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DEVELOPMENT AND DISEASE

Transcriptional regulation of *mitfa* accounts for the *sox10* requirement in zebrafish melanophore development

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SUMMARY

The transcription factor Sox10 is required for the specification, migration and survival of all nonectomesenchymal neural crest derivatives including melanophores. *sox10*$^{-/-}$ zebrafish lack expression of the transcription factor *mitfa,* which itself is required for melanophore development. We demonstrate that the zebrafish *mitfa* promoter has sox10 binding sites necessary for activity in vitro, consistent with studies using mammalian cell cultures that have shown that *Sox10* directly regulates *Mitf* expression. In addition, we demonstrate that these sites are necessary for promoter activity in vivo. We show that reintroduction of *mitfa* expression in neural crest cells can rescue melanophore development in *sox10*$^{-/-}$ embryos. This rescue of melanophores in *sox10*$^{-/-}$ embryos is quantitatively indistinguishable from rescue in *mitfa*$^{-/-}$ embryos. These findings show that the essential function of *sox10* in melanophore development is limited to transcriptional regulation of *mitfa*. We propose that the dominant melanophore phenotype in Waardenburg syndrome IV individuals with *SOX10* mutations is likely to result from failure to activate *MITF* in the normal number of melanoblasts.

Key words: Zebrafish, *Danio rerio*, Neural crest, Fate specification, Melanocyte, *sox10*, *colourless*, *mitf*, *nacre*, Survival, Transcriptional regulation

INTRODUCTION

During vertebrate embryogenesis neural crest cells delaminate from the dorsal neural tube, migrate throughout the body and differentiate into a remarkably diverse array of cell types (Le Douarin and Kalcheim, 1999; Smith et al., 1994). These neural crest fates can be broadly categorized as ectomesenchymal and nonectomesenchymal. The ectomesenchymal neural crest fates include cranial cartilage and fin mesenchyme (in fish) whereas the nonectomesenchymal fates include neurons and glia of the peripheral nervous system and pigment cells. Defects in neural crest development are a significant cause of human disease and the resulting syndromes are termed neurocristopathies (Le Douarin and Kalcheim, 1999). One such neurocristopathy is Waardenburg's Syndrome, in which individuals have dominant pigmentation defects. Waardenburg's Syndrome types IIa and IV are associated with haploinsufficiency for the transcription factor genes *MITF* and *SOX10*, respectively (Pingault et al., 1998; Tachibana et al., 1994; Tassabehji et al., 1994).

Zebrafish or mice homozygous for mutations in the *sox10* transcription factor gene [previously called *colourless* (*cls*) in zebrafish] have severe defects in all the nonectomesenchymal neural crest cell fates (Dutton et al., 2001; Herbarth et al., 1998; Kelsh and Eisen, 2000; Southard-Smith et al., 1998). In *cls/sox10*$^{-/-}$ zebrafish many neural crest cells undergo apoptotic cell death near the neural tube. They do so after failing to become specified or migrate (Dutton et al., 2001). Apoptotic death of cells on the neural crest migration pathways has also been reported in *Sox10*$^{-/-}$ mouse embryos (Kapur, 1999). In *cls/sox10*$^{-/-}$ zebrafish and in *Sox10*$^{-/-}$ mouse embryos some of the nonectomesenchymal neural crest cell fates such as melanocytes (also called melanophores in zebrafish) and peripheral glia are essentially absent whereas others such as the dorsal root ganglia sensory neurons do form but with fewer and disorganized cells (Britsch et al., 2001; Kelsh and Eisen, 2000; Sonnenberg-Riethmacher et al., 2001; Southard-Smith et al., 1998).

In mammalian systems it has been shown that in the case of the peripheral glia a major requirement of *Sox10* is to directly regulate expression of terminal differentiation genes such as P_0 and *Cx32* (*Gjb1* – Mouse Genome Informatics) (Bondurand et al., 2001; Peirano et al., 2000). *Sox10* also regulates expression of the neuregulin receptor gene, *Erbb3* (Britsch et al., 2001). Signaling through *Erbb3* promotes acquisition of the glial fate

by neural crest cells and is required for peripheral glial cell migration and survival (Paratore et al., 2001). However it is not known whether this *Erbb3* regulation by *Sox10* is direct.

In the case of melanocytes it is not clear to what extent *Sox10* is required for direct transcriptional regulation of terminal differentiation genes. One plausible hypothesis is that in the melanocyte lineage *Sox10* is simply required for direct activation of the *Mitf* transcription factor gene, which then acts as a master regulator of melanocyte cell fate. Evidence for the pivotal role of Mitf in melanocyte development has come from studies with both mammals and zebrafish. In mammalian systems Mitf transactivates expression of melanogenic enzyme genes such as *Tyr* and *Trp1* as well as the receptor tyrosine kinase gene *Kit*. Kit signaling potentiates Mitf activity in turn and is also required for melanocyte proliferation and survival in both zebrafish and mice (Goding, 2000; Hemesath et al., 1998; Hou et al., 2000; Opdecamp et al., 1997; Parichy et al., 1999; Steel et al., 1992; Yasumoto et al., 1997). In mammalian systems Mitf also directly regulates expression of the antiapoptotic factor gene *Bcl2* required for melanocyte survival (McGill et al., 2002). Similarly, ectopic *mitfa* (previously known as *nac*) expression in zebrafish embryos causes ectopic expression of the melanogenic enzyme gene *dct* (Lister et al., 1999). Forced expression of *Mitf* in cultured mouse fibroblasts can induce some aspects of melanocyte differentiation and ectopic *nac/mitfa* expression in zebrafish embryos causes ectopic abnormal melanized cells (Lister et al., 1999; Tachibana et al., 1996).

In cultured mammalian cells, Sox10 can directly activate expression from the mouse or human *Mitf* promoter (Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). *Sox10*$^{-/-}$ zebrafish or mouse embryos lack *Mitf* expression and *nac/mitfa*$^{-/-}$ zebrafish or *Mitf*$^{-/-}$ mouse embryos have melanocyte defects at least as severe as those in *Sox10*$^{-/-}$ mutant embryos (Dutton et al., 2001; Hodgkinson et al., 1993; Lister et al., 1999; Potterf et al., 2001). Thus loss of *mitf* expression would be sufficient to account for the melanocyte defect in *sox10*$^{-/-}$ mutant embryos.

Although regulation of *Mitf* expression is clearly part of the *Sox10* requirement in the melanocyte lineage it is also possible that there are other essential *Sox10* functions in this lineage. Unlike zebrafish, mice show a haploinsufficiency phenotype when heterozygous for *Sox10* mutations (Britsch et al., 2001). This phenotype includes a mild melanocyte deficiency. Melanocytes from these mice show little reduction in *Mitf* expression and yet transiently have a severe reduction in expression of the melanogenic enzyme gene *Dct* (Potterf et al., 2001). In addition, *Sox10* can transactivate expression from a *Dct* promoter construct in cultured cells (Britsch et al., 2001; Potterf et al., 2001). These findings could suggest a requirement for *Sox10* in regulating *Dct* expression that is not mediated via *Mitf*. A critical question is whether any such non-*Mitf*-mediated effects of *Sox10* have a significant role in melanocyte development.

