Development 133, 4619-4630 (2006) doi:10.1242/dev.02668

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

Thomas J. Carney[1], Kirsten A. Dutton[1], Emma Greenhill[1], Mariana Delfino-Machín[1], Pascale Dufourcq[2], Patrick Blader[2] and Robert N. Kelsh[1,*]

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; Herbarth 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

[1]Centre for Regenerative Medicine, Developmental Biology Programme, Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK. [2]Centre de Biologie du Développement, UMR5547, Université Paul Sabatier, bât. 4R3, 118, route de Narbonne, 31062 Toulouse, France.

*Author for correspondence (e-mail: bssrnk@bath.ac.uk)

Accepted 21 September 2006

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 DRGs (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 cls^{t3} or cls^{m618} were used interchangeably; they show equally strong DRG phenotypes (Dutton et al., 2001b) (G. Ford, K.A.D. and R.N.K., unpublished). The *–3.1ngn1:gfp* transgenic line has been described previously (Blader et al., 2003).

Constructs

Constructs used in this study include pCSHSP, hs>*sox10* and hs>$sox10^{m618}$(L142Q) (Dutton et al., 2001b). A *sox10* reporter construct, p-*4.9sox10:egfp*, was generated using 4.9kb of zebrafish *sox10* promoter sequence upstream of the start of translation, subcloned as a *BamH*I-*Spe*I fragment from a *sox10* containing PAC (RZPD, Germany). This fragment was inserted in front of the *egfp* gene in the vector XLT.GFP_{LT}.$CS2^{+}$ (kind gift of R. Moon), using conventional cloning methods (Sambrook, 1989).

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′-TGTGTGTGTGTGTGTGT-GTGAGCAC-3′; *Sox9b*.MO2: 5′-AGCTGCTGAAACACACACAGAT-CCT-3′; and 4MM *Sox9b*.MO2, 5′-AGCTcCTGAtACACAgACAGtTCCT-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 pCSHSP, hs>*sox10* or hs>$sox10^{m618}$(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 p-*4.9sox10:egfp* construct was linearised and 50-80 pg of DNA injected per embryo. Offspring of incrosses of raised 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 Tübingen 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 carriers for cls^{t3}, cls^{tw2} or cls^{ty22f}. The $sox10^{baz1}$ 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 used were *ngn1* (Blader et al., 1997), *sox10* (Dutton et al., 2001b) and *mbp* (Brosamle and Halpern, 2002).

Single or double antibody staining was performed largely as previously described (Ungos et al., 2003). Primary antibodies used were anti-Hu (1:700 mAb 16A11) (Marusich et al., 1994), anti-DM-GRASP (1:400 mAb zn-5) (Fashena and Westerfield, 1999), anti-Islet1 (1:200 mAb 4D5; Developmental Studies Hybridoma Bank DSHB), anti-Sox10 (Park et al., 2005), anti-phospho-histone H3 (1:1000; Upstate Biotechnology, NY), anti-Fluorescein (1:400, Molecular Probes, Eugene, OR) and anti-GFP (1:200; Molecular Probes). Fluorescent visualisation used Alexa488- or Alexa546-conjugated secondary antibodies diluted in blocking solution (1:750; Molecular Probes).

TUNEL was performed as previously described, using fluorescein-11 dUTP, imaged by fluorescent microscopy (Dutton et al., 2001b).

Cell transplantation

For chimaera experiments, donor embryos were injected with 0.1% 10,000 MW fluorescein 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 $sox10^{+/–}$ 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 on 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

