

# The transcription factor Sox10 is a key regulator of peripheral glial development

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The molecular mechanisms that determine glial cell fate in the vertebrate nervous system have not been elucidated. Peripheral glial cells differentiate from pluripotent neural crest cells. We show here that the transcription factor Sox10 is a key regulator in differentiation of peripheral glial cells. In mice that carry a spontaneous or a targeted mutation of *Sox10*, neuronal cells form in dorsal root ganglia, but Schwann cells or satellite cells are not generated. At later developmental stages, this lack of peripheral glial cells results in a severe degeneration of sensory and motor neurons. Moreover, we show that Sox10 controls expression of *ErbB3* in neural crest cells. *ErbB3* encodes a Neuregulin receptor, and down-regulation of *ErbB3* accounts for many changes in development of neural crest cells observed in *Sox10* mutant mice. Sox10 also has functions not mediated by ErbB3, for instance in the melanocyte lineage. Phenotypes observed in heterozygous mice that carry a targeted *Sox10* null allele reproduce those observed in heterozygous *Sox10<sup>Dom</sup>* mice. Haploinsufficiency of *Sox10* can thus cause pigmentation and megacolon defects, which are also observed in *Sox10<sup>Dom</sup>/+* mice and in patients with Waardenburg-Hirschsprung disease caused by heterozygous *SOX10* mutations.

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Neural crest cells detach from the dorsal neural tube and migrate over large distances in the embryo, using characteristic pathways. On arrival at their targets, they differentiate to form the majority of the peripheral nervous system, as well as other cell types and tissues including melanocytes (Le Douarin and Kalcheim 1999). Glial cells of the peripheral nervous system are generated by neural crest cells. These glia include satellite cells located in ganglia as well as Schwann cells, which ensheath peripheral axons. The differentiation of glial cells is thought to be regulated by the neurons they accompany. In vitro, transient activation of Notch signaling suffices to suppress the neurogenic differentiation of neural crest cells and accelerates glial differentiation (Morrison et al. 2000; Wakamatsu et al. 2000). Moreover, differentiation of neural crest cells into glia is promoted by Neuregulin-1, an EGF-like factor that activates ErbB receptors (Shah et al. 1994). Melanocytes represent another major derivative of the neural crest. They originate as nonpigmented

precursors and migrate along characteristic dorso-lateral pathways to the epidermis. Development of the melanocyte lineage has been well studied genetically, as mutations in genes essential for their development cause pigmentation defects easily identifiable in mice and man (Goding 2000).

Sox10 was found because of its sequence homology to transcription factors of the SRY family, which contain a DNA-binding domain of the high mobility group (HMG) box family (Kuhlbrodt et al. 1998a; Wegner 1999). As yet, >20 members of the Sox gene family have been identified in mammals, which play important roles in diverse developmental processes such as sex determination, chondrogenic differentiation, or hematopoiesis (Wegner 1999). *Sox10* expression is initiated in neural crest cells as they dissociate from the neural tube, and expression is maintained during neural crest cell migration. Expression continues in the glial and melanocyte lineages, but *Sox10* is turned off in many other neural crest cell derivatives (Herbarth et al. 1998; Kuhlbrodt et al. 1998a; Pusch et al. 1998). In the heterozygous state, spontaneous mutations of *Sox10* interfere with the development of melanocytes and of the enteric nervous system, causing pigmentation changes and megacolon. Such mutations have been identified in mice, the Dominant megacolon mutation (*Sox10<sup>Dom</sup>*), and in patients afflicted

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with Waardenburg syndrome type 4 (Herbarth et al. 1998; Kuhlbrodt et al. 1998b; Pingault et al. 1998; Southard-Smith et al. 1998, 1999). Moreover, myelination defects in the central and peripheral nervous systems were noted in certain patients with heterozygous *Sox10* mutations (Inoue et al. 1999; Pingault et al. 2000; Touraine et al. 2000). In accordance, Sox10 controls expression of myelin protein genes like *P<sub>0</sub>* and binds to the *P<sub>0</sub>* promoter (Peirano et al. 2000). Homozygous *Sox10<sup>Dom</sup>* mutant mice display severe deficits in the peripheral nervous system, which include a lack of enteric ganglia and a severe hypoplasia of the sympathetic ganglion chain (Herbarth et al. 1998; Southard-Smith et al. 1998; Kapur 1999).

