A direct role for Sox10 in specification of neural crest-derived sensory neurons

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sox10 is necessary for development of neural and pigment cell derivatives of the neural crest (NC). However, whereas a direct role for Sox10 activity has been established in pigment and glial lineages, this is more controversial in NC-derived sensory neurons of the dorsal root ganglia (DRGs). We proposed that sox10 functioned in specification of sensory neurons, whereas others suggested that sensory neuronal defects were merely secondary to absence of glia. Here we provide evidence that in zebrafish, early DRG sensory neuron survival is independent of differentiated glia. Critically, we demonstrate that Sox10 is expressed transiently in the sensory neuron lineage, and specifies sensory neuron precursors by regulating the proneural gene neurogenin1. Consistent with this, we have isolated a novel sox10 mutant that lacks glia and yet displays a neurogenic DRG phenotype. In conjunction with previous findings, these data establish the generality of our model of Sox10 function in NC fate specification.

KEY WORDS: Sox10, Neural crest, Fate specification, Determination, Dorsal root ganglion, Neurogenin, Zebrafish, Transgene, Waardenburg-Shah syndrome

INTRODUCTION

In the trunk and tail, sensory neurons are clustered with support cells (glia) to form a reiterated segmental series of dorsal root ganglia (DRGs) (Le Douarin and Kalcheim, 1999). Sensory afferent axons are myelinated by Schwann cells, whereas DRG neuron cell bodies are surrounded by satellite glia. Both DRG neurons and associated glia are generated from an ectodermally derived stem cell population, the neural crest (NC).

The transcription factor Sox10 is pivotal to NC ontogeny (Kelsh, 2006). In humans, Sox10 mutations are associated with Waardenburg-Shah syndrome, characterised by defects in enteric nervous system and pigmentation (Pingault et al., 1998; Southard-Smith et al., 1998), and with severe dysmyelination syndromes (Inoue et al., 1999; Pingault et al., 2000; Touraine et al., 2000). Similarly, mouse Sox10 mutations exhibit similar dominant defects, as well as an embryonic lethal recessive phenotype, primarily characterised by widespread defects in NC derivatives, including enteric and sympathetic ganglia, melanocytes, glia and DRG sensory neurons (Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). Subsequent studies have highlighted the importance of Sox10 for generation of certain derivatives from the NC and, in particular, for development of glia (Britsch et al., 2001; Kapur et al., 1998; Paratore et al., 2001; Southard-Smith et al., 1998). Thus, homozygous Sox10 mutants lack early markers of PNS glial progenitors (e.g. Erbb3 and Notch1), as well as glial differentiation markers (Britsch et al., 2001; Sonnenberg-Riethmacher et al., 2001). Complete lack of Sox10 function results in cell death before overt fate acquisition, and any surviving cells fail to subsequently differentiate as glia (Britsch et al., 2001; Kapur, 1999; Paratore et al., 2001). Consistent with these defects, in mammals and also in chick, Sox10 is expressed in undifferentiated NC and persistently in mature glia, but not in mature DRG neurons (Bondurand et al., 1998; Britsch et al., 2001; Cheng et al., 2000; Herbarth et al., 1998; Southard-Smith et al., 1998).

Zebrafish sox10, also known as colourless (cls), shows a strong conservation of gene expression pattern and function, and homozygous sox10 mutants display a phenotype strikingly similar to that of mouse Sox10 null homozygotes (Dutton et al., 2001a; Dutton et al., 2001b; Kelsh et al., 1996; Kelsh et al., 2000; Kelsh and Eisen, 2000). As in mouse, premigratory NC forms in normal numbers in zebrafish sox10 mutants (Dutton et al., 2001b). Counts of NC cells (NCCs) on the medial pathway demonstrated that DRG precursor cells migrate in normal numbers in sox10 mutants. We recently proposed, based in part on single-cell analysis of NCCs in sox10 mutants, that zebrafish sox10 has a primary role in NCC fate specification, specifically in all neuronal, glial and pigment cell lineages (Dutton et al., 2001b; Kelsh and Raible, 2002). We have subsequently provided strong support for this model for the melanocyte and enteric neuron lineages, showing that critical genes encoding transcription factors required for specification of these lineages (namely mitfa and phox2b) fail to be transcribed in the NC of sox10 mutants (Dutton et al., 2001b; Elworthy et al., 2003; Elworthy et al., 2005). Using both single-cell labelling studies and TUNEL, we have shown that NCCs die, but this occurs in a narrow window between 35 and 45 hours post fertilisation (hpf), after they fail to become fate specified (Dutton et al., 2001b; Kelsh and Raible, 2002).

The sox10 mutant sensory neuron phenotype is weaker than that of other derivatives, in both mouse and in zebrafish (Kapur, 1999; Kelsh and Eisen, 2000). In mice, DRG neurons are absent posteriorly whereas anterior DRGs are reduced in size, and initially contain apparently normal sensory neurons, yet these eventually die (Britsch et al., 2001; Kapur, 1999). In zebrafish, sensory neuron number is strongly reduced in the tail, but less affected in the trunk (Kelsh and Eisen, 2000). In mouse, based partly on comparison to mouse Erbb3 mutants (Riethmacher et al., 1997), loss of trunk motor and sensory neurons in Sox10 mutants was proposed to be a secondary consequence of the failure of differentiation of DRG satellite glia and Schwann cells, with neuronal death due to loss of glial trophic support (Britsch et al., 2001). Thus, in contrast to glial