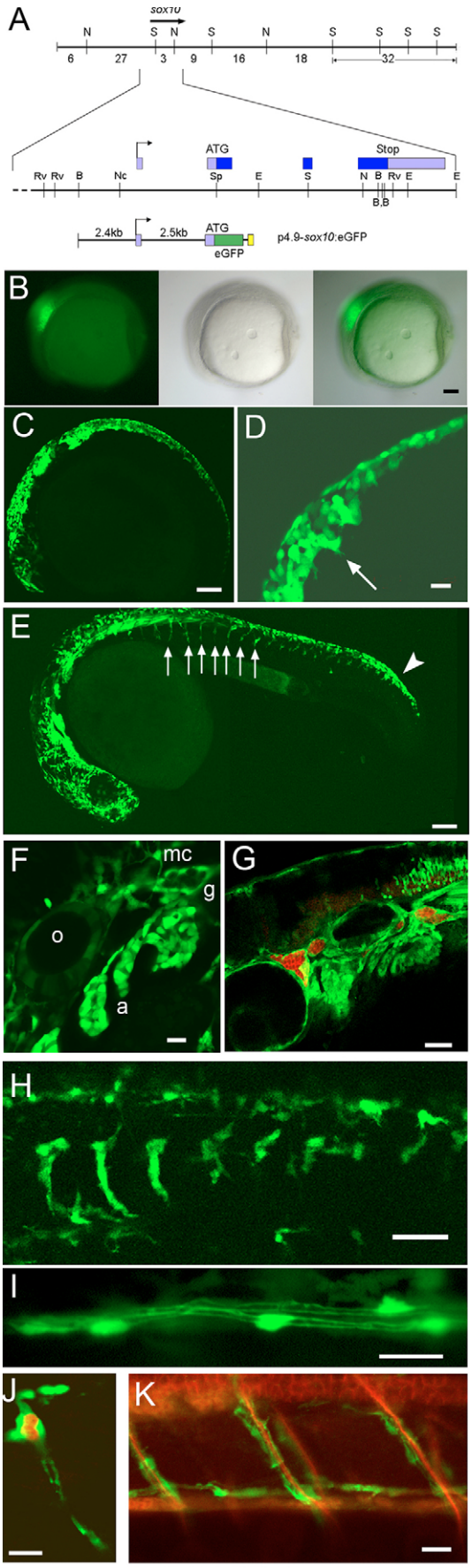

Fig. 1. Generation and characterisation of *sox10:egfp* transgenic fish. (**A**) Restriction maps of the sox10 genomic region. Exons are boxed, *sox10* untranslated sequences are indicated in light blue and coding region in dark blue. Lower panel shows a schematic of the construct used to generate transgenic fish. Yellow box shows the polyadenylation signal. Sizes in kb, N=*Not*I, S=*Sal*I, RV=*Eco*RV, B=*Bam*HI, Sp=*Spe*I, E=*Eco*RI, Nc=*Nco*I. (**B-H**) GFP in germline *sox10:eGFP* transgenic fish is seen in premigratory and migratory NCCs, in time and manner recapitulating the expression of *sox10*. Premigratory cranial NC at 11 hpf (B; fluorescence (left panel), DIC (middle) and combined (right)) and 17 hpf (C). (D) Cranial NCCs at 15 hpf to show resolution of fine cell morphology. (E) Premigratory (arrowhead) and migrating NCCs in head and on medial pathway in anterior trunk (arrows) at 24 hpf. (F) At 24 hpf, cranial GFP-expressing cells include migrating NC (mc), NC in branchial arches (a), glial precursors surrounding the cranial ganglia (g), and otic epithelium (o). (G) Double immunofluorescence imaging of GFP (green) and neurons (Hu, red) at 48 hpf. There is continued expression in otic epithelium and arches. (H) Tail of 40 hpf embryo to show nascent DRG and migrating NCCs. (**I-K**) GFP expression persists in peripheral glia, including DRG satellite glia (J), Schwann cells [posterior lateral line (I) and spinal nerves (J,K)], here at 5 dpf. (J) Double immunofluorescence demonstrates complementary expression of GFP (green) and Hu (red) in DRGs. (K) GFP$^+$ Schwann cells are intimately associated with spinal nerve axons (DM-GRASP, red). All are confocal images except (B) and are of live embryos except (G), (J) and (K). In this and all subsequent figures, embryos are shown in lateral view unless stated otherwise. Scale bars: 100 μm in B,C,E; 20 μm in D,F,I,J,K; 50 μm in G,H.

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

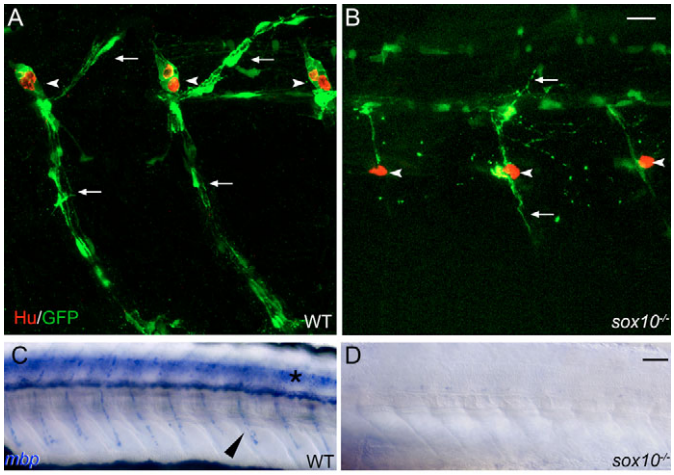

Fig. 2. Glial differentiation fails in *sox10* embryos. (**A**,**B**) Double immunofluorescent detection of trunk sensory neurons (Hu, red) and prospective glial cells (GFP, green) in anterior trunk in 5 dpf wild-type (A) and *sox10* mutant (B) embryos carrying the *sox10:egfp* transgene. Although GFP$^+$ 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.

morphology, 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 detection 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.

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 at 60 hpf, although rates were identical at 72 hpf. Thus, changes in proliferative rates cannot explain the *sox10* mutant DRG neuron phenotype.

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

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

	Wild type		*sox10*$^{-/-}$	
Stage	Embryos with Hu+; TUNEL+ DRG neuron/total embryos	Total number Hu+; TUNEL+ DRG neurons observed	Embryos with Hu+; TUNEL+ DRG neuron/total embryos	Total number Hu+; TUNEL+ DRG neurons observed
48 hpf	3/15	4	0/15	0
52 hpf	4/15	4	0/15	0
72 hpf	3/15	3	0/15	0
5 dpf	0/15	0	0/15	0
6 dpf	0/14	0	0/14	0