The spontaneous *Sox10* mutations characterized are nonsense or frameshift mutations. For instance, a frameshift mutation generated the murine *Sox10<sup>Dom</sup>* allele, which encodes a protein in which the N-terminal 193 amino acids of Sox10, including the HMG box, are preserved and fused to 99 amino acids encoded by a different reading frame (Herbarth et al. 1998; Southard-Smith et al. 1998). Similarly, known human *Sox10* mutations are predicted to generate truncated proteins that retain functional sequences, such as a homodimerization domain, a synergy region, or the DNA-binding domain (Kuhlbrodt et al. 1998b; Pingault et al. 1998; Bondurand et al. 1999; Inoue et al. 1999; Southard-Smith et al. 1999; Peirano and Wegner 2000; Pingault et al. 2000; Touraine et al. 2000). Indeed, the proteins encoded by many of the spontaneously mutated *Sox10* alleles have unaltered DNA-binding properties. It was therefore suggested that the developmental defects observed in Waardenburg-Hirschsprung disease are caused by a dominant-negative action of the mutant Sox10 protein (Kuhlbrodt et al. 1998b; Pingault et al. 1998; Southard-Smith et al. 1999).

*ErbB3* encodes a member of the family of EGF receptor tyrosine kinases, binds Neuregulins with high affinity, and requires ErbB2 as a coreceptor for signaling in vivo (Adlkofer and Lai 2000; Garratt et al. 2000). The expression of *ErbB3*, like that of *Sox10*, is initiated in neural crest cells as they dissociate from the neural tube and is maintained in glia but downregulated in other derivatives of neural crest cells (Meyer and Birchmeier 1995; Meyer et al. 1997). Additional, nonoverlapping expression domains of *Sox10* and *ErbB3* in other tissues exist. *ErbB3<sup>-/-</sup>* mice and other mutants of the Neuregulin signaling system display defects in development of neural crest cells and their derivatives, which include a lack of Schwann cells (Erickson et al. 1997; Riethmacher et al. 1997; Britsch et al. 1998; Morris et al. 1999; Woldeyesus et al. 1999; Garratt et al. 2000; Wolpowitz et al. 2000). An additional, conspicuous phenotype in such mice is the degeneration of sensory and motor neurons. ErbB3 is required cell autonomously for the development of Schwann cells, but not for survival of sensory and motor neurons (Riethmacher et al. 1997). The neurodegeneration observed in such mutant mice is thus caused by indirect mechanisms.

We report here the generation of a targeted *Sox10* mutation in mice, in which the complete open reading