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fates, Sox10 was attributed no direct role in sensory neuron development. Specification of DRG sensory neuron fate is critically dependent upon the Neurogenin (Ngn) gene family; encoding transcription factors, these are key regulatory genes for the sensory neuron lineage in both mouse and zebrafish (Blader et al., 1997; Perez et al., 1999). Ngn genes are expressed in a subset of NCCs early during migration, but are rapidly downregulated in the nascent DRG (Greenwood et al., 1999; Perez et al., 1999). DRG neuron specification was not examined in mouse Sox10 mutants (Britsch et al., 2001; Sonnenberg-Riethmacher et al., 2001). Hence, whether Sox10 mutants show an early reduction in nascent sensory neurons, in addition to later neuronal death due to absence of glial support remains untested. The DRG sensory neuron phenotype therefore provides a critical test of the model that Sox10 is required for fate specification of all nonskeletal NC derivatives.

Here, we analyse in detail the DRG phenotype in zebrafish Sox10 mutants. We generate a sox10:egfp line to allow in vivo observation of PNS glia, and show defects in both DRG-associated Schwann cells and satellite glia in Sox10 mutants. We show that neither motorneuron nor residual DRG neuron survival depends upon proximity to differentiated glia. Importantly, we show quantitatively that DRG neuron specification is defective in Sox10 mutants, and that Sox10 is able to induce ngn1 (neurog1 – Zebrafish Information Network) robustly and cell autonomously. Furthermore, we present evidence for early, but only transient, expression of Sox10 in DRG precursors. Finally, we introduce a new Sox10 allele that shows a neurogenic DRG phenotype, underscored by an excess of ngn1-positive cells, but again without glial support. Thus, Sox10 is required for specification of DRG neurons and hence plays an active role in the generation of all cell types of the zebrafish DRG.

MATERIALS AND METHODS

Fish husbandry
Embryos were obtained through natural crosses and staged according to Kimmel et al. (Kimmel et al., 1995). Sox10 alleles cl115 or cl660 were used interchangeably; they show equally strong DRG phenotypes (Dutton et al., 2001b). Both DNA and morpholino solutions were supplemented with 0.1% phenol red.

Constitutive Sox10 expression
Embryos were genotyped by PCR immediately following transplantation.

Morpholino and DNA injections
Morpholinos obtained from Gene Tools (Corvallis, OR) were used as described previously (Dutton et al., 2001a; Andermann et al., 2002). Sox9b morpholinos were as follows: Sox9b MO1, 5'-TGTGTTGTGTGTGTGTGTGTGTACACACGATC-3'; Sox9b MO2, 5'-AGCTGCTGAAACACACACAGATCCT-3'; and 4MM Sox9b MO2, 5'-AGCTGCTGAAACACACACAGATCCT-3' (mismatched bases shown in lower case). Only embryos injected with the Sox10 morpholino showing a clear melanophore phenotype were subsequently analysed. Plasmid DNA was prepared using the Wizard Midi system (Promega, Madison, WI) and diluted to a concentration of 25 ng/µl. Both DNA and morpholino solutions were supplemented with 0.1% phenol red.

Ectopic expression in zebrafish embryos by heat shock
Wild-type embryos were injected with 50-75 pg of pCSHP, hs>sox10 or hs>sox10(L142Q) and incubated at 28.5°C until 2 hpf, heat-shocked by incubation at 37°C for 2 hours and then fixed after a further 30 minutes at 28.5°C.

Generation of a transgenic line
To generate a stable transgenic line, the p4.9sox10:egfp construct was linearised and 50-80 pg of DNA injected per embryo. Offspring of injected fish were screened by fluorescent microscopy for GFP and a stable line, designated Tg(–4.9sox10:egfp)ba2, generated.

Mutagenesis and Sox10 allele screening
Forty adult male Tubingen fish were mutagenised using ENU according to the established protocol (Haffter et al., 1996). Fifteen surviving F0 males were crossed to AB wild-type females at weekly intervals and progeny raised as separate F1 families. New Sox10 alleles were isolated by non-complementation screen using carryers for cl115, cl660 or cl662. The sox10(L142Q) mutation was identified by directly sequencing RT–PCR products amplified from cDNA.

Whole-mount in situ hybridisation, antibody staining and TUNEL analysis
RNA in situ hybridisation was performed largely as previously described (Kelsh and Eisen, 2000), except that a tenfold concentration of Proteinase K was used on 5 days post fertilisation (dpf) embryos. Probes were used ngn1 (Blader et al., 1997), sox10 (Dutton et al., 2001b) and mbp (Brosamle and Halpern, 2002).

Cell transplantation
For chimera experiments, donor embryos were injected with 0.1% 10,000 MW fluoresein dextran (Molecular Probes) at the one- to two-cell stage. At approximately 30% epiboly, 15-20 cells were transplanted to shield stage hosts into the presumptive NC domain. Donors derived from sox10 heterozygous incrosses were genotyped by PCR immediately following transplantation. Host embryos were raised separately to 3 dpf, pooled according to donor genotype where appropriate, fixed and immunofluorescently stained for fluorescein and Hu. Donor-derived DRG neurons were counted and compared with a Mann–Whitney test.

Microscopy and statistical analysis
Fluorescent images were taken with a Zeiss Confocal microscope (LSM510) or an Eclipse E800 (Nikon) microscope using appropriate filters and a Spot digital camera (Diagnostic Instruments). Screening for GFP transgenics was performed on an MZ12-FL dissecting microscope (Leica) with fluorescent attachment. Statistical analysis was done with the Prism Statistical Package (GraphPad, San Diego, CA).