We show here that the direct regulation of *Mitf* expression by *Sox10* reported in cultured mammalian cells also occurs in developing melanophores in zebrafish embryos. We extend these studies by showing that forced expression of *nac/mitfa* in the neural crest of *cls/sox10*$^{-/-}$ mutant zebrafish embryos is sufficient to rescue melanophore development. Furthermore, we show that rescue of melanophores in *cls/sox10*$^{-/-}$ embryos is quantitatively indistinguishable from rescue in *nac/mitfa*$^{-/-}$ embryos. Together, these data suggest that regulation of *nac/mitfa* by *cls/sox10* can fully account for the *cls/sox10* requirement in the zebrafish melanophore lineage.

MATERIALS AND METHODS

Fish

Embryos were obtained through natural crosses and staged according to Kimmel et al. (Kimmel et al., 1995). We used three *cls* alleles (*m618*, *t3* and *tw2*) which all have equally strong phenotypes (Dutton et al., 2001). We used the *nac*^{w2} allele (Lister et al., 1999) except where it is stated that we used the *nac*^{b692} allele (Lister et al., 2001).

PCR genotyping

Embryos were tested for heterozygosity or homozygosity of the *nac* mutations by PCR on genomic DNA. The *nac*^{w2} test used PCR primers cattcttgggttcatggatgcaggac and ggcaggcttgaggggcaggag followed by digestion with *Dra*I which cleaves the mutant allele (Lister et al., 1999). The *nac*^{b692} test used PCR primers gcagaagtaagagccctggc and acggatcatttgacttgggaattaaag followed by digestion with BsrD1 which cleaves the mutant allele.

Whole-mount in situ hybridization

Embryos were processed for whole-mount in situ hybridization with *nac/mitfa* digoxigenin-labeled riboprobe as in Dutton et al. (Dutton et al., 2001).

Cell culture and luciferase assays

Promoter truncations were made from plasmid nac>luc (Dorsky et al., 2000) using the restriction sites indicated in Fig. 3. Mutation to the M1 sequence (see Table 1) was made by replacing the *Spe*1-*Age*1 region with the annealed oligonucleotides ctagtaaccc-atcgtcggcggtaggcttttgtcgaatcgga and ccggtccgattcgacaaaagcctacc-gccgacgatgggtta. The QuickChange kit (Stratagene) was used for mutation to the M2, M3 or M4 sequences (see Table 1). pCS2sox10 and pCS2sox10L142Q were constructed by cloning the *Cla*I/*Xba*I fragments from hs>*sox10* or hs>*sox10L142Q* (Dutton et al., 2001) into pCS2+.

Transfection of NIH3T3 cells and luciferase assays were performed essentially as described previously (Lister et al., 2001). Transfections were performed on cells in 24-well dishes, with each well receiving 300 ng *sox10* expression vector, 100 ng *mitfa*-promoter>luciferase reporter, and 50 ng pCMV-βgal as internal control.

Electrophoretic mobility shift assays

The pCls/Sox10-GST expression plasmid was constructed by cloning a PCR product amplified from hs>*sox10* (Dutton et al., 2001) (using primers cgggatcccgatgtcggcggaggagcacag and gcgaattcaggaaccc-ggtttgccgtt) between the BamH1 and EcoR1 sites of pGEX-3X (Amersham Pharmacia). Cls/Sox10-GST fusion protein was expressed in *E. coli* BL21(RIL) (Stratagene) and affinity purified using glutathione agarose following the manufacturer's instructions (Amersham Pharmacia). Approximate relative concentrations of Cls/Sox10-GST protein were estimated by comparison to a dilution series of bovine serum albumin (BSA) standard using Coomassie-stained polyacrylamide gel electrophoresis (PAGE). The SpeAge DNA probe was oligonucleotides ctagtaacccatcgtcaaaga-ggcttttgtcgaatcgga and ccgattcgacaaaagcctctttgagacgacgatgggttact annealed together, end labeled with [γ_^{32}P] ATP using T4 polynucleotide kinase and native PAGE purified. For electrophoretic mobility shift assays (EMSA), a 20 μl reaction mixture (containing Cls/Sox10-GST protein, 2000 c.p.m. of [^{32}P]DNA, 330 ng poly(dG-dC)•poly(dG-dC) (Amersham Pharmacia), 50 mM NaCl, 3% (w/v) Ficol (Amersham Pharmacia), 10 mM HEPES (pH 7.9), 5 mM $MgCl_2$,

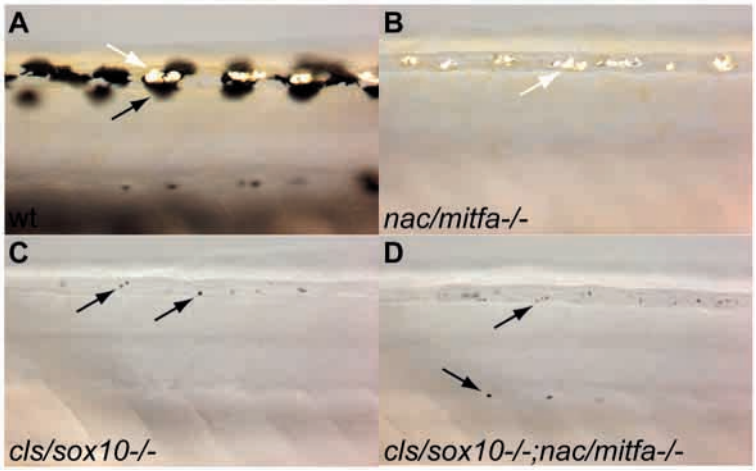

Fig. 1. Pigment cell defects in *cls/sox10*$^{-/-}$, *nac/mitfa*$^{-/-}$ and *cls/sox10*$^{-/-}$;*nac/mitfa*$^{-/-}$ mutant embryos. Lateral views of the dorsal trunk of 3 dpf wild type (A), *nac/mitfa*$^{b692/b692}$ (B), *cls/sox10*$^{t3/t3}$ (C) and *cls/sox10* $^{t3/t3}$;*nac/mitfa* $^{b692/b692}$ (D) embryos. Wild-type embryos have large flat melanophores (black arrow), *cls/sox10*$^{-/-}$ and *cls/sox10*$^{-/-}$;*nac/mitfa*$^{-/-}$ embryos have a few tiny rounded melanophores (black arrows), but *nac/mitfa*$^{-/-}$ embryos lack melanophores. Iridophores (white arrows) are not reduced in *nac/mitfa*$^{-/-}$ embryos but are severely reduced in *cls/sox10*$^{-/-}$ and *cls/sox10*$^{-/-}$;*nac/mitfa*$^{-/-}$. Double mutant embryos were identified by PCR genotyping.