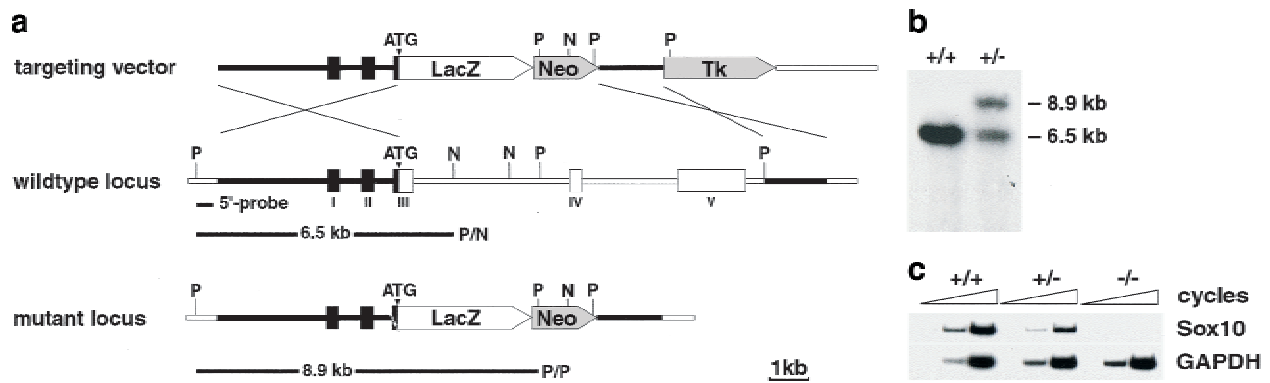
frame of *Sox10* is replaced by *lacZ* sequences (*Sox10<sup>lacZ</sup>*). In a heterozygous state, the *Sox10<sup>lacZ</sup>* mutation causes phenotypes that reproduce those of the spontaneous *Sox10<sup>Dom</sup>* allele. Thus, haploinsufficiency can account for megacolon and pigmentation defects. In homozygous *Sox10* mutant mice, sensory neurons form in dorsal root ganglia, but satellite cells or Schwann cells do not develop, demonstrating a key role of this transcription factor in the development of peripheral glial cells. The similarities in expression patterns of *Sox10* and *ErbB3* prompted us to investigate a genetic interaction between the two genes. We demonstrate here that appropriate *ErbB3* expression in neural crest cells, but not in other tissues like muscle or skin, requires Sox10. In accordance, *Sox10* and *ErbB3* mutant mice share phenotypes. These include a conspicuous degeneration of sensory and motor neurons. This finding allows us to assign, unequivocally, a trophic function to glial cells in the maintenance of neurons.

## Results

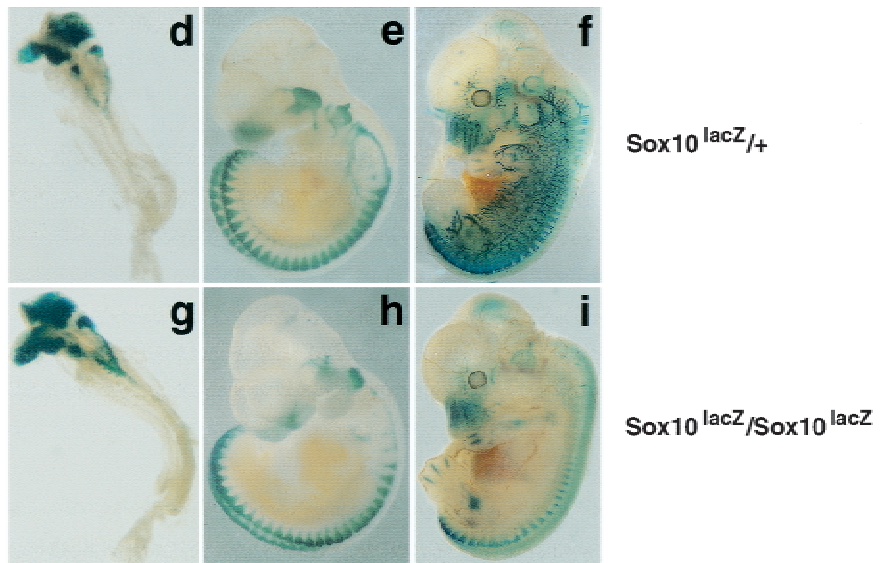
### Generation of *Sox10<sup>lacZ</sup>* mice

To mutate *Sox10*, a targeting vector was constructed that deleted the coding sequences and fused *lacZ* in frame to the *Sox10* initiation codon (Fig. 1a). The targeting vector was introduced into ES cells and homologous recombination events were identified (Fig. 1b; data not shown). Mutant ES cells were used to generate chimeras that transmitted the *Sox10<sup>lacZ</sup>* allele to their offspring (see also Materials and Methods). Heterozygous *Sox10<sup>lacZ</sup>* mutant mice were crossed with C3HeB/FeJLe-a/a (C3H) mice; this strain was chosen because of its widespread use in the analysis of phenotypes of the *Sox10<sup>Dom</sup>* allele (Lane and Liu 1984; Herbarth et al. 1998; Southard-Smith et al. 1998, 1999). In the first three backcross generations, heterozygous *Sox10<sup>lacZ</sup>* mice were indistinguishable from wild-type littermates. In the fourth generation, they became recognizable by pigmentation defects, and most displayed a white belly spot. Further experiments described here were performed with mice of the fourth or subsequent generations of backcrosses with C3H mice.