RESULTS

Generation of a sox10:egfp transgenic line
To better understand the role of Sox10 in NC development, we generated a stable transgenic line carrying a construct driving GFP from 4.9 kb of the zebrafish sox10 promoter (see Materials and methods; Fig. 1A). In this line, GFP expression in premigratory NCCs faithfully replicated the endogenous sox10 expression pattern (Dutton et al., 2001b), being visible from around the one-somite stage in two stripes lateral to the anterior neural plate (data not shown); subsequently GFP expression was seen in both premigratory and migratory NCCs (Fig. 1B-E), and reveals details of cell morphology (Fig. 1C-E). At 24 hpf, expression mostly resulted from perdurance of GFP from premigratory stages, but was
strong in all cells, including in branchial arch cartilage progenitors (Fig. 1E,F) (see also Wada et al., 2005). GFP was later detected in NCCs on both the lateral (not shown) and the medial (Fig. 1H) NC migration pathways and in satellite cells of the cranial ganglia (Fig. 1G). GFP was prominent in all PNS glial precursors, including those of Schwann cells of the posterior lateral line and spinal nerves (Fig. 1I,J,K) and satellite glia within the DRGs (Fig. 1J, Fig. 4C). At 5 dpf, GFP expression clearly revealed the compacted morphology of the satellite glia in the DRGs, but was absent from Hu+ cells within. Expression in peripheral glia was strongly maintained to 10 dpf in posterior lateral line nerve Schwann cells (data not shown). Similar GFP expression patterns have been observed in multiple other sox10 transgenic lines (T.J.C., J. Dutton and R.N.K., unpublished). Thus, this transgenic line (allele designation Tg(–4.9sox10:egfp) ba2), which we here call sox10:egfp, labels all DRG glia and NC precursors.

**DRG-associated peripheral glia are defective in sox10 mutants**

We examined the sox10 mutant glial phenotype, especially the DRG and spinal nerves using the sox10:egfp line. In 5 dpf wild-type embryos, both posterior lateral line and spinal nerves showed numerous GFP+ glial cells closely associated with axons (Fig. 1I,K, Fig. 2A). As shown before, the posterior lateral line nerve in sox10 mutants lacked all GFP+ glial progenitors (data not shown) (Kelsh et al., 2000). In contrast, GFP+ cells were associated with spinal nerves in all segments of sox10 mutants (Fig. 2B). Thus, even in sox10 mutants, NCCs migrate along the medial pathway and remain in position along the spinal nerves. However, these GFP+ cells were not normally differentiated. First, whereas in the wild type these cells showed a consistent smooth
morbidity, with Schwann cells organised along the middle of each somite segment and satellite glia compacted around the sensory neurons (Fig. 2A), in sox10 mutants both the morphology and organisation of cells associated with the nerve was disrupted, with GFP+ branches diverging from the medial position. Further, no morphologically normal satellite glia could be seen in any somite segment (Fig. 2B). Finally, mRNA in situ hybridisation of myelin basic protein (mbp), currently the only zebrafish Schwann cell differentiation marker (Brosamle and Halpern, 2002), revealed expression in wild-type spinal nerve Schwann cells (Fig. 2C), but was undetectable in sox10 mutants (Fig. 2D). Thus, GFP+ NCCs near the spinal nerves failed to differentiate as Schwann cells in sox10 mutants. We also observed an absence of mbp expression in the oligodendrocytes of the CNS (Fig. 2D). Thus, as in mouse sox10 mutants, differentiated PNS glia and CNS oligodendrocytes are absent in zebrafish sox10 mutants.

**Fig. 2. Glial differentiation fails in sox10 embryos.** (A,B) Double immunofluorescent detection of trunk sensory neurons (Hu, red) and prospective glial cells (GFP, green) in anterior trunk in 5 dpf wild-type (A) and sox10 mutant (B) embryos carrying the sox10:egfp transgene. Although GFP+ cells occupy similar positions in sox10 mutant and wild-type embryos (arrows), the elongated morphology of Schwann cells and the compact clustered arrangement of satellite glial cells in DRGs (arrowheads) of wild-type embryos (A) are absent in sox10 mutants (B). Neurons are often displaced ventrally in sox10 mutant embryos, but sensory neuron number may be approximately normal at this axial position (B). (C,D) In situ hybridisation with mbp reveals absence of differentiated Schwann cells in sox10 embryos (D) compared with wild-type siblings (arrowhead, C). Likewise, oligodendrocyte differentiation (*, C) fails in sox10 mutants (D). Scale bars: 20 µm in A; 50 µm in B.

**Table 1. Apoptosis of DRG sensory neurons in sox10 mutants**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Embryos with Hu+</th>
<th>Total number Hu+ neurons observed</th>
<th>Embryos with Hu+</th>
<th>Total number Hu+ neurons observed</th>
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<tr>
<td>48 hpf</td>
<td>3/15</td>
<td>4</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>52 hpf</td>
<td>4/15</td>
<td>4</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>72 hpf</td>
<td>3/15</td>
<td>3</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>5 dpf</td>
<td>0/15</td>
<td>0</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>6 dpf</td>
<td>0/14</td>
<td>0</td>
<td>0/14</td>
<td>0</td>
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</tbody>
</table>