0.5 mM EDTA, 0.1 mM dithiothreitol, 1 mg/ml BSA and sometimes specific competitor oligonucleotide) was incubated on ice for 20 minutes then electrophoresed on a gel (5% (w/v) polyacrylamide (37:1), 0.5% TBE) at 120 V, at 4°C, for 3 hours. Dried gels were exposed to Biomax MS film (Kodak) for autoradiography.

Embryo injections

One- or two-cell stage embryos were injected with plasmids and/or RNA using standard methods as in Dutton et al. (Dutton et al., 2001). RNA was produced using the mMESSAGE mMACHINE kit (Ambion) from hs>*sox10* or hs>*sox10(L142Q)* templates (Dutton et al., 2001) linearized with Asp718.

Plasmids nac>GFP and nac>nac were generated as follows: the SV40 promoter of pGL3-Promoter (Promega) was replaced by a fragment of the *mitfa* promoter from the plasmid pNP-P+ (Lister et al., 2001) via *Sal*I and *Hin*dIII sites to make pGL3-NP. The luciferase gene of pGL3-NP was then excised with *Hin*dIII and *Xba*I and replaced with GFP (from pCS2-BE-GFP) or *mitfa* (from pHS-MT3A.1) (Lister et al., 1999). Plasmids nac>GFP and nac>nac were mutated to the M1, M2, M3, M4, M1M3 and M3M4 sequences by replacing the appropriate *nac* promoter fragments with those from the corresponding Fspnac>luc constructs (see above). cls>nac was constructed by PCR amplifying the *nac/mitfa* coding sequence with N-terminal myc tags from pHS-MT3A.1 (Lister et al., 1999) and cloning the PCR fragment into the Xba1 site of CS26.8. CS26.8 has the Sal1-Xba CMV promoter fragment of pCS2+ replaced by 6.8 kb of sequence extending upstream from the *cls/sox10* translational start site.

GFP fluorescence was scored in gastrulas using an MZ12 dissecting microscope (Leica). GFP fluorescence was scored in 24 hours-post-fertilization (hpf) embryos using an Axioplan 2 microscope (Zeiss) with the embryos anesthetized using 0.003% MS222 (Sigma) and mounted between bridged coverslips. Melanophore rescue was scored at 48 hpf or at 72 hpf in the case when the *cls/sox10*$^{-/-}$ iridophore phenotype was also being scored. Melanophores were only scored as rescued if they had wild-type morphology.

Photography

Live embryos were anesthetized with 0.003% MS222 (Sigma), mounted in methylcellulose or between bridged coverslips and photographed using a Spot digital camera mounted on an Eclipse E800 microscope (Nikon) or Axioplan 2 microscope (Zeiss) with DIC optics. Embryo whole-mount in situ hybridization specimens were photographed using a Spot digital camera mounted on a MZ12 microscope (Leica) with epi-illumination. The GFP fluorescent gastrula image was captured using a LSM510 confocal microscope (Zeiss) with DIC and confocal fluorescence images superimposed.

RESULTS

nac/mitfa$^{-/-}$;*cls/sox10*$^{-/-}$ double mutant embryos have minute melanophores

cls/sox10$^{-/-}$ embryos show no *nac/mitfa* expression detectable by in situ hybridization and *nac/mitfa*$^{-/-}$ embryos have a complete absence of melanophores (Dutton et al., 2001; Lister et al., 1999). Although *cls/sox10*$^{-/-}$ embryos never have any normal melanophores, they do have a small number of tiny rounded cells expressing melanin (Kelsh et al., 1996; Kelsh et al., 2000). To determine whether these melanized cells result from residual *mitfa* expression below the sensitivity of in situ hybridization, we examined *nac/mitfa*$^{-/-}$;*cls/sox10*$^{-/-}$ double mutant embryos.

Intercrossing *nac/mitfa*$^{+/b692}$;*cls/sox10*$^{+/t3}$ parents gave embryos with three different phenotypes: wild-type (Fig. 1A), embryos with the typical *nac/mitfa*$^{-/-}$ phenotype of complete loss of all melanophores but no reduction in iridophores (Fig. 1B), and embryos with the typical *cls/sox10*$^{-/-}$ phenotype of a severe reduction in all pigment types including iridophores but a persistence of tiny melanized spots (Fig. 1C,D). All embryos classified as having a *cls* phenotype were similar, having at least five tiny melanophores, and importantly we did not observe any embryos with both a complete absence of these tiny melanized cells and loss of iridophores. The numbers of embryos with these specific phenotypes, 168 wild type: 59 *nac*: 67 *cls*, fits the ratio of 9:3:4 expected if embryos with the genotype *cls*$^{-/-}$;*nac*$^{-/-}$ exhibit the *cls* phenotype (p=0.64 by chi-square analysis). We confirmed that some of these embryos were indeed *nac/mitfa*$^{-/-}$ homozygotes by PCR genotyping. Of the 27 such embryos we tested, four were *nac/mitfa*$^{-/-}$;*cls/sox10*$^{-/-}$ (Fig. 1D), 14 were *nac/mitfa*$^{+/-}$;*cls/sox10*$^{-/-}$ and nine were *nac/mitfa*$^{+/+}$;*cls/sox10*$^{-/-}$.

To test whether this surprising result was also observed with other *nac/mitfa* and *cls/sox10* alleles we crossed *nac/mitfa*$^{w2/w2}$;*cls/sox10*$^{+/tw2}$ and *nac/mitfa*$^{+/w2}$;*cls/sox10*$^{+/tw2}$ parents. This gave 36 wild-type embryos, 39 embryos with the typical *nac/mitfa*$^{-/-}$ phenotype and 18 embryos with a severe reduction in all pigment types. These 18 embryos each had at least five tiny melanophores and PCR genotyping showed that of the 17 such embryos we tested, 12 were *nac/mitfa*$^{-/-}$;*cls/sox10*$^{-/-}$ and five were *nac/mitfa*$^{+/-}$;*cls/sox10*$^{-/-}$.