A significant proportion of heterozygous *Sox10<sup>lacZ</sup>* mice were lost during the first postnatal weeks. At the time of weaning, heterozygous mice were under-represented, constituting 38.2% of the progeny (259 animals examined), but perinatally the expected Mendelian ratio was observed. Dissection demonstrated the occurrence of megacolon in a fraction of the heterozygous *Sox10<sup>lacZ</sup>* animals (data not shown). Megacolon, which reduces postnatal survival, has also been reported previously for heterozygous *Sox10<sup>Dom</sup>* mice (Lane and Liu 1984; Southard-Smith et al. 1999). Among offspring of heterozygous intercrosses, no *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* animals were observed. To determine the time of death, embryos were genotyped at various stages of development. Live *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* embryos were found in ratios close to the expected Mendelian frequencies before and at day



**Figure 1.** Targeted deletion of *Sox10* and expression of the *Sox10<sup>lacZ</sup>* allele in mice. (a) Schematic representation of the targeting strategy employed to disrupt the *Sox10* gene. Shown are the targeting vector (top), the wild-type *Sox10* locus (middle) and the mutated locus after homologous recombination (bottom). Noncoding and coding exons are represented by black or open boxes, respectively. Restriction sites for *SpeI* (P) and *NcoI* (N) as well as the location of the 5' probe are indicated. (b) Southern blot analysis of DNA from adult heterozygous (+/-) and wild-type (+/+) mice after digestion with *NcoI/SpeI*. The expected size of the fragments detected by the probe used for hybridization are shown (a). (c) RT-PCR analysis of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) embryos at E12.5 using primers specific for the coding region of *Sox10*. Results from 20, 23, and 26 cycles are shown for each genotype. *Sox10<sup>lacZ</sup>/+* (d-f) and *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* (g-i) embryos at E8.5 (d,g), E10.5 (e,h), and E12.5 (f,i) after X-gal staining.



16.5 of development (E16.5), but the ratio was lower at E18.5 (13.9%, 72 animals examined). *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* mice did not exhibit any gross morphological abnormalities at birth, their hearts did beat, but they were cyanotic, did not breathe, and their lung alveoli did not expand. Moreover, their forelimb posture was abnormal, and they did not move or react to tactile stimulation. A failure to breathe appears to cause the perinatal death of *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* mice. *Sox10* expression was analyzed by semiquantitative RT-PCR (Fig. 1c), Northern blot analysis, and in situ hybridization (data not shown). As expected, we were unable to detect *Sox10* transcripts in *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* mice. Compared with wild-type littermates, *Sox10* mRNA levels were reduced by approximately half in the heterozygotes.

*Expression of β-galactosidase in Sox10<sup>lacZ</sup> mice*

The *lacZ* sequence present in the *Sox10<sup>lacZ</sup>* allele enables the visualization of its expression by β-galactosidase staining (Fig. 1d-f). At E8.5, β-galactosidase staining was detected in the cephalic fold and in neural crest cells emerging from the rostral neural tube (Fig. 1d). At E10.5,

β-galactosidase staining was prominent in neural crest cells migrating to and in the gut, in sensory ganglia of the head and trunk, in the primary sympathetic ganglion chain, or in cells localized along the developing axon tracts. Staining was also observed in the otic and nasal placodes (Fig. 1e). At E12.5, β-galactosidase staining in Schwann cell precursors located along peripheral nerves was prominent. Staining was also detected in single cells located subcutaneously, that is, melanoblasts, and in oligodendrocyte precursors of the central nervous system (Fig. 1f; data not shown). These sites of *lacZ* expression are identical to those reported for *Sox10* (Kuhlbrodt et al. 1998a; Pusch et al. 1998). Additional weak β-galactosidase staining was noted in mesenchymal condensations of the digits, radius, and ulna. Possibly, low levels of expression in these structures precluded their previous identification.