**DRG sensory neurons are reduced, but not dying, in sox10 mutants**

Similar to mouse sox10 mutants, DRG sensory neurons are strongly reduced in zebrafish sox10 mutants. Using Hu antigen expression as a marker, wild-type 5 dpf fish have DRGs positioned in a reiterated series alongside ventrolateral spinal cord (Fig. 3A). As noted before (Kelsh and Eisen, 2000), sox10 siblings showed both abnormal placement of and a strong reduction in numbers of DRG neurons and ganglia (Fig. 3B,C). For example, at 5 dpf, sox10 mutants show only approximately 25% of the number of neurons of their wild-type siblings. Individual segments showed different DRG patterns, with Hu+ cell number entirely absent, reduced or normal; we could detect no clear pattern to the distribution of each of these, although fewer cells were detected posteriorly. A time-course study showed that sensory neuron number increased with time in wild-type embryos, but remained consistently reduced in mutants (Fig. 3C). In mouse sox10 mutants, DRG sensory neurons degenerate, and this phenotype is attributed to lack of Schwann cell-derived trophic support (Britsch et al., 2001). To test whether apoptosis of newly generated Hu+ sensory neurons made a major contribution to the sensory neuron phenotype of zebrafish sox10 mutants, we used TUNEL combined with immunofluorescent detection of Hu to label sensory neurons between 48 hpf and 6 dpf. All Hu+ DRG neurons throughout the trunk and tail of each embryo were examined for TUNEL. Although small numbers of apoptosing Hu+ neurons were seen in cranial ganglia and in the brain of both wild-type and sox10 mutant embryos, apoptosing DRG sensory neurons were seen only occasionally (0-2 TUNEL+ Hu+ DRG neuron per embryo) in wild type and not at all in sox10 mutants at these stages (Table 1). Thus, although a strong DRG sensory neuron sox10 phenotype is conserved in zebrafish, in contrast to mouse sox10 mutants (Britsch et al., 2001), apoptotic loss of differentiated sensory neurons is unlikely to explain the sensory neuron phenotype prior to 6 dpf.

Previous analyses indicated that NCC numbers were not reduced in sox10 mutant embryos up to 30 hpf (Dutton et al., 2001b). To test whether changes in proliferation of DRG neurons might contribute to the sox10 mutant phenotype, we used double immunofluorescent detection of phosphoHistone H3 to detect proliferation combined with Hu to label neurons (Table 2 and Fig. S2 in the supplementary material). Intriguingly, we observed a significantly elevated level of DRG neuron proliferation in sox10 mutant embryos as early as 24 hpf, although rates were identical at 72 hpf. Thus, changes in proliferative rates cannot explain the sensory neuron phenotype of zebrafish sox10 mutants, we used double immunofluorescent detection of trunk sensory neurons (Hu, red) and prospective glial cells (GFP, green) in anterior trunk in 5 dpf wild-type (A) and sox10 mutant (B) embryos carrying the sox10:egfp transgene. Although GFP+ cells occupy similar positions in sox10 mutant and wild-type embryos (arrows), the elongated morphology of Schwann cells and the compact clustered arrangement of satellite glial cells in DRGs (arrowheads) of wild-type embryos (A) are absent in sox10 mutants (B). Neurons are often displaced ventrally in sox10 mutant embryos, but sensory neuron number may be approximately normal at this axial position (B). (C,D) In situ hybridisation with mbp reveals absence of differentiated Schwann cells in sox10 embryos (D) compared with wild-type siblings (arrowhead, C). Likewise, oligodendrocyte differentiation (*, C) fails in sox10 mutants (D). Scale bars: 20 µm in A; 50 µm in B.

Both motor and sensory neuron death in Sox10 mouse mutant embryos has been attributed to a common mechanism of lack of glial support. To compare directly numbers of differentiated sensory and motor neurons in sox10 mutants at various stages, we used antibodies against DM-GRASP (zn-5) and Islet1. At 5, 8 and 10 dpf, DRG sensory neuron expression of DM-GRASP was absent in all...
segments of sox10 mutants, but readily seen in wild-type siblings (Fig. 3D,E; data not shown). At 60 hpf Isl1+ DRG sensory neurons were reduced in sox10 mutants by 36% compared with their wild-type siblings (Fig. 3F-H). Importantly both markers indicated that motor neurons are present in normal numbers (Fig. 3D-G,I) at all stages examined up to 7.5 days after onset of sensory neuron phenotype. These observations further demonstrate that DRG sensory neurons are reduced in sox10 mutants and that sensory and motor neurons show disparate phenotypes.

Surviving DRG neurons do not require intimate association with GFP+ cells

If the sox10 mutant DRG neuron phenotype resulted purely from absence of glia, then any remaining DRG neurons would be expected to be obtaining trophic support from associated residual glial cells. As shown above, we see no evidence of differentiated glial cells, but even if we allow that some GFP+ cells may have partial glial character, we still do not see tight association of residual DRG neurons and GFP+ cells. Although in the wild type, GFP+ satellite glia and Schwann cells were consistently associated with each neuronal cluster (Fig. 4C), sox10 mutants were more variable, but often showed neurons without surrounding glia (Fig. 4A,B). Hence, the remaining sensory neurons in sox10 mutants are unlikely to be receiving trophic support from neighbouring glia, although we cannot rule out a longer-range trophic effect from residual glial precursors.