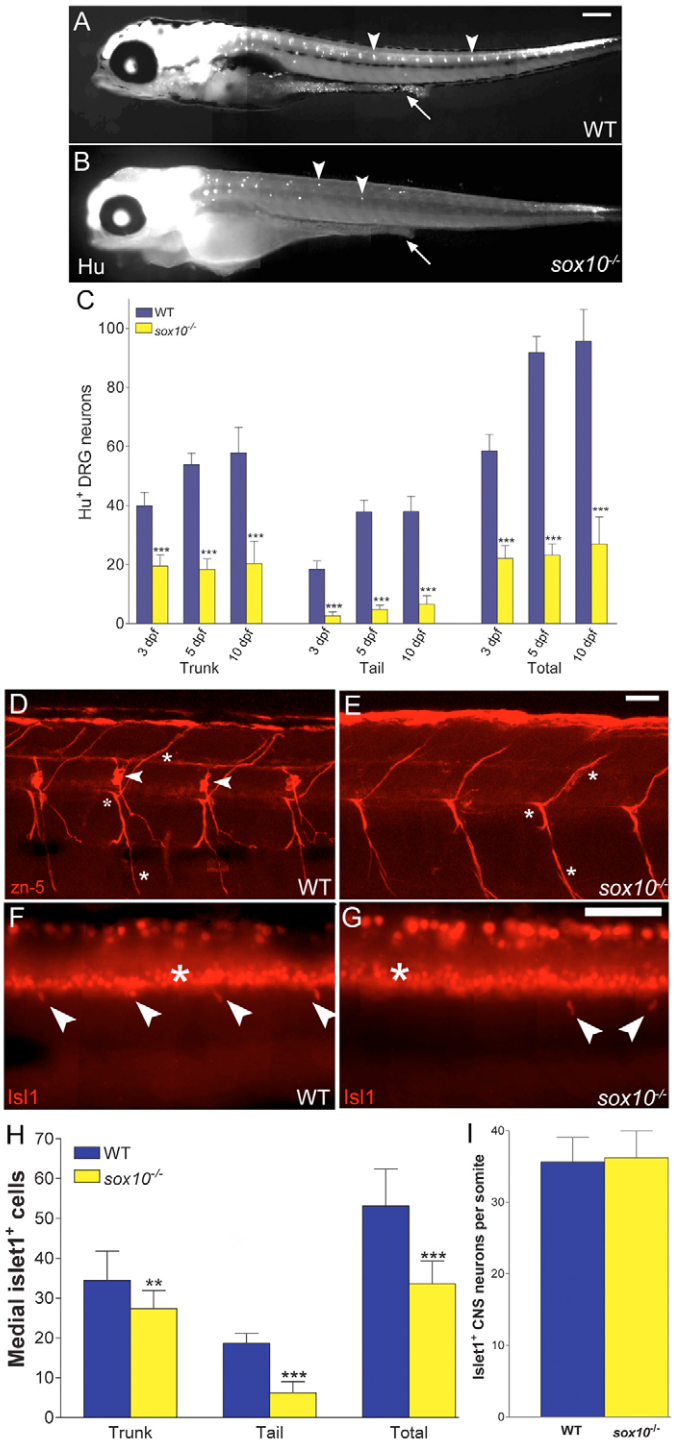

Fig. 3. *sox10* mutants display a DRG neuron phenotype. Immunofluorescent detection of Hu at 5 dpf (**A**,**B**), DM-GRASP (zn-5 antibody) at 8 dpf (**D**,**E**) and Isl-1 at 60 hpf (**F**,**G**) in wild-type (A,D,F) and *sox10* (B,E,G) embryos demonstrates the reduction in DRG sensory neurons (arrowheads) in the mutant. Quantitation (mean+s.d.) of Hu (**C**) and Isl-1 (H) phenotypes shows increasing severity posteriorly. Significant reduction in sensory neurons is noted at all stages (** $P<0.01$, *** $P<0.0001$; Student's *t*-test). $n=12$ embryos for each of wild-type and *sox10* mutants in (C) and $n=15$ (wild type) and $n=13$ (*sox10*) in (**H**). There is an absence of enteric neurons in *sox10* mutants (arrow, B). However, DM-GRASP expression in secondary motorneuron processes (*,D) is unchanged in *sox10* mutants (E). Likewise, Islet1 also labels ventral spinal cord motorneurons (*,F,G). Quantification (**I**) of these cells at 5 dpf per somite segment equivalent shows no significant difference between wild-type and *sox10* embryos ($P=0.609$; Student's *t*-test). Three consecutive segment-lengths in the tail were counted in seven embryos of each genotype. Scale bars: 200 μm in A,B; 50 μm in D-G.

segments of *sox10* mutants, but readily seen in wild-type siblings (Fig. 3D,E; data not shown). At 60 hpf Islet1$^+$ 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. 5D,E). 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.

Table 2. DRG neuron proliferation in *sox10* mutants

Stage (hpf)	Genotype	Number embryos scored	Number pH3+ neurons/ Hu+ DRG neurons scored	% proliferating DRG neurons
60	Wild type	18	279/501	55.7
	sox10$^{-/-}$	18	174/205	84.9*
72	Wild type	10	295/328	89.9
	sox10$^{-/-}$	8	94/100	94.0

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

5D). 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 dependant on *ngn1* for their development.

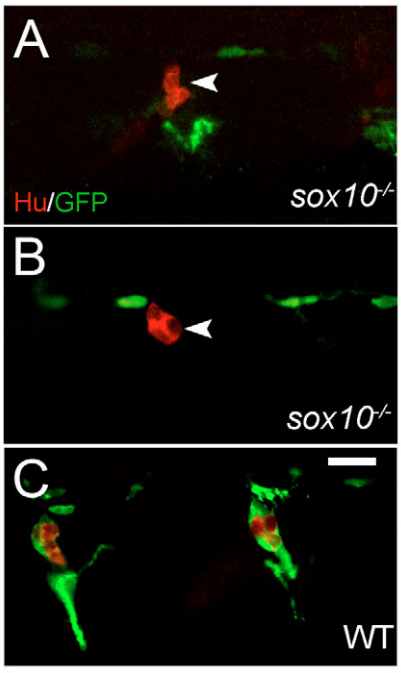

Fig. 4. DRG sensory neurons persist in *sox10* embryos without differentiated glial support. Double immunofluorescent detection of DRG neurons (Hu, red) and glia (GFP, green) in 5 dpf embryos showing two examples of *sox10* mutant DRG neuron clusters (arrowheads) lacking intimate association with GFP$^+$ cells (**A**,**B**), contrasting with consistently intimate arrangement with satellite cells in wild types (**C**). Scale bar: 20 μm.