A comparison of β-galactosidase staining in heterozygous and homozygous *Sox10<sup>lacZ</sup>* mice revealed changes in the distribution of neural crest cells (Fig. 1d-i). At E8.5, generation and emigration of neural crest cells were unaltered (Fig. 1g). Differences in the distribution of β-galactosidase-positive cells became apparent at

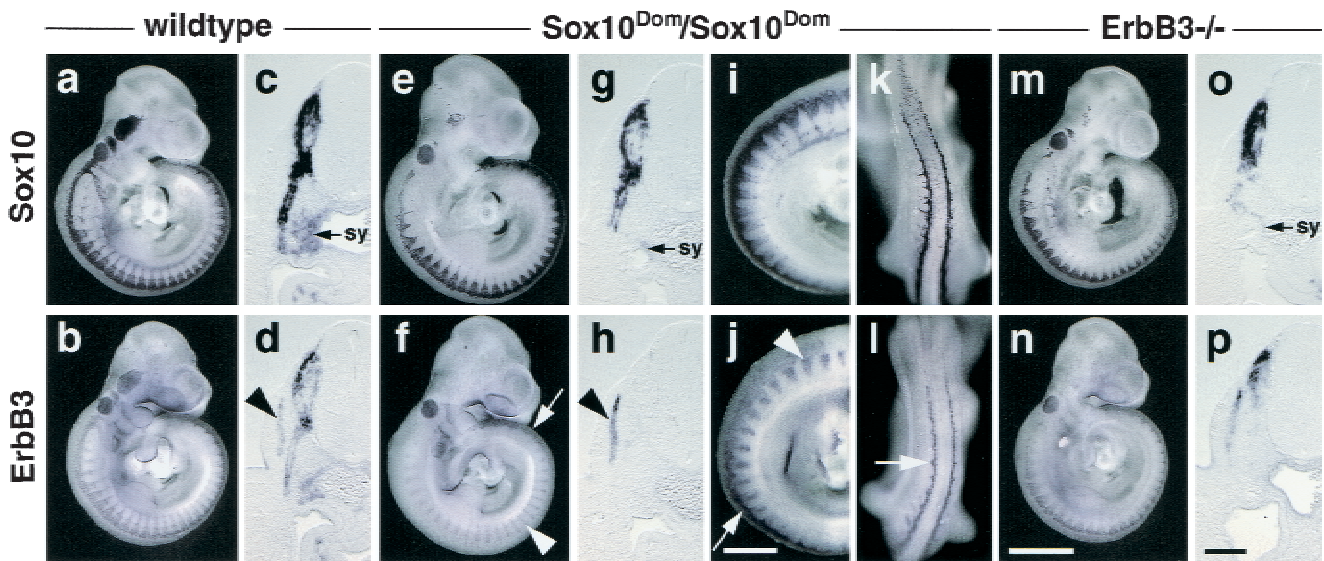
E10.5, that is, reduced numbers of stained cells in cranial ganglia and along their projections in *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* embryos (Fig. 1, cf. h and e).  $\beta$ -Galactosidase-positive cells in the gut were absent and their numbers were reduced in the sympathetic primordium. At E12.5, differences were pronounced: In dorsal root ganglia, we noted a rostro-caudal gradient in the staining intensity in *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* mice not present in the heterozygous mice (Fig. 1i). Most conspicuous was the severe reduction of  $\beta$ -galactosidase-positive cells along peripheral nerves. In contrast, staining in the olfactory epithelium, the otic vesicles, and the central nervous system persisted, as did the staining associated with developing cartilage. Many of these changes in *Sox10<sup>lacZ</sup>/Sox10<sup>lacZ</sup>* mice reflect phenotypes described previously in *Sox<sup>Dom</sup>/Sox<sup>Dom</sup>* mice and, in general, we did not observe phenotypic differences in the two mutants (Herbarth et al. 1998; Southard-Smith et al. 1998; Kapur 1999).