Early markers of sensory neuron specification are reduced in sox10 mutants

Together, these observations argued against the proposal that DRG sensory neurons were depleted in zebrafish sox10 mutants as a secondary consequence of loss of peripheral glial cells. Consequently, we tested whether sox10 might be required directly for specification of sensory neurons, by examining ngn1 expression. In wild type, ngn1 expression was seen in a reiterated series of cells lying ventrolateral to the spinal cord, whereas sox10 mutants show frequent gaps in this pattern (Fig. 5A,B). Quantitation of these cells at 36 hpf revealed significant deficiencies (52% reduction compared with wild-type siblings) of ngn1+ cells in sox10 embryos, even at this very early stage (Fig. 5C). To rule out the possibility that ngn1+ cells were simply obscured due to delayed migration, we used a ngn1:GFP reporter line, which shows strong GFP expression in nascent DRG neurons (Fig. 5D) (Blader et al., 2003). We used a sox10 morpholino (Dutton et al., 2001a) to knock down Sox10 in this transgenic reporter line. As a positive control for successful Sox10 knock down, we noted the characteristic strong reduction in pigment cell numbers, consistent with the sox10 phenotype (compare left panels in Fig. 5E,F). At 48 hpf, uninjected embryos or embryos injected with a mismatch morpholino showed normal pigmentation and a reiterated series of GFP+ cells alongside the neural tube in a position consistent with forming DRG neurons (Fig. 5G,H).
In *sox10* morpholino-injected embryos showing a strong pigment phenotype, we observed significant loss (85% reduction) of GFP+ DRG neuron precursors at all axial levels (Fig. 5F), although the dorsal CNS GFP expression domain remained unaffected (Fig. 5E). There was no sign of misplaced cells, arguing against defects in sensory neuron migration. Instead, we conclude that *ngn1* transcription fails in the absence of Sox10 function.

**Residual Hu+ DRG neurons in sox10 mutants form through an Ngn1-dependant mechanism**

Residual DRG sensory neurons are a notable aspect of mouse and zebrafish *sox10* mutants. All sensory neurons in wild-type embryos are *ngn1* dependent (Cornell and Eisen, 2002). To test whether the remaining Hu+ DRG neurons in *sox10* mutants were also *ngn1* dependent or somehow formed independently of *ngn1*, we used morpholino-mediated *Ngn1* knock down. *sox10* mutants injected with a *ngn1* morpholino consistently showed a significant reduction (by 91%) of Hu+ DRG neurons at 5 dpf compared with uninjected *sox10* mutants (Fig. 5G-I). Thus, the remaining Hu+ neurons in *sox10* mutants were dependent on *ngn1* for their development.

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**Table 2. DRG neuron proliferation in sox10 mutants**

<table>
<thead>
<tr>
<th>Stage (hpf)</th>
<th>Genotype</th>
<th>Number embryos scored</th>
<th>Number pH3+ neurons/ Hu+ DRG neurons scored</th>
<th>% proliferating DRG neurons</th>
</tr>
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<tbody>
<tr>
<td>60</td>
<td>Wild type ∆/∆</td>
<td>18</td>
<td>279/501</td>
<td>55.7</td>
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<tr>
<td></td>
<td><em>sox10</em> ∆/+</td>
<td>18</td>
<td>174/205</td>
<td>84.9*</td>
</tr>
<tr>
<td>72</td>
<td>Wild type ∆/∆</td>
<td>10</td>
<td>295/328</td>
<td>89.9*</td>
</tr>
<tr>
<td></td>
<td><em>sox10</em> ∆/+</td>
<td>8</td>
<td>94/100</td>
<td>94.0</td>
</tr>
</tbody>
</table>

*Significant at P<0.0001, unpaired t-test, two-tailed.

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**Residual Hu+ DRG neurons in sox10 mutants are Sox9b dependent**

We then asked whether functional redundancy between SoxE genes might explain the relatively weak sensory neuron phenotype in *sox10* mutants by knocking down *sox9b*. *sox9b* is expressed transiently in premigratory NCCs (Chiang et al., 2001; Li et al., 2002; Yan et al., 2005). Injection of 9 ng of *sox9b* morpholino into wild-type embryos gave the previously reported small head and down-curved tail phenotype (Chiang et al., 2001; Li et al., 2002; Yan et al., 2005). Interestingly, these embryos also showed a small reduction of Hu+ DRG neurons and some disorganisation of the remaining cells (Fig. 6A,B) compared with uninjected siblings. When injected into *sox10* mutant embryos, this same dose of *sox9b* morpholino resulted in a dramatic phenotype, showing the small head and curved tail, but also strongly accentuating the *sox10* mutant DRG neuron phenotype (Fig. 6C,D). We saw similar DRG neuron phenotypes with either of two non-overlapping *sox9b* morpholinos, whereas an 18 ng dose of a 4 bp-mismatch morpholino had no effect (Fig. 6E).

**GFP perdurance reveals activity of the sox10 promoter in DRG sensory neuron precursors**

If the effect on DRG neurons seen in *sox10* mutants does not reflect a lack of glial trophic support, perhaps Sox10 acts autonomously in the sensory neuron lineage. This requires that DRG neuron precursors express Sox10 at some stage. Consequently, we used double immunofluorescent detection of Hu antigen and GFP in *sox10:egfp* embryos to mark *sox10* expression cell autonomously. Given the perdurance of GFP, we anticipated that the strong GFP expression in premigratory NCCs would allow use of GFP as a lineage tracer to determine if the *sox10* promoter was active at some stage in the development of DRG neurons. At 5 dpf, GFP signal is absent from DRG neurons (Fig. 1J). However, 48 hpf embryos consistently showed double-labelled NCCs in nascent DRGs (Fig. 7D). These Hu+ GFP+ DRG neurons were surrounded by Hu+ GFP+ cells, which likely represent glial lineages (Fig. 7E). We conclude that the *sox10* promoter was transiently active in DRG sensory neuron progenitors, and thus infer that these cells expressed Sox10 transiently.