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),

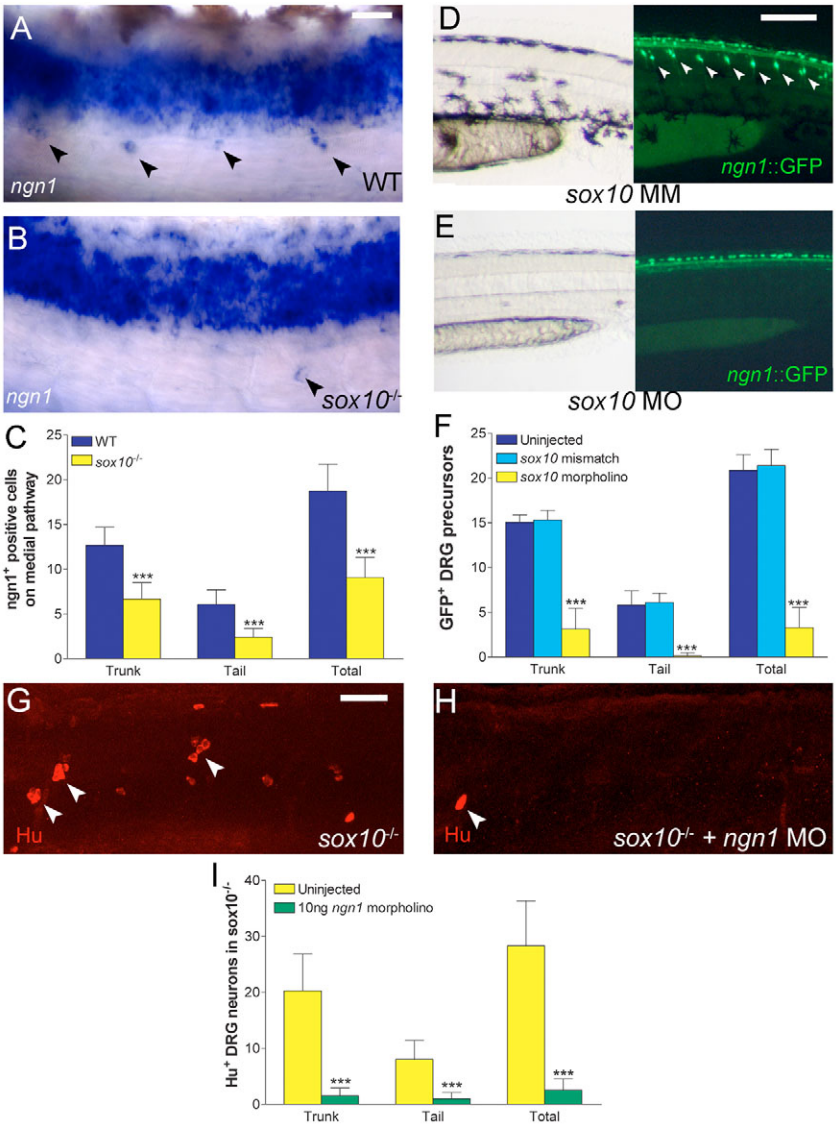

Fig. 5. Specification of a subset of DRG neurons fails in *sox10* mutants. (**A-C**) Whole mount in situ hybridisation of 36 hpf wild-type (A) and *sox10* mutant (B) embryos for *ngn1*. Quantitation (mean+s.d., C) showed highly significant reduction in mutants (*** $P<0.0001$; Student's *t*-test; $n=15$ for both data sets). (**D-F**) Injection of *sox10* morpholino (MO; E), but not 3 bp-mismatch morpholino (MM; D), into *ngn1:gfp* transgenic fish results in reduction of both melanophores (left panels) and DRG sensory neuron precursors (arrowheads, right panels) at 48 hpf. Mismatch morpholino-injected embryos are indistinguishable from uninjected embryos. Quantification (mean+s.d., F) showed highly significant effect in *sox10* morphants (*** $P<0.0001$; ANOVA with Tukey's Post Test; $n=22$ for *sox10* MO, $n=20$ for both *sox10* MM and uninjected data sets). Numbers of GFP$^+$ cells (F) are slightly greater than numbers of *ngn1*$^+$ cells (C) consistent with the later stage examined and perdurance of GFP in cells in which the promoter is no longer active. (**G-I**) Ngn1 knock down using a previously characterised morpholino in *sox10* mutant embryos results in near-complete loss of escaper DRG sensory neurons, even in the trunk (H), compared with uninjected *sox10* mutants (G). This reduction was highly significant (I) (*** $P<0.0001$; Student's *t*-test) ($n=16$ for each data set). Scale bars: 20 μm in A,B; 50 μm in G,H; 100 μm in D,E.

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>*sox10*^{m618}(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, *sox10*^{baz1}, that showed a unique phenotype compared with all reported mouse or zebrafish *sox10* alleles. Like other zebrafish *sox10* alleles, *sox10*^{baz1} is fully recessive and homozygous lethal. *sox10*$^{baz1/baz1}$ 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 *sox10*$^{baz1/baz1}$ 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