These results suggest that the less severe melanophore defect observed in *cls/sox10*$^{-/-}$ embryos as compared to *nac/mitfa*$^{-/-}$

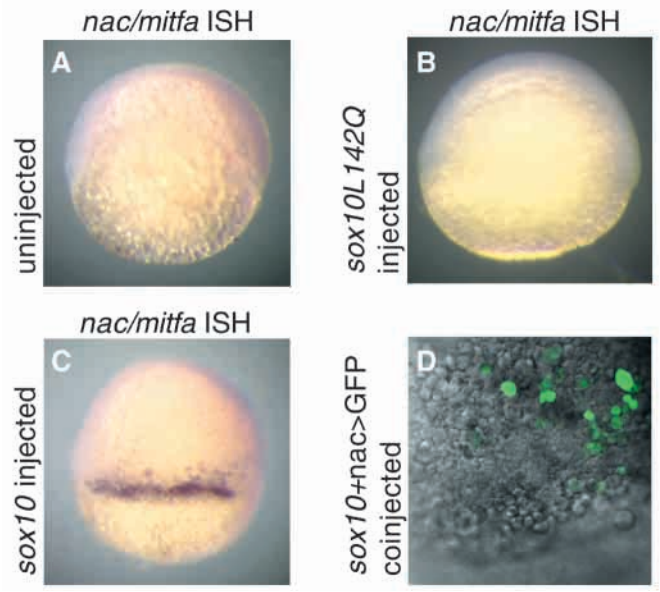

Fig. 2. Precocious *nac/mitfa* expression in 6 h.p.f embryos following injection with *cls/sox10* RNA. Lateral views of uninjected (A), *cls/sox10L142Q* RNA injected (250 pg per embryo; B) and *cls/sox10* RNA injected (250 pg per embryo; C) 6 hpf embryos following *in situ* hybridization with a *nac/mitfa* probe. Spots and/or patches of *nac/mitfa* expression were detected in 39% of *cls/sox10* RNA injected embryos (*n*=136) but not in any of the uninjected embryos (*n*=58) nor in any of the *cls/sox10L142Q* RNA injected embryos (*n*=92). (D) Superimposed fluorescent confocal and DIC images of an animal/lateral view of a 6 hpf embryo coinjected with *cls/sox10* RNA (250 pg per embryo) and nac>GFP reporter plasmid (150 pg per embryo) show cells with GFP fluorescence. GFP fluorescence was observed in 75% (*n*=224) of embryos coinjected with *cls/sox10* RNA and nac>GFP.

embryos cannot be attributed to residual *nac/mitfa* expression in *cls/sox10*$^{-/-}$ mutant embryos.

Ectopic *cls/sox10* expression in the embryo can induce ectopic *nac/mitfa* expression

In zebrafish embryos *cls/sox10* has been shown to be necessary for *nac/mitfa* expression (Dutton et al., 2001). In mammalian cells *Sox10* has also been reported to directly activate *Mitf* expression (Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). We used forced ectopic expression of *cls/sox10* to test whether *cls/sox10* was also sufficient to induce *nac/mitfa* expression in the zebrafish embryo. Embryos injected with *cls/sox10* RNA were probed for *nac/mitfa* expression by in situ hybridization. *cls/sox10* RNA injection induced *nac/mitfa* transcription at 6 hpf (Fig. 2C), 12 hours before the onset of endogenous *nac/mitfa* expression (Lister et al., 1999). The induced *nac/mitfa* expression was unevenly distributed as patches or spots, with the pattern of expression varying greatly from embryo to embryo. Ectopic *nac/mitfa* expression was not seen when embryos were injected with point mutant *cls/sox10L142Q* RNA (Fig. 2B), the mutation in the *cls*^{m618} allele (Dutton et al., 2001). These results show that *cls/sox10* can induce *nac/mitfa* expression in embryonic contexts other than the neural crest cells where *nac/mitfa* is normally expressed.

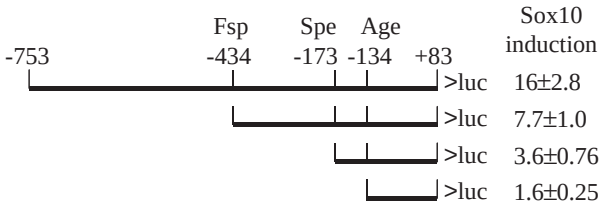

Fig. 3. Distribution of cls/sox10 response elements in the nac/mitfa promoter. Schematic diagram represents the 836 b.p. *nac/mitfa* promoter luciferase reporter construct (nac>luc) and derivative constructs with truncations of the *nac/mitfa* promoter. Co-transfection of the cls/sox10 expression plasmid pCS2sox10 into NIH3T3 cells with these constructs led to higher levels of induction with the full length promoter and incrementally lower levels with incremental 5′ truncations of the promoter. Sox10 induction was measured as: (luciferase activity with co-transfected pCS2sox10)/(luciferase activity with co-transfected pCS2sox10L142Q). The values shown are means±s.e.m. from at least four repetitions of each experiment.

nac/mitfa upstream sequence responds to *cls/sox10*

To establish whether *cls/sox10* acts directly or indirectly on *nac/mitfa* transcription it was necessary to identify sequence elements in the *nac/mitfa* promoter mediating *cls/sox10* responsiveness. Dorsky et al. (Dorsky et al., 2000) showed that an 836 b.p. *nac/mitfa* promoter (extending from –753 to +83 b.p. relative to the transcriptional start site) was able to direct expression from a GFP reporter plasmid (nac>GFP) to melanophores. We found that this reporter responded to *cls/sox10* RNA coinjection (Fig. 2D), but not *cls/sox10L142Q* RNA coinjection (*n*=146 embryos), in gastrula embryos, recapitulating the ectopic expression of *nac/mitfa*. This indicates that this 836 b.p. region of the *nac/mitfa* promoter contains sequence elements responsible for the *cls/sox10* response in zebrafish embryos. We used a cell line transfection assay to further localize sequence elements in the *nac/mitfa* promoter responsible for *cls/sox10* responsiveness. In transfected NIH3T3 cells a luciferase reporter construct with the 836 b.p. *nac/mitfa* promoter (nac>luc) was activated by a co-transfected zebrafish *cls/sox10* expression construct (pCS2sox10). All *cls/sox10* transfections were compared with the baseline value obtained by co-transfection with the point mutant construct pCS2sox10L142Q. Successive 5′ truncations of the *nac/mitfa* promoter resulted in incremental decreases in the level of induction in response to *cls/sox10* (Fig. 3). Thus elements conferring response to *cls/sox10* appeared to be widely distributed throughout the 836 b.p. *nac/mitfa* promoter. We chose to focus on the most proximal regions that conferred *cls/sox10* response. A promoter with a 5′ truncation to the *Spe*1 site (at –173 b.p.) could still respond to *cls/sox10*, and was significantly different than control transfection (p=0.01), but further truncation to the *Age*1 site (at –134 b.p.) prevented significant response (indistinguishable from control, *P*>0.1). These results tentatively localized a sequence element(s) responsible for some of the response to *cls/sox10* to this 41 b.p. region of the *nac/mitfa* promoter.