We then used a direct approach to confirm Sox10 expression in sensory neuron precursors by using a polyclonal serum that recognises zebrafish Sox10 (Park et al., 2005) on the *ngn1:GFP* reporter line, as *ngn1* is the earliest available marker for sensory neuron precursors. We assessed cells for co-expression of GFP and Sox10 and noted that 7/26 (27%) and 10/52 (19%) GFP+ nascent DRG sensory neurons were expressing detectable Sox10 protein in 40 and 42 hpf embryos, respectively (Fig. 7F). Interestingly, the GFP+;Sox10- cells tended to show rather stronger GFP expression, suggesting that they were more mature, consistent with the suggestion that Sox10 is rapidly downregulated in neuronal precursors.

**Ectopic Sox10 expression induces ectopic ngn1**

We then asked if Sox10 expression was sufficient to induce *ngn1* expression. Embryos were injected with heat-shock constructs driving Sox10 or various control constructs (Dutton et al., 2001b)
heat shocked at 37°C for 2 hours, then fixed and examined for ectopic ngn1 transcripts by in situ hybridisation. Embryos injected with the hs:sox10 construct reproducibly contained many cells with strong ngn1 expression, whereas uninjected embryos or embryos injected with an empty heat-shock vector showed none (Fig. 7A,B). Interestingly, embryos injected with a heat-shock construct driving expression of a mutant Sox10 protein [hs>sox10m618 (L142Q)] (Dutton, et al., 2001b) only weakly induced ngn1, presumably due to low level residual activity of this protein. Injected, but not heat-shocked, embryos showed greatly reduced ngn1 induction (data not shown). Further, double in situ hybridisation analysis showed that cells expressing ngn1 almost always also contained detectable sox10 message (Fig. 7C). Thus, Sox10 functioned cell autonomously to induce ngn1 expression.

Previously we have demonstrated that sox10 acts cell autonomously in all pigment cell lineages (Kelsh and Eisen, 2000). We used cell transplantation from sox10 mutants or their wild-type siblings into wild-type embryos to test whether the wild-type environment is able to support sox10 mutant cells as well as wild-type cells. sox10 mutant donor cells generated significantly fewer sensory neurons compared with wild-type sibling donor cells in wild-type host embryos, consistent with a cell-autonomous role in DRG sensory neurons (see Table S1 in the supplementary material). In addition, experiments transferring wild-type cells into sox10 mutant hosts provided two clear cases of hosts containing a DRG with multiple wild-type glial cells, but which failed to rescue sensory neuron survival, a result inconsistent with the trophic support model, but consistent with our fate specification model (see Fig. S1 in the supplementary material).

A new sox10 allele with a neurogenic sensory neuron phenotype

In a sox10 allele screen we recovered one allele, sox10baz1, that showed a unique phenotype compared with all reported mouse or zebrafish sox10 alleles. Like other zebrafish sox10 alleles, sox10baz1 is fully recessive and homozygous lethal. sox10baz1 embryos have a strong melanophore phenotype similar to other reported sox10 alleles, although other pigment cell types are less affected, with xanthophores and iridophores mildly reduced (Fig. 8A,B; data not shown). In situ hybridisation using mbp probe showed the complete absence of PNS Schwann cells in sox10baz1 mutants, similar to the phenotype of strong sox10 alleles (Fig. 8C,D). Hence, glial and melanophore phenotypes were strongly hypomorphic, and xanthophore and iridophore phenotypes more weakly so. In striking contrast to any
previously described sox10 phenotype, 5 dpf sox10baz1/baz1 embryos were hypermorphic for DRG sensory neurons. Thus, instead of the strong decrease in Hu+ DRG sensory neurons seen in other strong sox10 mutant alleles, sox10baz1/baz1 embryos showed a 98% increase in their number compared with wild-type siblings (Fig. 8E-H,K). This phenotype was specific to sensory neurons, because as with the other sox10 alleles, sox10baz1/baz1 mutants also had no enteric neurons (Fig. 8E, arrow). The supernumerary DRG neurons survived until at least 5 dpf, despite the absence of fully differentiated Schwann cells. To confirm its identity as a new sox10 allele, we sequenced sox10 cDNA isolated from sox10baz1/baz1 embryos. We found a G to A substitution at position 724 (Fig. 8M), which creates a Valine to Methionine substitution within the HMG domain of the Sox10 protein at amino acid position 117 (Fig. 8M). This position is fully conserved between human, mouse and chicken Sox10 protein sequences, and is also within one of two nuclear localisation sequences in the HMG box.

To better understand the underlying cause of the DRG phenotype, we asked whether the increase in DRG sensory neurons was detectable at the time of their specification or if it occurred later through an independent mechanism. We saw a 90% increase in ngn1+ cells on the medial pathway outside the neural tube in sox10baz1/baz1 embryos compared with their wild-type siblings at 36 hpf (Fig. 8I,J and L). Therefore, the sox10baz1/baz1 phenotype strongly and independently supports an early role for Sox10 in regulating the specification of DRG neurons in the correct numbers.

