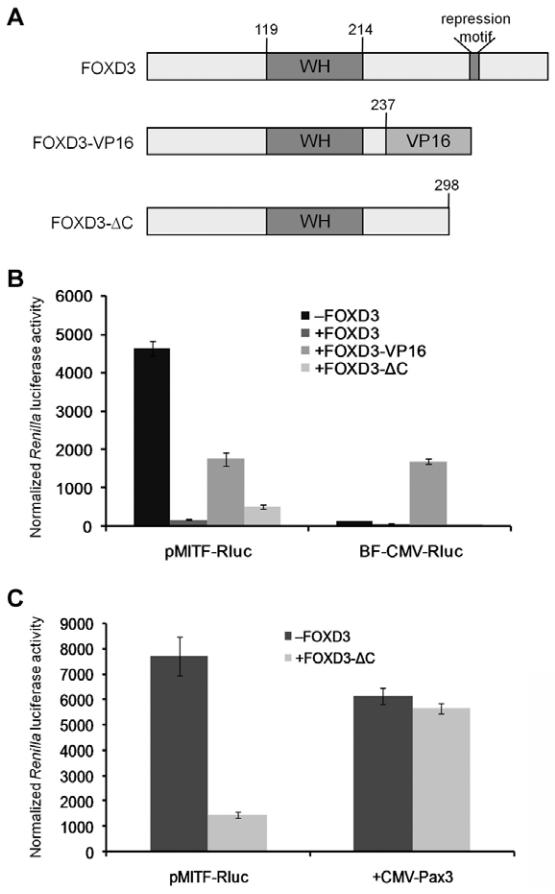

Fig. 6. FOXD3 mutants with altered function repress *MITF*. (**A**) FOXD3 and mutant derivatives FOXD3-VP16 and FOXD3-ΔC (see text for details). WH, winged-helix domain. (**B**) pMITF-Rluc or BF-CMV-Rluc [which contains eight brain factor binding sites that are known to bind FOXD3 (Freyaldenhoven et al., 1997a; Freyaldenhoven et al., 1997b) plus a minimal CMV promoter] was transfected into B16-F10 cells with the FOXD3 variants. Both mutants repress transcription from the *MITF-M* promoter. (**C**) B16-F10 cells were transfected with pMITF-Rluc (250 ng), pCS2-βGal (100 ng) and, in some cases, pFOXD3-ΔC (100 ng) or CMV-PAX3 (40 ng). *Renilla* luciferase activity was normalized to β-galactosidase activity. PAX3 reduces FOXD3-ΔC-mediated repression of expression from the *MITF* promoter. The data are representative of three separate trials. Error bars indicate the 95% confidence interval.

MITF is crucial for specification of melanoblasts by showing that loss of MITF affects cell fate, causing the cells to switch to a glial phenotype. Second, we show that FOXD3 represses expression of *MITF* and that this expression is crucial in regulating the switch between glial and melanocyte lineages. Third, we show that FOXD3 represses *MITF* by binding to PAX3 and preventing it from activating *MITF* transcription. Based on previous experiments demonstrating that FOXD3 regulates the switch to a melanogenic lineage, we present a model to explain the progressive specification of NC cells during development.

Mechanism of repression

Perhaps the most surprising result from the present work is the mechanism by which FOXD3 represses *MITF*. FOXD3 is known to bind DNA to regulate expression in some circumstances. A Groucho-interacting motif in the C-terminal domain of the protein is responsible for transcriptional repression in at least some cases in which FOXD3 binds DNA (Yaklichkin et al., 2007). In our studies, removal of this motif is not sufficient to eliminate the repression of *MITF* by FOXD3. Mouse FOXD3 is also known to interact with the transcription factor OCT4 (POU5F1) through its DNA-binding domain in the absence of DNA (Guo et al., 2002). Here we have shown that FOXD3 can also interact with PAX3 to prevent its binding to the *MITF* promoter. This mechanism for inhibition of PAX3 function is not without precedent. PAX3 is known to interact with other transcription factors through its paired domain and homeodomain to either enhance (Lang and Epstein, 2003) or repress (Magnaghi et al., 1998; Wiggan et al., 1998; Hollenbach et al., 1999; Hollenbach et al., 2002) transcription. It is not yet known which domains of FOX3 and PAX3 are involved in their interaction.

Whether FOXD3 similarly inhibits the binding of PAX3 to other promoters is not known. In the previously reported example of OCT4 and FOXD3, OCT4 was able to inhibit the binding of FOXD3 to the mouse *Foxa1* and *Foxa2* promoters, but not the osteopontin enhancer (Guo et al., 2002). We predict that this FOXD3-mediated inhibition of PAX3 binding is similarly sequence dependent.

Although *MITF* is upregulated in the nascent NC by both PAX3 and SOX10, neither one alone is sufficient for *MITF* expression. Loss of Sox10 in zebrafish results in downregulation of *mitfa* and loss of melanophores and can be rescued by misexpression of *mitfa* (Elworthy et al., 2003). No MITF-positive cells are observed in SOX10-negative mouse NC cells (Hou et al., 2006). Downregulation of PAX3 by IL6RIL6 in B16/F10.9 cells results in loss of *Mitf* expression and loss of melanogenic enzymes (Kamaraju et al., 2002). Likewise, mutations in either the *Pax3* or *Sox10* gene result in a pigment cell phenotype very similar to that of *Mitf* mutants (Tachibana et al., 2003). Clearly, loss of PAX3-mediated transcriptional activation is sufficient to reduce *MITF* expression enough to dramatically affect melanoblast specification and differentiation.