#### *Sox10* controls expression of *ErbB3*

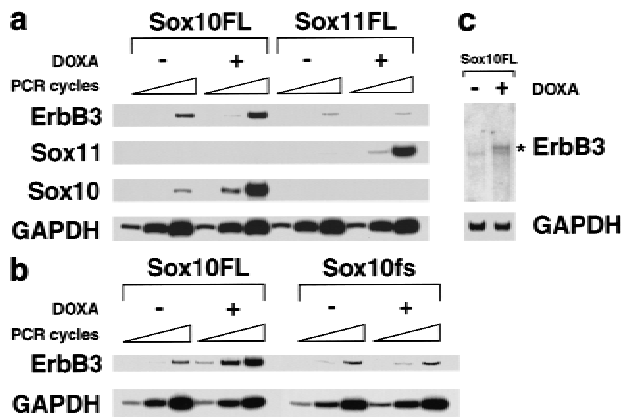
*Sox10* and *ErbB3* are expressed in similar patterns in neural crest cells (Fig. 2a–d) and overlaps in phenotypes of *Sox10* and *ErbB3* mutants exist (see also below). We therefore tested whether the two genes interact genetically. When *ErbB3* is used as a probe to detect neural crest cells in *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* mice, expression is markedly down-regulated (Fig. 2f,h,j). Other genes expressed by neural crest cells, like *p75<sup>NTR</sup>*, *Cadherin-6*, or *Sox10* are not down-regulated; note that *Sox10* probes reveal the distribution of neural crest cells in *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>*, as a transcript is produced from the mutant

allele (Fig. 2e–k; data not shown). The severe down-regulation of *ErbB3* is pronounced at E10.5, but *ErbB3*-positive neural crest cells are present caudally, where neural crest cells are still generated (arrow in Fig. 2f). Similarly, on E9.0 a rostro-caudal gradient in down-regulation was noted in *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* mutants. Rostrally, *ErbB3* expression was down-regulated, but caudally, neural crest cells that just emerged from the neural tube still express *ErbB3* (arrows in Fig. 2j,l). Thus, *ErbB3* expression is initiated in neural crest cells of *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* mutants, but later, when the cells have moved away from their origin, it is not maintained. *ErbB3* is not only expressed in neural crest cells and their derivatives, but also in the myotome (arrowhead in Fig. 2d); the latter expression domain is not shared with *Sox10* and myotome expression of *ErbB3* is not affected in *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* mice (arrowheads in Fig. 2f,h,j).

To test the effect of *Sox10* on *ErbB3* expression in vitro, *Sox10* was expressed in N2A neuroblastoma cells in an inducible manner using the *tet-on* system, and transcript levels were measured by semiquantitative RT-PCR (Peirano et al. 2000). In the presence of doxycycline, *Sox10* transcript levels increased, as did the levels of transcripts from the endogenous *ErbB3* gene (Fig. 3a). This was confirmed by Northern blot analysis (Fig. 3c). In contrast, expression of *Sox11* had no effect on *ErbB3* mRNA levels, nor did the expression of a cDNA that encodes the *Sox10*fs variant derived from the *Sox10<sup>Dom</sup>* allele (Fig. 3a,b). Whether the effect of *Sox10* on *ErbB3* expression is direct is unclear. The kinetics of induction favor such a model, but reporter gene assays in transiently transfected N2A cells failed to identify a *Sox10*-responsive element in 40 kb of genomic *ErbB3*



**Figure 2.** *Sox10* controls expression of *ErbB3*. Whole-mount in situ hybridizations of wild-type (a–d), *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* (e–l), and *ErbB3*<sup>-/-</sup> (m–p) embryos with probes specific for *Sox10* (top), and *ErbB3* (bottom). Shown are lateral (a,b,e,f,m,n) views of E10.5 embryos and the corresponding vibratome sections (c,d,g,h,o,p) on forelimb levels. Lateral (i,j) and dorsal (k,l) views of *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* embryos at E9.0. Arrows in f, j, and l point toward *ErbB3*-expressing cells close to the neural tube in *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* embryos; arrows in c, g, and o point toward the anlage of the primary sympathetic ganglion chain (sy); arrowheads in d, f, h, and j point toward the myotome that also expresses *ErbB3*. Bars (a,b,e,f,m,n) 1 mm; (c,d,g,h,o,p) 100  $\mu$ m; (i–l) 300  $\mu$ m.