**DISCUSSION**

**Primary versus secondary loss of sensory neurons in sox10 mutants**

Studies of the Sox10 mutant DRG phenotype at trunk axial levels in mouse showed that although nascent DRG size was approximately normal at E10, subsequently the constituent NCCs failed to show any glial cell markers, showed reduced DRG neurons, and later lost motoneurons (Britsch et al., 2001; Sonnenberg-Riethmacher et al., 2001). These authors noted the similar phenotype to that of Erbb3 mutants and proposed that DRG neuron and motoneuron deficits were likely secondary effects of loss of glial support. However, these same studies did not assess neuronal number quantitatively, nor did they evaluate the tail where the DRG deficit is more severe (Kapur, 1999). Thus, a role for Sox10 in fate specification of sensory neurons, as recently hypothesised (Dutton et al., 2001b; Kelsh and Raible, 2002), was not eliminated by these studies.

The zebrafish DRG phenotype shows many similarities to that in mouse, suggesting that an essential role for sox10 in DRG development is conserved throughout vertebrates. Thus, here we have shown a similar absence of glial differentiation, combined with expression of sensory neuron markers in residual DRG neurons in sox10baz1/baz1 mutants. We have also previously demonstrated that undifferentiated cells contributing to the nascent DRG of sox10 mutants undergo apoptosis (Dutton et al., 2001b). However, although we have shown a quantitative reduction in DRG sensory neurons, we have also shown that there is no elevated apoptotic loss of Hu+ DRG sensory neurons up to 6 dpf in sox10 mutants. Furthermore, using markers of motoneuron cell bodies and secondary motoneuron axons we see normal numbers of motoneurons to at least 10 dpf. We suggest that the timing of onset of glial dependency for trophic support of sensory and motoneurons may be relatively later in zebrafish than in mice. Consistent with this, residual sensory neurons were seen in the absence of cells showing normal glial differentiation, and were often distant from even undifferentiated GFP+ NCCs remaining on the medial pathway. Reconstitution of a wild-type glial environment in chimaeras was...
Sensory neuron specification requires sox10

Sox10 and sensory neuron fate specification

Although specification of individual cell fates from NCCs is a key process in NC development, our understanding remains incomplete. Recent work has shown that both Wnt and Shh signalling and the Ngn transcription factors have key roles in mouse and/or zebrafish sensory neuron specification (Cornell and Eisen, 2002; Lee et al., 2004; Ma et al., 1999; Perez et al., 1999). We tested the mechanistic basis for the sensory neuron phenotype in sox10 mutants, especially the more severe phenotype in the tail and, in particular, our hypothesis that sox10 might have a role in DRG sensory neuron fate specification (Dutton et al., 2001). In contrast to the predictions of the trophic support model, our fate specification model predicted that sox10 mutants should show very early defects in sensory neuron markers, with fewer cells expressing the sensory neuron specification gene ngn1 at its time of onset. Furthermore, sensory neurons would be derived from sox10-expressing NCCs, and sox10 expression should be sufficient, at least in some cells, to drive ngn1 transcription cell autonomously. We have tested all of these predictions and have shown here that all are fulfilled. We conclude, therefore, that sox10 is required for specification of sensory neurons from the NC, acting at the level of initiation of ngn1 transcription. Thus, sox10 functions directly in sensory neuron development, just as has been previously demonstrated for the melanocyte lineage (Bondurand et al., 2000; Dutton et al., 2001b; Elworthy et al., 2003; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000). In the case of melanocytes, Sox10 acts directly on the mitf/mitfa promoter to activate expression (Bondurand et al., 2000; Potterf et al., 2000; Verastegui et al., 2000; Elworthy et al., 2003). Regulation of ngn1 has been investigated in the CNS (Blader et al., 2004; Blader et al., 2003), but not in DRG sensory neuron precursors, so it remains to be tested whether Sox10 directly activates the ngn1 promoter, although we note that our ngn1:gfp transgene studies localise a putative responsive region within the ngn1 promoter and that in this fragment lie multiple Sox-binding sites (P.D. and P.B., unpublished).

Fig. 7. Cell-autonomous induction of ngn1 by Sox10. (A) In situ hybridisation reveals robust ngn1 induction by 4 hours after injection of heat-shock construct driving wild-type sox10 (hs>sox10) into one-cell stage wild-type embryos followed by 2 hour heat shock at 37°C from 2 hpf (A). Heat-shocked uninjected embryos and embryos injected with an empty heat-shock construct, never contained ngn1+ cells at this stage. Injection of a heat-shock vector driving expression of the Sox10m618 mutant protein (hs>sox10m618(L142Q)) gave only very weak induction of ngn1. (B) Relative proportions of embryos showing very strong, strong, weak or no induction of ngn1 after each treatment. (C) Double in situ hybridisation of injected embryos with sox10 (red/fluorescence) and ngn1 (blue) showed that ngn1+ cells had detectable sox10 message (99/103 cells scored in ten embryos). Nuclear localisation of ngn1 message suggests that nuclear export of these transcripts was limited within the timeframe of this experiment. (D,E) Double immunofluorescence confocal images (single focal plane) of 48 hpf wild-type sox10:eGFP embryos showed perduring GFP protein (green, left panel) in Hu+ (red, middle panel) nascent DRG neurons (merged, right panel). At this stage, 54/71 (76%) DRG neurons in five wild-type embryos were detectably GFP+, colocalisation has been confirmed in an independent sox10:eGFP line (data not shown). Hu7/GFP+ glial precursors (green) surrounding the Hu7/GFP+ neurons (yellow) were revealed using a larger confocal pinhole size (E). (F) Double immunofluorescence confocal images (single focal plane) of 40 hpf wild-type ngn1:eGFP embryo showing overlap of weak (arrowed) GFP (green, left panel) with Sox10+ (red, middle) nascent DRG neurons (merged, right panel). Cells expressing higher levels of GFP, likely to be more mature neurons, lack Sox10 expression (asterisks). Scale bars: 100 μm in A; 10 μm in C; 50 μm in D; 20 μm in E,F.
Whereas knock down of ngn1 results in absence of DRG neuron precursors, the strong sox10 mutant alleles in zebrafish consistently show only a partial reduction. Other mechanisms specifying DRG sensory neurons may also contribute to the weaker phenotype of these sox10 mutants. In particular, we have shown here that functional redundancy with sox9b, which is also expressed in premigratory NC, plays a role (Chiang et al., 2001; Li et al., 2002; Yan et al., 2005). Regardless of how they are formed, we have shown here that the residual sensory neurons in sox10 mutants are ngn1-dependent. It is likely that the residual ngn1+ cells seen on the medial migration pathway in sox10 mutants generate the remaining Hu+ neurons seen at later stages.