In a recent report by Ignatius and colleagues (Ignatius et al., 2008), zebrafish Foxd3 was shown by EMSA to bind to the *mitfa* promoter, contrary to our findings. They did not report any functional analysis of Foxd3 on the *mitfa* promoter, comparable to the *Renilla* luciferase assays that we report here. The apparent contradiction between their results and ours might be due to differences between species or differences in experimental conditions. Under some conditions, we were also able to see some binding of FOXD3-GST to the *MITF* promoter, but further experimentation showed that this binding was spurious. It will be interesting to see whether functional analyses of the zebrafish *mitfa* promoter, similar to those we have undertaken for the chick FOXD3 mutants, will reveal whether the repression of *MITF* by FOXD3 is broadly similar across all vertebrates.

A model for specification

We previously demonstrated that FOXD3 represses melanogenesis and that FOXD3-expressing NC cells, whether naturally expressed or experimentally induced, do not exhibit melanocyte characteristics (Kos et al., 2001). In conjunction with the fact that other *MITF*-inducing factors (SOX10, PAX3, Wnt signaling) are expressed at the time when neural and glial cells are emigrating from the neural tube, we propose a model for the delayed expression of *MITF* and the fate switch that occurs as NC migration progresses. According to our model, expression of *MITF* is essential for specification of NC cells as melanoblasts. Although components sufficient for *MITF* expression are present in early-migrating NC cells, *MITF* is not expressed owing to the presence of FOXD3. Therefore, these cells

are not capable of melanogenesis. The later downregulation of *FOXD3* permits melanogenesis and, in effect, switches these cells from a neural/glial fate to a melanocyte fate.

The signals responsible for *FOXD3* downregulation are not known. Preliminary in silico analysis (Ovcharenko et al., 2004) of the genomic *FOXD3* locus revealed several evolutionary conserved regions containing binding sites for transcription factors known to be expressed in the NC. Of particular interest are PAX3 and MSX1 because both have been shown to be genetically upstream of *FOXD3* (Dottori et al., 2001; Tribulo et al., 2003). In addition, MSX1 initiates the expression of *Pax3* in *Xenopus* (Monsoro-Burq et al., 2005). Also present are binding sites for TCF/LEF and SMAD family transcription factors. LEF1 is a binding partner of β-catenin and is a target of Wnt signaling. The BMP signaling pathway alters gene expression through SMAD family transcription factors. *FOXD3* was demonstrated in a recent report to be regulated by both Wnt and BMP signaling in the chick (Pohl and Knochel, 2001; Taneyhill and Bronner-Fraser, 2005). *FOXD3* is induced during the initial generation of the NC by intermediate levels of BMP signaling (Tribulo et al., 2003), whereas low or high levels inhibit NC formation. It is therefore possible that a change in the level of BMP signaling is responsible for the downregulation of *FOXD3* and for the consequent melanoblast specification.

It was recently reported that *foxd3* is repressed by Hdac1 in zebrafish and that the prolonged expression of *foxd3* in *colgate* (*hdac1*$^{-/-}$) mutants results in reduced melanogenesis (Ignatius et al., 2008). However, the authors did not determine whether Hdac1 acts directly at the *foxd3* locus or further upstream in the genetic cascade.

MITF: master regulator of melanoblast specification?

Melanoblasts are specified from the NC in a progressive fashion (Weston, 1991; Le Douarin and Dupin, 2003) in which initially pluripotent cells are progressively fate restricted through successive cell divisions until a specified melanoblast is generated. The molecular events governing melanoblast specification are not well understood. It was known that downregulation of *FOXD3* is necessary for the specification of melanoblasts and that misexpression of FOXD3 is sufficient to direct NC cells to a neural/glial fate (Kos et al., 2001), but the downstream targets of FOXD3 that mediate molecular changes during specification were not known. Based on the work presented here and by Ignatius and colleagues (Ignatius et al., 2008), we now know that *MITF* is one of those downstream targets. The repression of *MITF* by FOXD3 is sufficient to explain how FOXD3 represses melanogenesis, as MITF is required for the expression of many melanogenic genes. However, it is not known whether FOXD3 represses dorsolateral migration through MITF as well. When we electroporated our MITF shRNA construct into the neural tubes of chick embryos, we saw numerous GFP-positive cells migrating in the dorsolateral pathway (data not shown). In addition, data from our laboratory has shown that misexpression of EDNRB2 can drive non-melanogenic avian NC cells into the dorsolateral pathway (Harris et al., 2008), suggesting that pathfinding ability and lineage specification might be separable characteristics of NC cells. FOXD3 can repress both melanogenic and pathfinding characteristics of melanoblasts, so we suggest in our model that FOXD3 lies upstream of two gene networks: one, headed by *MITF*, that drives the expression of genes responsible for melanogenesis, and another that is responsible for the ability of melanoblasts to exploit the dorsolateral pathway. Further experiments to determine which other genes are regulated by FOXD3 and which gene products are responsible for the migratory properties of melanoblasts will be required to confirm this model.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/cgi/content/full/136/11/1849/DC1

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