Cls/Sox10 binds *nac/mitfa* promoter sequences in vitro

The 41 b.p. critical region of the *nac/mitfa* promoter between the *Spe*1 and *Age*1 sites contains a sequence element (site S1)

Table 1. Potential Sox binding sites in the *nac*/*mitfa* promoter and mutated versions

Position in promoter	Oligonucleotides used to test Sox10 binding	
–157 b.p.	S1	5′ccatcgtCTCAAAGaggctt3′ 3′gtagcaGAGTTTCtccgaaa5′
	M1 (mutated S1)	5′ccatcgtCGGCGGTaggctt3′ 3′gtagcaGCCGCCAtccgaaa5′
–247 b.p.	S2	5′gagaacaAACAATGttttat3′ 3′tcttgtTTGTTACaaaatac5′
	M2 (mutated S2)	5′gagaacaACCGCGGttttat3′ 3′tcttgtTGGCGCCaaaatac5′
–262 b.p.	S3	5′tgctagtGATTGTAtgccgg3′ 3′cgatcaCTAACATacggcct5′
	M3 (mutated S3)	5′tgctagtGGTACCAtgccgg3′ 3′cgatcaCCATGGTacggcct5′
–284 b.p.	S4	5′ttagaccAACAGTGctagtg3′ 3′atctggTTGTCACgatcact5′
	M4 (mutated S4)	5′ttagaccACCCGGGctagtg3′ 3′atctggTGGGCCCgatcact5′

similar to the consensus sox binding site WWCAAWG (Mertin et al., 1999) (Table 1). We used an in vitro DNA binding assay to establish whether Cls/Sox10 could be acting by binding to site S1. An EMSA showed that a Cls/Sox10-GST fusion protein (with Cls/Sox10 residues 1-189) binds to the *Spe*1-*Age*1 fragment that contains site S1 (Fig. 4B). However, when site S1 is mutated this binding is greatly reduced. Similarly, binding to the *Spe*1-*Age*1 fragment is effectively competed by a 19 b.p. double-stranded oligonucleotide with the site S1 sequence but not by an equivalent oligonucleotide with the site S1 mutated (Fig. 4C).

The sequence upstream of the *Spe*1 site contains additional sequence elements similar to the consensus sox binding site (sites S2, S3 and S4; Table 1). We tested 19 b.p. double-stranded oligonucleotides corresponding to these sequence elements for their ability to compete with the *Spe*1-*Age*1 fragment in the EMSA binding assay with Cls/Sox10-GST fusion protein. These oligonucleotides also effectively competed for Cls/Sox10-GST protein binding whereas equivalent oligonucleotides with the Sox consensus binding sites mutated did not compete as effectively (Fig. 4D). However, the short and degenerate nature of the sox binding site consensus sequence means that it occurs frequently, making it difficult to identify functional sox response elements by sequence alone. It was thus important to test what relevance these binding sites had for *cls/sox10* responsiveness in vivo.

A Cls/Sox10 binding site is needed for the *cls/sox10* response of the *nac/mitfa* promoter

In order to test whether sox binding sites S1, S2, S3 or S4 could act as *cls/sox10* response elements, we mutated each of them in a luciferase reporter construct (Fspnac>luc) with a *nac/mitfa* promoter truncated to the Fsp1 site (–434 b.p.). The mutations used were the same as those used to disrupt binding to these sites in vitro (see Fig. 4; Table 1). In co-transfection assays with pCS2Sox10, mutation of site S1 (to make FspM1nac>luc) was found to reduce the plasmid's response to *cls/sox10* in NIH3T3 cells (Fig. 5). Mutation of sites S2, S3 or S4 or both S3 and S4 had only a slight effect in this assay (Fig. 5). Similarly, mutation of both S1 and S3 did not have more of an effect than mutating S1 alone (Fig. 5).

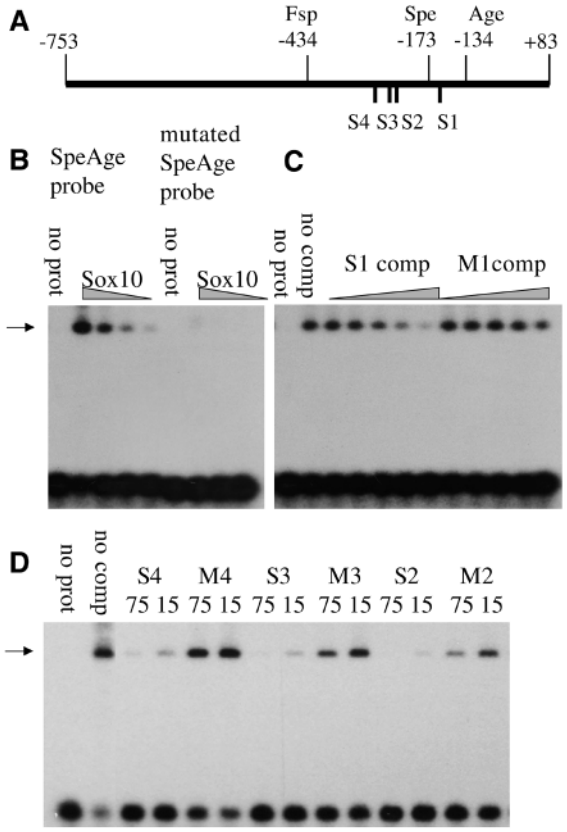

Fig. 4. Electrophoretic mobility shift assays (EMSA) showing binding of Cls/Sox10-GST fusion protein to sites in the *nac/mitfa* promoter. (A) Schematic diagram of the 836 b.p. *nac/mitfa* promoter showing the positions of the putative sox binding sites S1, S2, S3 and S4. (B) The *Spe*1-*Age*1 fragment of the *nac/mitfa* promoter (SpeAge probe) shows a band of reduced electrophoretic mobility (black arrow) with ~20 nM, 10 nM, 5 nM and 2.5 nM Cls/Sox10-GST fusion protein (Sox10) which is not seen without the Cls/Sox10-GST protein (no prot). When site S1 is mutated in this DNA fragment (mutated SpeAge probe) binding under these same Cls/Sox10-GST protein concentrations is greatly reduced. (C) Binding of ~10 nM Cls/Sox10-GST protein to the *Spe*1-*Age*1 fragment of the *nac/mitfa* promoter is effectively competed by an oligonucleotide with site S1 (S1 comp) but not by the mutated site oligonucleotide M1 (M1 comp). Shown are binding reactions with a serial five-fold dilution series of this competitor oligonucleotide giving 0.13 to 75 pmoles per reaction and also controls with no specific competitor (no comp) and with no Cls/Sox10-GST protein (no prot). (D) Binding of ~10 nM Cls/Sox10-GST protein to the *Spe*1-*Age*1 fragment of the *nac/mitfa* promoter is effectively competed by oligonucleotides with binding sites S2, S3 or S4 but less effectively by the mutated versions M2, M3 or M4. Shown are binding reactions with 75 pmoles (75) or 15 pmoles (15) of these competitor oligonucleotides and also controls with no specific competitor (no comp) and with no Cls/Sox10-GST protein (no prot).