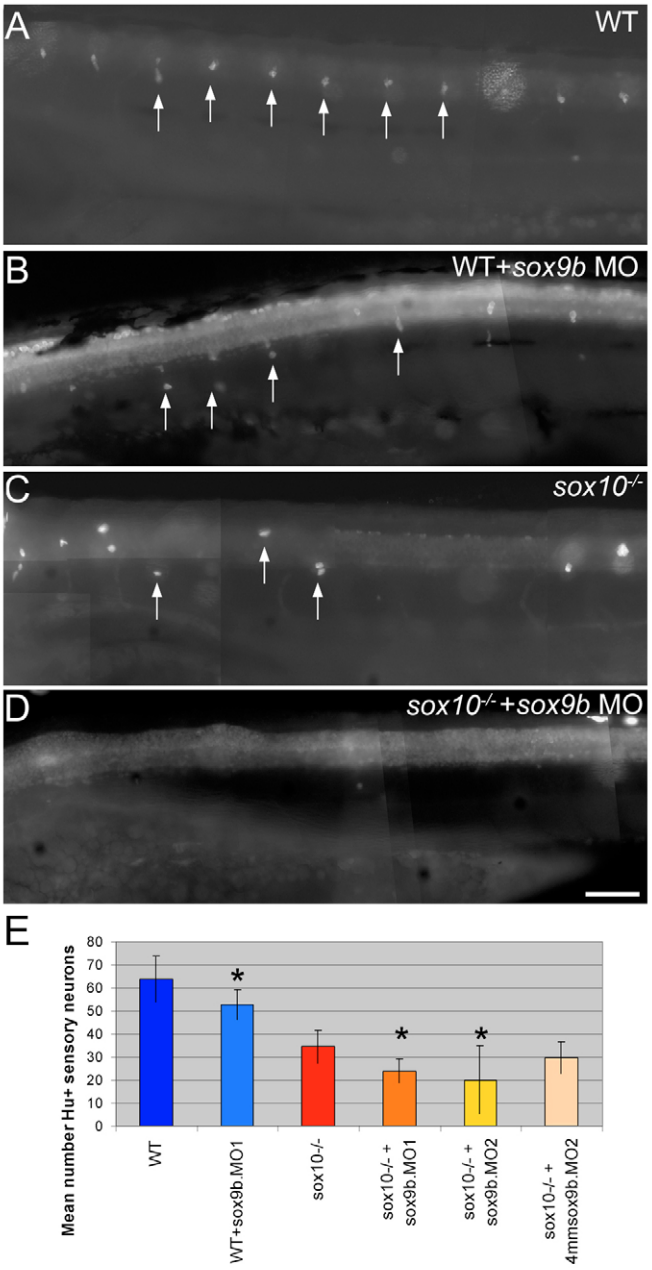

Fig. 6. Functional redundancy between *sox9b* and *sox10* in DRG neuron development. *sox9b* morpholino knock-down results in decreased Hu$^+$ sensory neuron number in 5 dpf wild-type (**A**,**B**) and *sox10* mutant (**C**,**D**) embryos. *Sox9b* morpholino injection into wild type (B) disrupts the regular DRG pattern of uninjected wild-type siblings (A), resulting in reduced numbers of DRGs (arrows), fewer DRG neurons and disorganised patterning of DRGs. These features are also typical of *sox10* mutant embryos (C), although the latter show a more severe phenotype. Injection of *sox9b* morpholino into *sox10* mutants results in a dramatic reduction in DRG sensory neurons (D). (A-D) Lateral views of trunk. (**E**) Quantitation (mean+s.d.) of total number of trunk Hu$^+$ sensory neurons in 5 dpf wild-type and *sox10* mutant embryos after injection with *sox9b* morpholinos as shown (n=10 for all conditions). Asterisk indicates statistically significant differences from respective controls (P<0.05; Student's t-test). Scale bar: 50 μm.

previously described *sox10* phenotype, 5 dpf *sox10$^{baz1/baz1}$* 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, *sox10$^{baz1/baz1}$* 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, *sox10$^{baz1/baz1}$* 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 *sox10$^{baz1/baz1}$* 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 *sox10$^{baz1/baz1}$* embryos compared with their wild-type siblings at 36 hpf (Fig. 8I,J and L). Therefore, the *sox10$^{baz1/baz1}$* 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 motorneurons (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 motorneuron 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 zebrafish *sox10* 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 motorneuron cell bodies and secondary motorneuron axons we see normal numbers of motorneurons to at least 10 dpf. We suggest that the timing of onset of glial dependency for trophic support of sensory and motorneurons 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

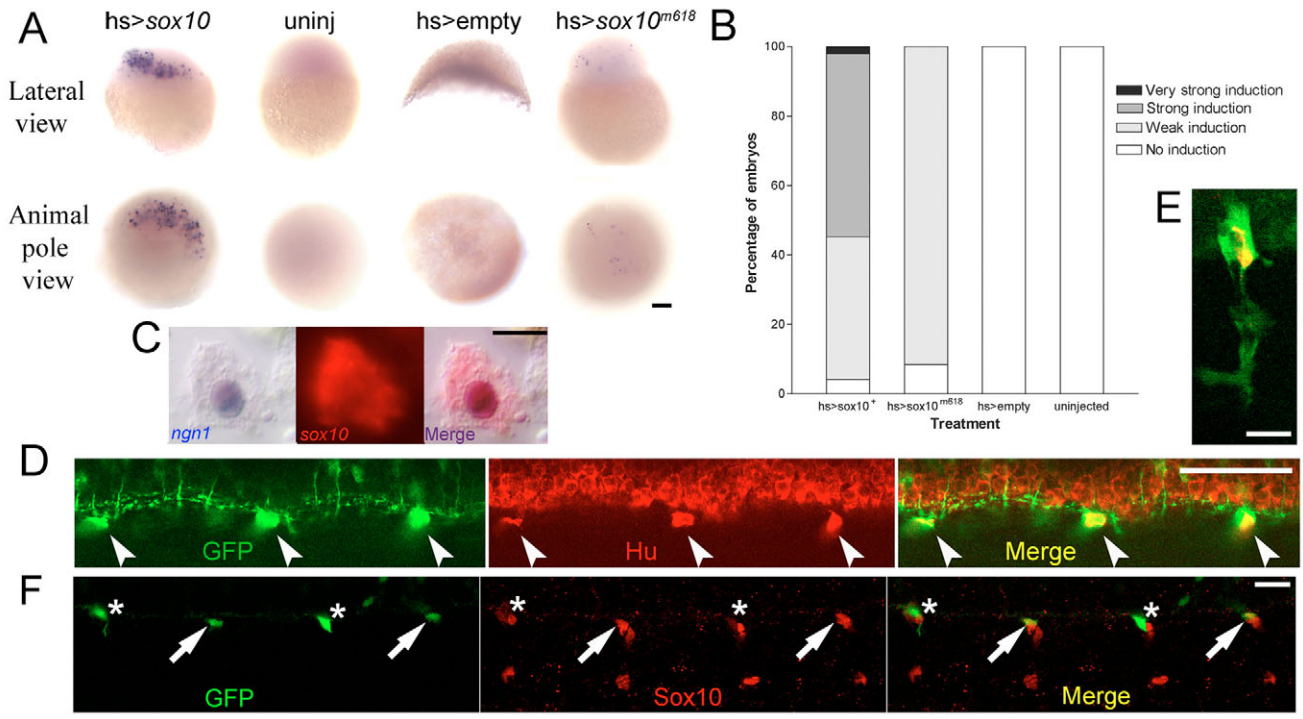

Fig. 7. Cell-autonomous induction of *ngn1* by Sox10. (**A**) In situ hybridisation reveals robust *ngn1* induction by 4 hours after injection of heat-shock construct driving wild-type *sox10* (*hs>sox10*) into one-cell stage wild-type embryos followed by 2 hour heat shock at 37°C from 2 hpf (A). Heat-shocked 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 Sox10^{m618} mutant protein (hs>*sox10*^{m618}(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). Hu^{-}/GFP^{+} glial precursors (green) surrounding the Hu^{+}/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.

insufficient to rescue the *sox10* mutant sensory neuron phenotype. Furthermore, *sox10* mutant NCCs generate sensory neurons with decreased efficiency in a wild-type environment. In conclusion, although zebrafish *sox10* mutants show equally dramatic sensory neuron defects to mouse *Sox10* mutants, secondary effects due to absence of differentiated glial cells do not explain the sensory neuron defects. Instead our data strongly indicate a direct role for Sox10 in sensory neuron specification.