**Figure 3.** Sox10 increases endogenous *ErbB3* expression in N2A cells. (a,b) RT-PCR analysis of mRNA obtained from N2A cells that express Sox10, Sox11, or a mutant version of Sox10 (FL, wild-type cDNAs; Sox10fs, *Sox10<sup>Dom</sup>* mutant; Kuhlbrodt et al. 1998b) in an inducible manner. Doxycycline (DOXA) was used to induce the expression of the transcription factors (+), which was compared to the uninduced state (-). ErbB3 transcript levels were compared in the various transfectants by semiquantitative PCR, using increasing numbers of amplification cycles (20, 23, and 26); in addition, the expression of *GAPDH*, *Sox10*, and *Sox11* was monitored. (c) Northern blot analysis on 2  $\mu$ g of poly(A)<sup>+</sup> RNA from uninduced (left) and doxycycline-treated (right) N2A cells that express Sox10 in an inducible manner. The ErbB3-specific signal is indicated by an asterisk.

DNA (M. Wegner, R. Peirano, and D. Riethmacher, unpubl.).

*Common phenotypes in ErbB3 and Sox10 mutant mice*

If Sox10 is essential for correct expression of *ErbB3*, phenotypes of *ErbB3* mutant mice should be present in *Sox10* mutants, which is indeed the case. Cranial ganglia and their projections can be visualized in control mice by in situ hybridization using a Sox10 probe, but only remnants are present in *ErbB3<sup>-/-</sup>* and *Sox10* mutant mice (Fig. 2a,e,m). Also, the morphology of cranial ganglia in both mutants is changed in a similar manner when analyzed by histology or with antineurofilament antibodies (Meyer and Birchmeier 1995; Erickson et al. 1997; Meyer et al. 1997; Riethmacher et al. 1997; Herbarth et al. 1998). This reflects the absence of those portions of cranial ganglia that derive from neural crest cells. Furthermore, Sox10-positive cells are abundant along the axon tracts in control embryos at E10.5 and are significantly reduced in numbers in *ErbB3* and *Sox10* mutants (Fig. 2, cf. e,g and m,o to control in a,c). This phenotype appears somewhat less severe in *Sox10* than in *ErbB3* mutants, which we attribute to the transient expression of *ErbB3* in neural crest cells of *Sox10* mutants. Finally, the sympathetic primordium is severely hypoplastic in *ErbB3* and *Sox10* mutants, and a rostro-caudal gradient in the severity is apparent (arrows in Fig. 2c,g,o; cf. Britsch et al. 1998; Kapur 1999). Thus, various early deficits in neural