We used an in vivo melanophore rescue assay to test whether these sox binding sites controlled expression in neural crest cells in the zebrafish embryo. As shown by Dorsky et al. (Dorsky et al., 2000), a plasmid with the *nac/mitfa* cDNA under control of the 836 b.p. *nac/mitfa* promoter (nac>nac) can rescue melanophores when injected into *nac/mitfa*$^{-/-}$ embryos. Mutation of sox binding site S1 in this plasmid's promoter

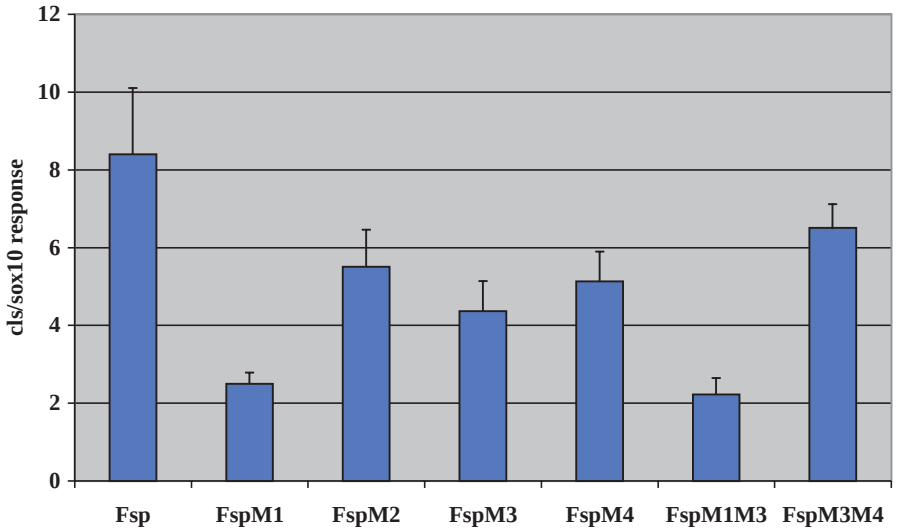

Fig. 5. *cls/sox10* response of *nac/mitfa* promoter luciferase reporter constructs with mutated Cls/Sox10 binding sites (see Table 1). *cls/sox10* response is measured as: (luciferase activity in NIH3T3 cells co-transfected with reporter and pCS2Sox10)/(activity with reporter and pCS2Sox10L142Q). Mean values from five or more repetitions of each experiment are shown for Fspnac>luc (Fsp), FspM1nac>luc (FspM1), FspM2nac>luc (FspM2), FspM3nac>luc (FspM3), FspM4nac>luc (FspM4), FspM1M3nac>luc (FspM1M3) and FspM3M4nac>luc. Bars indicate s.e.m.

(making M1nac>nac) greatly reduced the plasmid's effectiveness at melanophore rescue (Table 2). Mutation of sox binding site S3 (making M3nac>nac) caused a less dramatic reduction in effectiveness, whereas mutations of sites S2 or S4 (making M2nac>nac and M4nac>nac) had little effect (Table 2). We combined the S1 and S3 mutations (making M1M3nac>nac) which had more effect than mutating S1 alone (P<0.0001 by chi-square analysis). Combining the S3 and S4 mutations (making M3M4nac>nac) had no more effect than mutating S3 alone (P>0.5). These results show that the ability of a binding site to act as a response element in vivo is not accurately reflected by binding affinity in vitro, because sites S2 and S4 compete effectively for Cls/Sox10-GST protein binding in vitro (Fig. 4C) and yet show little evidence of being *cls/sox10* response elements in vivo (Table 2). Presumably other characteristics such as the context of the binding site in the promoter are just as important in defining a site as active in vivo.

We used the GFP reporter plasmid nac>GFP to further test the effect of mutating sox binding sites S1 and S3. As shown by Dorsky et al. (Dorsky et al., 2000), the 836 b.p. *nac/mitfa* promoter in nac>GFP directs expression of GFP to prospective pigment cells in injected embryos at 24 hpf. This assay differs from the nac>nac melanophore rescue assay in that it assesses promoter function in melanoblasts at an earlier developmental stage. Mutation of sox binding site S1 in nac>GFP (making M1nac>GFP) markedly reduced GFP reporter expression (Table 3). Mutation of site S3 (making M3>GFP) also reduced GFP reporter expression and combining the two mutations (making M1M3nac>GFP) had more effect than mutating S1 alone. The mutant rescue and GFP expression assays are different and so it is not prudent to compare the magnitude of the effects observed with each. However, both assays show similar trends in which mutating site S1 has a major effect, mutating site S3 has less of an effect, and mutating both sites has more effect than mutating S1 alone.

These results demonstrate that the *nac/mitfa* promoter contains a Cls/Sox10 protein binding site (site S1) that acts as a *cls/sox10* response element and that is necessary for adequate *nac/mitfa* expression in developing melanophores in the zebrafish embryo. The Cls/Sox10 protein binding site S3 also contributes to activation of *nac/mitfa* expression but to a lesser extent. These results suggest that in zebrafish neural crest cells in the embryo, Cls/Sox10 activates *nac/mitfa* expression by directly binding to the *nac/mitfa* promoter.

Table 2. Melanophore rescue at 48 hpf from *nac/mitfa* promoter constructs with mutated Cls/Sox10-binding sites

Injected plasmid*	Number of *nac/mitfa*$^{-/-}$ embryos injected	Number of embryos with one or more rescued melanophores (%)
nac>nac	658	373 (57)
M1nac>nac	568	50 (8.8)
M2nac>nac	429	235 (55)
M3nac>nac	405	94 (23)
M4nac>nac	371	163 (44)
M3M4nac>nac	338	75 (22)
M1M3nac>nac	485	10 (2.1)

*50 pg plasmid injected per embryo.

Table 3. GFP expression in wild type embryos from a *nac/mitfa* promoter construct is reduced by mutating a Cls/Sox10-binding site in the promoter of the plasmid

Injected plasmid*	Number of embryos injected	Number of embryos with GFP fluorescent crest cells at 24 hpf (%)
nac>nac	132	87 (66)
M1nac>nac	179	29 (16)
M3nac>nac	139	55 (40)
M1M3nac>nac	153	10 (6.5)

*25 pg plasmid injected per embryo.