**Combinatorial factors and fate specification**

Levels of Sox10 protein within cultured DRG explants alter the cell’s response to environmental cues, and can subsequently bias fate acquisition in different cellular contexts. DRG cells from Sox10 mutant heterozygous mice display excess sensory neurogenesis compared with wild-type cells when cultured under conditions allowing cell contacts (Paratore et al., 2001). This led to the prediction that, at least under certain conditions, excess DRG neurogenesis might be seen in mice with reduced Sox10 activity. The zebrafish sox10baz1 mutant shows in vivo the excess neurogenesis phenotype predicted from these in vitro studies. At a molecular level, this mutation is a point mutant generating a single amino acid substitution in the DNA binding domain. Phenotypically, it generally behaves as a hypomorphic allele, having a somewhat weaker phenotype than the strong sox10 alleles published before (Kelsh et al., 1996; Malicki et al., 1996). The mechanistic basis for the hypermorphic sensory neuron phenotype remains unclear, but the neurogenic nature suggests an involvement of the Notch-Delta signalling system, already implicated in sensory neuronal and glial fate specification (Morrison et al., 2000; Wakamatsu et al., 2000), and may give insight into the interactions...
between the Sox10-Ngn1 and Notch-Delta pathways. The sox10
mutant phenotype gives strong confirmation that Hu+ sensory
neurons do not require differentiated glia for survival and that levels
of Sox10 activity help regulate levels of sensory neuron specification.

This study extends the evidence for a general model of Sox10
function in NC derivative specification (Kelsh, 2006). Key lineage
specification transcription factors for each of the sensory and enteric
neuron and melanocyte cell types have been shown to require Sox10
for their expression in zebrafish (this work) (Elworthy et al.,
2003; Elworthy et al., 2005), and in mouse similar defects in
sympathetic neuron and melanocyte fate specification have been
shown (Kim et al., 2003; Lee et al., 2000; Potter et al., 2000;
Verastegui et al., 2000). It will be of interest to use quantitative
analysis of mouse Sox10 mutants to test whether DRG sensory
neuron specification is affected.

Given that Sox10 is required for multiple diverse cell types, it is
clear that this transcription factor alone is insufficient to explain
the logic of NC fate specification. In general, fate specification of NC
derivatives requires both intrinsic factors and extrinsic signals (Kelsh
and Raible, 2002; Le Douarin and Kalcheim, 1999). In the case of
DRG sensory neurons, both Shh and Wnt signalling also influence
selection of that fate (Lee et al., 2004; Ungos et al., 2003), yet these
same signals are required for fate specification of melanocytes
(Bondurand et al., 2000; Dorsky et al., 2000; Elworthy et al., 2003;
Lee et al., 2000; Potter et al., 2000; Takeda et al., 2000; Verastegui
et al., 2000; Yasumoto et al., 2002). There clearly remain further
factors for both melanocyte and sensory neuron specification to
identify. As Sox proteins are known to interact with other partner
proteins (Wegner and Stolt, 2005), these are likely to have crucial
influences on the response of NCCs to intrinsic and extrinsic factors
mediating fate choice. Identification of the full complement of these
factors will be necessary for a comprehensive understanding of the
logic of NC fate specification.

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Phox2b function in the enteric nervous system is conserved in zebrafish and is
sox9b is an early neural crest marker.

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Table S1. Cell autonomy of sox10 action in DRG sensory neurons

<table>
<thead>
<tr>
<th>Experimental series</th>
<th>Genotype</th>
<th>Number of transplants</th>
<th>Number of DRG with donor contribution</th>
<th>Total donor DRG neurons</th>
<th>Mean+s.d. number neurons/chimeric DRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>WT → WT sibling</td>
<td>43</td>
<td>46</td>
<td>53</td>
<td>1.15±0.47</td>
</tr>
<tr>
<td></td>
<td>WT → sox10&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>18</td>
<td>21</td>
<td>30</td>
<td>1.43±0.75</td>
</tr>
<tr>
<td>B</td>
<td>WT sibling → WT</td>
<td>68</td>
<td>54</td>
<td>87</td>
<td>1.61±0.98</td>
</tr>
<tr>
<td></td>
<td>sox10&lt;sup&gt;−/−&lt;/sup&gt; → WT</td>
<td>30</td>
<td>14</td>
<td>7</td>
<td>0.50±0.52*</td>
</tr>
</tbody>
</table>

*Significant, P<0.0001; Mann-Whitney U-test.
WT, wild type.