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., 2001b; Kelsh and Raible, 2002). 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).

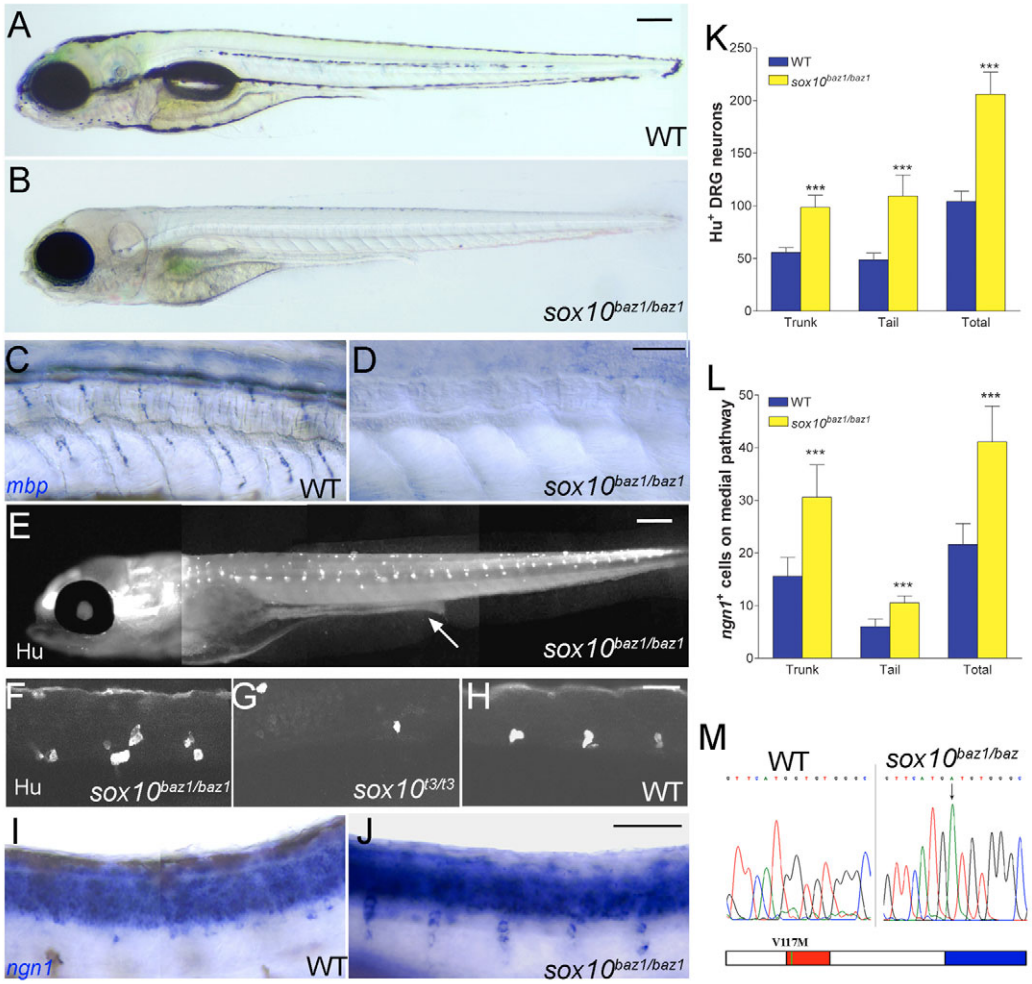

Fig. 8. *sox10*^{baz1} mutants show a unique, neurogenic DRG phenotype. (**A-H**) 5 dpf *sox10*$^{baz1/\ baz1}$ showed severe reductions in NC-derived melanophores (B) and *mbp*$^{+}$ Schwann cells of spinal nerves (D) compared with wild-type siblings (A,C). Note similar strength of these phenotypes to *sox10*$^{m618/m618}$ (see Fig. 2C-D). In contrast, Hu^{+} DRG sensory neurons were more abundant in *sox10*$^{baz1/baz1}$ embryos (E, low power; F, higher confocal magnification view of three segments of tail) than in wild type (H), and thus much more than in typical strong *sox10* alleles (G). (**I**,**J**) *sox10*$^{baz1/\ baz1}$ embryos similarly displayed an increased number of *ngn1*$^{+}$ cells on the medial migration pathway at 36 hpf (J) compared with wild-type (I). (**K**,**L**) Quantitation of these Hu^{+} (K) and *ngn1*$^{+}$ (L) DRG-associated cells in *sox10*$^{baz1/baz1}$ and siblings (***P<0.0001, Student's t-test; n=10 and n=15 embryos counted for each data set, respectively). (**M**) Sequencing identified molecular lesion in *sox10*^{baz1} as a G to A transition at cDNA position 724 (upper panel), producing a Valine to Methionine substitution at amino acid 117 within the Sox10 protein (lower panel). DNA binding domain (red) and transactivation domain (blue) are indicated. Scale bars: 200 μm in A,B,E; 50 μm in C,D,F-J.

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 *sox10*^{baz1} 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* mutant 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 *sox*^{baz1} 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 melanophore 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; Potterf 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; Potterf 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 NCC fate specification.

We thank members of our laboratories for helpful discussions and advice throughout the course of this work. We thank Bruce Appel for the kind gift of the Sox10 antibody. We thank the Wellcome Trust and MRC (R.N.K.), the University of Bath and ORS scheme (T.J.C.) and the CNRS, FRM and French Ministère de la Recherche (P.B.) for financial support that contributed to this work.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/23/4619/DC1

References

Andermann, P., Ungos, J. and Raible, D. W. (2002). Neurogenin1 defines zebrafish cranial sensory ganglia precursors. *Dev. Biol.* **251**, 45-58.