Forced *nac/mitfa* expression rescues the *cls/sox10*$^{-/-}$ melanophore phenotype

cls/sox10$^{-/-}$ mutant embryos lack *nac/mitfa* expression and *nac/mitfa*$^{-/-}$ mutant embryos lack melanophores (Dutton et al., 2001; Lister et al., 1999). This prompted us to investigate whether activation of *nac/mitfa* transcription could account for

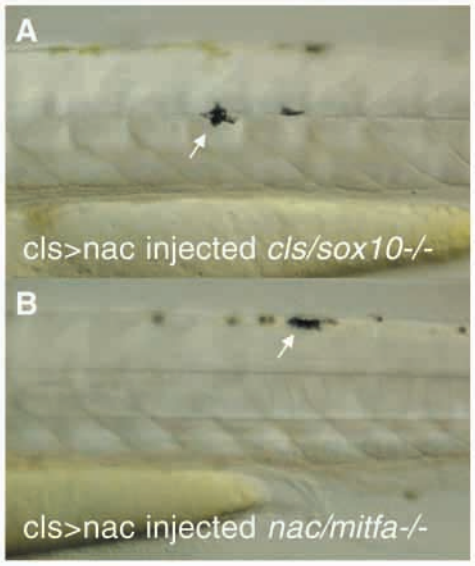

Fig. 6. In vivo melanophore rescue by forced *nac/mitfa* expression in the premigratory neural crest. Lateral views of the posterior trunk of 2 dpf, *cls/sox10*$^{-/-}$ (A) and *nac/mitfa*$^{-/-}$ (B) embryos that had been injected with cls>nac (15 pg per embryo). Rescued melanophores (arrows) have normal position and morphology.

the required role of *cls/sox10* in the melanophore lineage. We tested this by forcing *nac/mitfa* expression in *cls/sox10*$^{-/-}$ embryos, thus bypassing the role of *cls/sox10* in activating *nac/mitfa* expression. Because ectopic expression of *mitf* can confer some melanophore characteristics upon other cell types (Lister et al., 1999; Tachibana et al., 1996), we wanted to express *nac/mitfa* specifically in neural crest cells. We constructed a plasmid with the *nac/mitfa* cDNA under control of a *cls/sox10* promoter (cls>nac). The *cls/sox10* promoter used had previously been shown to target expression of a GFP reporter plasmid to the endogenous sites of *cls/sox10* expression such as neural crest and otic vesicle (T.J.C., J. Dutton and R.N.K., unpublished). Injected cls>nac was able to rescue melanophores with normal morphology and migratory ability in *cls/sox10*$^{-/-}$ mutant embryos and in *nac/mitfa*$^{-/-}$ mutant embryos (Fig. 6). In both genotypes, and in agreement with previous rescue studies of *mitf/nac*$^{-/-}$ (Lister et al., 1999), only a few melanophores were rescued in each embryo, presumably because of the highly mosaic distribution of injected DNA typical for zebrafish injection experiments. These results show that reintroduction of *nac/mitfa* expression rescues the differentiation, migration and survival deficiencies of *cls/sox10*$^{-/-}$ neural crest cells in the melanophore lineage. We were also able to rescue melanophores by expression of *nac/mitfa* using a hsp70 promoter construct (Lister et al., 1999) (data not shown).

We tested whether forced expression of *nac/mitfa* was as effective at rescuing melanophores in *cls/sox10*$^{-/-}$ embryos as in *nac/mitfa*$^{-/-}$ embryos. We injected cls>nac into embryos from intercrossed *cls/sox10*$^{+/-}$;*nac/mitfa*$^{+/-}$ double heterozygous parent fish to compare rescue in *cls/sox10*$^{-/-}$ and *nac/mitfa*$^{-/-}$ siblings that were laid, injected and raised together. Because both *cls/sox10*$^{-/-}$ and *nac/mitfa*$^{-/-}$ embryos have melanophore defects, we used the iridophore phenotype of the *cls/sox10*$^{-/-}$ embryos to distinguish them from embryos

Table 4. Melanophore rescue from forced *nac/mitfa* expression in embryos from intercrossed *cls/sox10*$^{+/-}$,*nac/mitfa*$^{+/-}$ double heterozygous parent fish

	Number of embryos injected* with cls>nac	Number of mutant embryos with one or more rescued melanophores (%)
Wild-type embryos	450	Not applicable
Embryos with melanophore and iridophore defects	210	68 (32)
Embryos with melanophore but not iridophore defects	161	48 (30)

*15 pg plasmid injected per embryo.

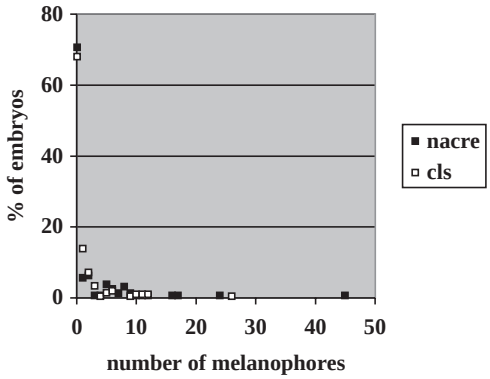

Fig. 7. Melanophore rescue by forced *nac/mitfa* expression in the neural crest of embryos from intercrossed *cls/sox10*$^{+/-}$,*nac/mitfa*$^{+/-}$ double heterozygous parent fish. The plot shows what number of melanophores were rescued in what percentage of *cls/sox10*$^{-/-}$ embryos (white squares) and embryos mutant for *nac/mitfa* but not *cls/sox10* (black squares). The numbers of each class of embryo are shown in Table 4. A Mann-Whitney rank sum test shows no significant difference in the extent of rescue of these two classes of mutant (P=0.876).

mutant for *nac/mitfa* alone (see Fig. 1). As mentioned above, double homozygous *cls/sox10*$^{-/-}$;*nac/mitfa*$^{-/-}$ embryos have melanophore and iridophore defects as in *cls/sox10*$^{-/-}$ embryos and this is reflected in the ratio of phenotypes (Table 4). The *cls/sox10*$^{-/-}$ embryos were rescued to the same extent as the embryos mutant for *nac/mitfa*$^{-/-}$ alone, both in terms of the proportion of embryos showing any rescued melanophores and in terms of the number of rescued melanophores per embryo (Table 4, Fig. 7). This result indicates that in the melanophore lineage, *cls/sox10* is required only to induce *nac/mitfa* expression.

DISCUSSION

cls/sox10$^{-/-}$*; nac/mitfa*$^{-/-}$ embryos have a less severe melanophore phenotype than *nac/mitfa*$^{-/-}$

Previous reports indicated that *nac/mitfa*$^{-/-}$ embryos lack all melanophores whereas *cls/sox10*$^{-/-}$ embryos still have a few tiny, rounded, melanized cells that fail to migrate (Kelsh and Eisen, 2000; Kelsh et al., 2000; Lister et al., 1999). We report

here that the presence of these melanized cells cannot be attributed to putative residual *nac/mitfa* expression in *cls/sox10*$^{-/-}$ embryos because they are also found in *cls/sox10*$^{-/-}$*; nac/mitfa*$^{-/-}$ embryos. The stronger phenotype of *nac/mitfa*$^{-/-}$ embryos may, therefore, imply the presence of a *cls/sox10*-dependent activity that inhibits melanophore development. Obviously, in normal development any such effect must be greatly outweighed by the positive activation of melanophore development mediated by *cls/sox10*. The source of any such inhibitory activity is completely unknown. However, *nac/mitfa*$^{-/-}$ embryos have an increased number of iridophores (Lister et al., 1999) and so it is conceivable that there might be some mechanism for mutual repression between pigment cell types. Sox10 is expressed in neural crest lineages other than that giving rise to melanophores, and perhaps the inhibitory activity functions to prevent expression of melanogenic genes in these cell types.