Blader, P., Fischer, N., Gradwohl, G., Guillemot, F. and Strahle, U. (1997). The activity of Neurogenin1 is controlled by local cues in the zebrafish embryo. *Development* **124**, 4557-4569.

Blader, P., Plessy, C. and Strahle, U. (2003). Multiple regulatory elements with spatially and temporally distinct activities control neurogenin1 expression in primary neurons of the zebrafish embryo. *Mech. Dev.* **120**, 211-218.

Blader, P., Lam, C. S., Rastegar, S., Scardigli, R., Nicod, J. C., Simplicio, N., Plessy, C., Fischer, N., Schuurmans, C., Guillemot, F. et al. (2004). Conserved and acquired features of neurogenin1 regulation. *Development* **131**, 5627-5637.

Bondurand, N., Kobetz, A., Pingault, V., Lemort, N., Encha-Razavi, F., Couly, G., Goerich, D. E., Wegner, M., Abitbol, M. and Goossens, M. (1998). Expression of the *SOX10* gene during human development. *FEBS Lett.* **432**, 168-172.

Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Le Caignec, C., Wegner, M. and Goossens, M. (2000). Interaction among SOX10 PAX3 and MITF, three genes altered in Waardenburg syndrome. *Hum. Mol. Genet.* **9**, 1907-1917.

Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C. and Wegner, M. (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15**, 66-78.

Brosamle, C. and Halpern, M. E. (2002). Characterization of myelination in the developing zebrafish. *Glia* **39**, 47-57.

Cheng, Y. C., Cheung, M., Abu-Elmagd, M. M., Orme, A. and Scotting, P. J. (2000). Chick *Sox10*, a transcription factor expressed in both early neural crest cells and central nervous system. *Dev. Brain Res.* **121**, 233-241.

Chiang, E. F., Pai, C. I., Wyatt, M., Yan, Y. L., Postlethwait, J. and Chung, B. (2001). Two sox9 genes on duplicated zebrafish chromosomes: expression of similar transcription activators in distinct sites. *Dev. Biol.* **231**, 149-163.

Cornell, R. A. and Eisen, J. S. (2002). Delta/Notch signaling promotes formation of zebrafish neural crest by repressing Neurogenin 1 function. *Development* **129**, 2639-2648.

Dorsky, R. I., Raible, D. W. and Moon, R. T. (2000). Direct regulation of nacre, a zebrafish MITF homolog required for pigment cell formation, by the Wnt pathway. *Genes Dev.* **14**, 158-162.

Dutton, K., Dutton, J. R., Pauliny, A. and Kelsh, R. N. (2001a). A morpholino phenocopy of the colourless mutant. *Genesis* **30**, 188-189.

Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., Geisler, R., Haffter, P. and Kelsh, R. N. (2001b). Zebrafish *colourless* encodes *sox10* and specifies non- ectomesenchymal neural crest fates. *Development* **128**, 4113-4125.

Elworthy, S., Lister, J. A., Carney, T. J., Raible, D. W. and Kelsh, R. N. (2003). Transcriptional regulation of mitfa accounts for the sox10 requirement in zebrafish melanophore development. *Development* **130**, 2809-2818.

Elworthy, S., Pinto, J. P., Pettifer, A., Cancela, M. L. and Kelsh, R. N. (2005). Phox2b function in the enteric nervous system is conserved in zebrafish and is sox10-dependent. *Mech. Dev.* **122**, 659-669.

Fashena, D. and Westerfield, M. (1999). Secondary motoneuron axons localize DM-GRASP on their fasciculated segments. *J. Comp. Neurol.* **406**, 415-424.

Greenwood, A. L., Turner, E. E. and Anderson, D. J. (1999). Identification of dividing, determined sensory neuron precursors in the mammalian neural crest. *Development* **126**, 3545-3559.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J., Jiang, Y. J., Heisenberg, C. P. et al. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. *Development* **123**, 1-36.

Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., Lemort, N., Goossens, M. and Wegner, M. (1998). Mutation of the Sry-related *Sox10* gene in *Dominant megacolon*, a mouse model for human Hirschsprung disease. *Proc. Natl. Acad. Sci. USA* **95**, 5161-5165.

Inoue, K., Tanabe, Y. and Lupski, J. R. (1999). Myelin deficiencies in both the central and the peripheral nervous systems associated with a SOX10 mutation. *Ann. Neurol.* **46**, 313-318.

Kapur, R. P. (1999). Early death of neural crest cells is responsible for total enteric aganglionosis in *Sox10*^{Dom}/*Sox10*^{Dom} mouse embryos. *Pediatr. Dev. Pathol.* **2**, 559-569.

Kelsh, R. N. (2006). Sorting out Sox10 functions in neural crest development. *BioEssays* **28**, 788-798.

Kelsh, R. N. and Eisen, J. S. (2000). The zebrafish *colourless* gene regulates development of non- ectomesenchymal neural crest derivatives. *Development* **127**, 515-525.

Kelsh, R. N. and Raible, D. W. (2002). Specification of zebrafish neural crest. In *Pattern Formation in Zebrafish* (ed. L. Solnicka-Kresel), pp. 216-236. Berlin, Heidelberg, New York: Springer-Verlag.

Kelsh, R. N., Brand, M., Jiang, Y. J., Heisenberg, C. P., Lin, S., Haffter, P., Odenthal, J., Mullins, M. C., van Eeden, F. J. M., Furutani-Seiki, M. et al. (1996). Zebrafish pigmentation mutations and the processes of neural crest development. *Development* **123**, 369-389.

Kelsh, R. N., Dutton, K., Medlin, J. and Eisen, J. S. (2000). Expression of zebrafish *fkd6* in neural crest-derived glia. *Mech. Dev.* **93**, 161-164.

Kim, J., Lo, L., Dormand, E. and Anderson, D. J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. *Neuron* **38**, 17-31.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.

Le Douarin, N. M. and Kalcheim, C. (1999). *The Neural Crest*. Cambridge: Cambridge University Press.

Lee, H. Y., Kleber, M., Hari, L., Brault, V., Suter, U., Taketo, M. M., Kemler, R. and Sommer, L. (2004). Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. *Science* **303**, 1020-1023.

Lee, M., Goodall, J., Verastegui, C., Ballotti, R. and Goding, C. R. (2000). Direct regulation of the Microphthalmia promoter by Sox10 links Waardenburg-Shah syndrome (WS4)-associated hypopigmentation and deafness to WS2. *J. Biol. Chem.* **275**, 37978-37983.

Li, M., Zhao, C. T., Wang, Y., Zhao, Z. X. and Meng, A. M. (2002). Zebrafish sox9b is an early neural crest marker. *Dev. Genes Evol.* **212**, 203-206.

Ma, Q. F., Fode, C., Guillemot, F. and Anderson, D. J. (1999). NEUROGENIN1 and NEUROGENIN2 control two distinct waves of neurogenesis in developing dorsal root ganglia. *Genes Dev.* **13**, 1717-1728.

Malicki, J., Schier, A. F., SolnicaKrezel, L., Stemple, D. L., Neuhauss, S. C. F., Stainier, D. Y. R., Abdelilah, S., Rangini, Z., Zwartkruis, F. and Driever, W. (1996). Mutations affecting development of the zebrafish ear. *Development* **123**, 275-283.

Marusich, M. F., Furneaux, H. M., Henion, P. D. and Weston, J. A. (1994). Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.* **25**, 143-155.

Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G. and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell* **101**, 499-510.

Paratore, C., Goerich, D. E., Suter, U., Wegner, M. and Sommer, L. (2001). Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development* **128**, 3949-3961.

Park, H. C., Boyce, J., Shin, J. and Appel, B. (2005). Oligodendrocyte specification in zebrafish requires notch-regulated cyclin-dependent kinase inhibitor function. *J. Neurosci.* **25**, 6836-6844.

Perez, S. E., Rebelo, S. and Anderson, D. J. (1999). Early specification of sensory neuron fate revealed by expression and function of neurogenins in the chick embryo. *Development* **126**, 1715-1728.

Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D. E., Prehu, M. O., Puliti, A., Herbarth, B., Hermans-Borgmeyer, I., Legius, E., Matthijs, G. et al. (1998). SOX10 mutations in patients with Waardenburg-Hirschsprung disease. *Nat. Genet.* **18**, 171-173.

Pingault, V., Guiochon-Mantel, A., Bondurand, N., Faure, C., Lacroix, C., Lyonnet, S., Goossens, M. and Landrieu, P. (2000). Peripheral neuropathy with hypomyelination, chronic intestinal pseudo-obstruction and deafness: a developmental "neural crest syndrome" related to a SOX10 mutation. *Ann. Neurol.* **48**, 671-676.

Potterf, S. B., Furumura, M., Dunn, K. J., Arnheiter, H. and Pavan, W. J. (2000). Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. *Hum. Genet.* **107**, 1-6.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-730.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Sonnenberg-Riethmacher, E., Miehe, M., Stolt, C. C., Goerich, D. E., Wegner, M. and Riethmacher, D. (2001). Development and degeneration of dorsal root ganglia in the absence of the HMG-domain transcription factor Sox10. *Mech. Dev.* **109**, 253-265.

Southard-Smith, E. M., Kos, L. and Pavan, W. J. (1998). Sox10 mutation disrupts neural crest development in DOM Hirschsprung mouse model. *Nat. Genet.* **18**, 60-64.

Takeda, K., Yasumoto, K., Takada, R., Takada, S., Watanabe, K., Udono, T., Saito, H., Takahashi, K. and Shibahara, S. (2000). Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. *J. Biol. Chem.* **275**, 14013-14016.

Touraine, R. L., Attie-Bitach, T., Manceau, E., Korsch, E., Sarda, P., Pingault, V., Encha-Razavi, F., Pelet, A., Auge, J., Nivelon-Chevallier, A. et al. (2000). Neurological phenotype in Waardenburg syndrome type 4 correlates with novel SOX10 truncating mutations and expression in developing brain. *Am. J. Hum. Genet.* **66**, 1496-1503.

Ungos, J. M., Karlstrom, R. O. and Raible, D. W. (2003). Hedgehog signaling is directly required for the development of zebrafish dorsal root ganglia neurons. *Development* **130**, 5351-5362.

Verastegui, C., Bille, K., Ortonne, J. P. and Ballotti, R. (2000). Regulation of the microphthalmia-associated transcription factor gene by the Waardenburg syndrome type 4 gene, SOX10. *J. Biol. Chem.* **275**, 30757-30760.

Wada, N., Javidan, Y., Nelson, S., Carney, T. J., Kelsh, R. N. and Schilling, T. F. (2005). Hedgehog signaling is required for cranial neural crest morphogenesis and chondrogenesis at the midline in the zebrafish skull. *Development* **132**, 3977-3988.

Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-2821.

Wegner, M. and Stolt, C. C. (2005). From stem cells to neurons and glia: a Soxist's view of neural development. *Trends Neurosci.* **28**, 583-588.

Yan, Y. L., Willoughby, J., Liu, D., Crump, J. G., Wilson, C., Miller, C. T., Singer, A., Kimmel, C., Westerfield, M. and Postlethwait, J. H. (2005). A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development. *Development* **132**, 1069-1083.

Yasumoto, K., Takeda, K., Saito, H., Watanabe, K., Takahashi, K. and Shibahara, S. (2002). Microphthalmia-associated transcription factor interacts with LEF-1, a mediator of Wnt signaling. *EMBO J.* **21**, 2703-2714.

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

Experimental series	Genotype	Number of transplants	Number of DRG with donor contribution	Total donor DRG neurons	Mean+s.d. number neurons/ chimeric DRG
A	WT → WT sibling	43	46	53	1.15+0.47
	WT → *sox10*$^{-/-}$	18	21	30	1.43+0.75
B	WT sibling → WT	68	54	87	1.61+0.98
	sox10$^{-/-}$ → WT	30	14	7	0.50+0.52*

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