Role of *sox10* in nonectomesenchymal crest fate specification

Several groups have shown that *Sox10* can directly activate *Mitf* expression in cultured mammalian cells (Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). We found that the zebrafish *nac/mitfa* promoter is also directly activated by zebrafish Cls/Sox10 and that this direct regulation is necessary for expression from the zebrafish *nac/mitfa* promoter in neural crest cells in the developing embryo. Most significantly we found that this activation of *nac/mitfa* expression can account quantitatively for all of the *cls/sox10* requirement in the melanophore lineage. Studies in zebrafish and in mice have revealed defects in neural crest cell fate specification, migration, survival and differentiation in *sox10* mutants. We have previously proposed that the complex phenotype of *cls/sox10* mutants might be explained by a primary defect in specification of nonectomesenchymal crest fates, with defects in migration, survival and differentiation being secondary consequences of this (Dutton et al., 2001; Kelsh and Raible, 2002). Our demonstration here that *cls/sox10* directly activates *nac/mitfa*, a key gene in melanophore fate specification, and that this is vital for melanophore rescue in *nac/mitfa* mutants, is clearly consistent with our model.

Although not usually interpreted in the same way, the mouse *Sox10* mutant phenotype is plainly consistent with the model proposed. For example, the recent demonstration that *Mitf* regulates the antiapoptotic gene *Bcl2* provides a molecular explanation for the apoptosis of melanoblast progenitors in *Sox10* mutants (McGill et al., 2002). Furthermore, in mice the regulation of *Erbb3* (directly or indirectly) by *Sox10* (Britsch et al., 2001) provides evidence that *Sox10* regulates glial fate specification, because neuregulin signaling has been shown to direct neural crest stem cells to a glial fate (Shah and Anderson, 1997; Shah et al., 1994).

At first glance, our findings with the melanophore lineage contrast with the body of work establishing that *Sox10* directly activates a variety of differentiation genes in developing glia. However, these findings are consistent with the observation that *cls/sox10* expression is downregulated in melanoblasts but retained in developing peripheral glia (Dutton et al., 2001), and suggests that in addition to its roles in nonectomesenchymal fate specification, *sox10* is also required for glial cell differentiation.

Only a subset of *sox10*-expressing neural crest cells express *mitfa* and become melanophores. Dorsky et al. (Dorsky et al., 2000) showed that wnt signaling also directly activated *nac/mitfa* expression. These findings are consistent with a model for *cls/sox10* function in the melanophore lineage in which *sox10* is required in conjunction with Wnt signaling to activate *nac/mitfa* expression in neural crest cells (Kelsh and Raible, 2002). *nac/mitfa* then in turn specifies the melanophore fate by activating expression of differentiation genes such as *dct* and genes such as *spa/kit* required for survival and migration. The NIH3T3 cell transfection work described here was conducted in the absence of any known Wnt signaling. Furthermore, eliminating the Tcf/Lef binding sites as described by Dorsky et al. (Dorsky et al., 2000) from the *nac/mitfa* promoter reporter construct did not prevent the observed *cls/sox10* response in NIH3T3 cells (data not shown). Recently, Saito et al. (Saito et al., 2002) have shown that LEF-1 activates transcription from the *MITF* promoter in Hela cells much more effectively when bound together as a complex with the MITF-M protein itself. Future studies using coexpression of *sox10*, *mitfa* and Wnt signaling components could help to reveal how Wnt signaling and *sox10* interact to establish *mitfa* expression. Work by others using mammalian systems has also shown that the transcription factors *Pax3*, *OC-2* and *CREB* transactivate *Mitf* transcription (Bertolotto et al., 1998; Jacquemin et al., 2001; Potterf et al., 2000; Watanabe et al., 1998).

SOX10, *MITF* and human disease

Our demonstration that *sox10* function in melanophores may be limited to regulation of *mitfa* helps to explain the similar pigmentation defects of the Waardenburg Syndromes IIa and IV. Waardenburg Syndromes IIa and IV are associated with human haploinsufficiency for *MITF* and *SOX10*, respectively (Pingault et al., 1998; Tachibana et al., 1994; Tassabehji et al., 1994). Although zebrafish *cls/sox10* mutants have no dominant phenotype, our results suggest a model for the aetiology of Waardenburg Syndrome IV. We propose that in heterozygous *SOX10* mutant humans, activation of *MITF* by SOX10 is less efficient, resulting in specification of fewer melanoblasts. Consistent with this, in heterozygous *Sox10* mutant mice, which share the dominant pigment defects of human individuals, *Kit*-positive melanoblasts are reduced in number (Potterf et al., 2001); although not reported in these studies, we predict that the number of *Mitf*-expressing cells would be reduced in these mice compared to wild-types.

That we can, in zebrafish, account quantitatively for the role of *sox10* in the melanophore lineage by its activation of *mitfa* is perhaps surprising in view of the reports that the mouse *Dct* promoter can be directly regulated by Sox10 (Britsch et al., 2001; Potterf et al., 2001). However, these studies used co-transfection assays in cultured cells and thus leave open the question of whether *Dct* is regulated directly by Sox10 in the developing neural crest. Our findings strongly suggest that even if Sox10 does directly regulate *dct* expression in vivo, this requirement may be dispensable for melanophore development. Such an interpretation is consistent with the phenotype in heterozygous *Sox10* mutant mice. Thus, a transient reduction in *Dct* expression seen in developing melanoblasts was attributed to an effect of the reduced levels of Sox10 (Potterf et al., 2001), although an alternative explanation that sub-wild-type levels of *Mitf* expression result

in lowered *Dct* expression cannot be ruled out; indeed, more recent studies in culture show that MITF interacts with LEF-1 to directly coactivate the *DCT* promoter (Yasumoto et al., 2002). However, regardless of the mechanism mediating this reduction in detectable *Dct* expression, the *Dct* phenotype rapidly recovers, suggesting that in melanophores in which *Mitf* expression is above a threshold level, the requirement for *Sox10* is only transient and non-essential. The alternative explanation, that the precise contributions of *Sox10* and *Mitf* in melanocyte development may not be fully conserved between zebrafish and mice, is less attractive because of the striking similarities in the genetic control of melanocyte development already demonstrated between mouse and zebrafish (Rawls et al., 2001).

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