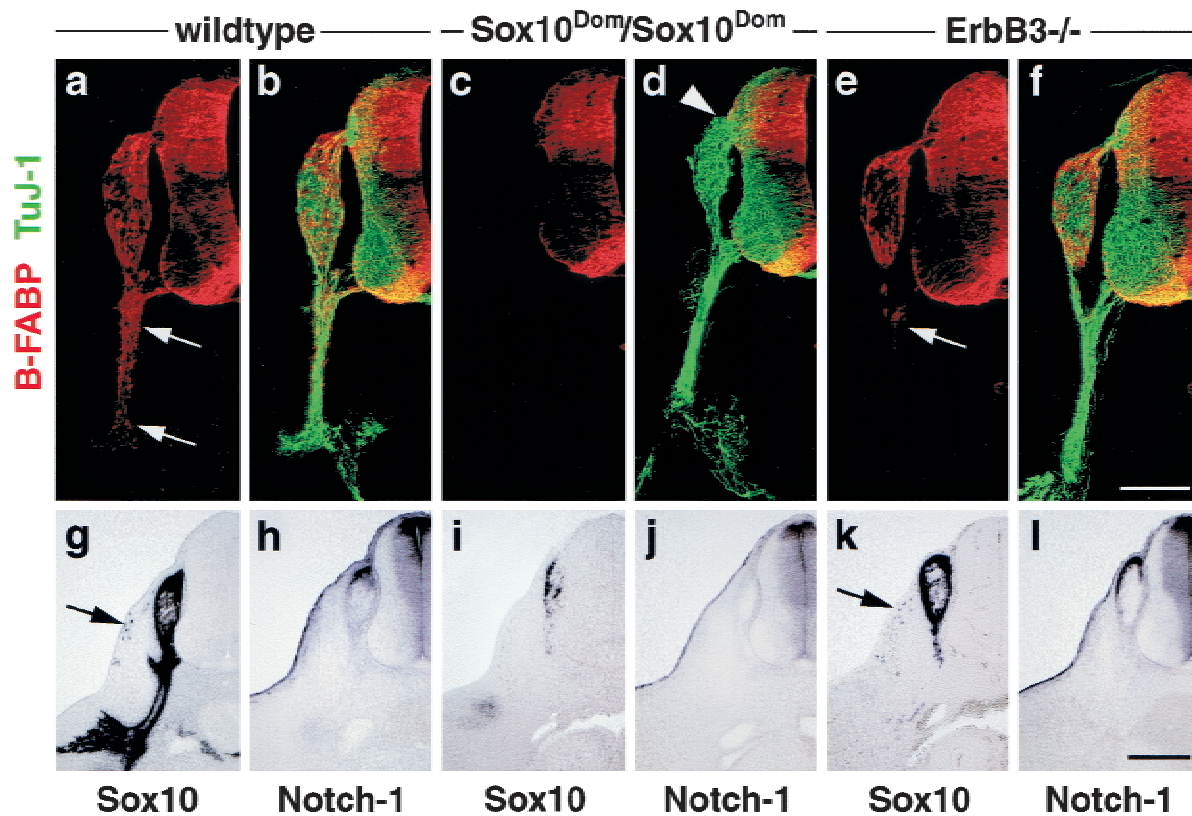
crest cells and their derivatives present in *ErbB3* mutants are also found in *Sox10* mutants. However, additional phenotypes exist in the *Sox10* mutants, for instance, the absence of enteric neural crest cells, that are not observed in *ErbB<sup>-/-</sup>* mice.

*Sox10 is essential for differentiation of peripheral glia*

Many genes expressed by neural crest cells are turned off in differentiated neurons but remain expressed in developing glial cells. Genes that display such expression patterns include *Sox10*, *ErbB3*, and *Cadherin-6*. However, markers that distinguish neural crest cells from newly formed glia are scarce. Brain-specific fatty acid binding protein (B-FABP; Kurtz et al. 1994) is nondetectable in neural crest cells but is easily detected in various peripheral glial populations. For example, whereas no B-FABP-positive cells along spinal nerves or in dorsal root ganglia are found at E10.5 in mice on lumbar axial levels by immunohistochemistry, they are abundant at E11.5 (Fig. 4a,b; data not shown). In sympathetic ganglia, B-FABP-positive cells appear on E12.5. Nonoverlapping cell populations stain with B-FABP- and neuron-specific antibodies, respectively. Several days later, the S100-antigen appears in glial cells and is coexpressed with B-FABP, for instance, in satellite cells of dorsal root ganglia or in Schwann cells (not shown). B-FABP distinguishes glia from neural crest cells and neurons during early stages of development of the peripheral nervous system.

Sensory neurons in dorsal root ganglia form on a similar temporal schedule in control, *Sox10*, and *ErbB3* mutant mice, as analyzed by the use of TuJ-1 antibodies (Fig. 4b,d,f; data not shown). In control mice at E11.5, B-FABP-positive cells are abundant in dorsal root ganglia and along spinal nerves on lumbar axial levels (Fig. 4a,b). In homozygous *Sox10* mutants, B-FABP-positive cells are detected neither in the dorsal root ganglia nor along the spinal nerves (Fig. 4c,d). This does not reflect simply a delay of glial differentiation, as B-FABP-positive cells are also absent at E12.5 (Fig. 5h). In contrast, in *ErbB3* mutants B-FABP-positive cells are abundant in the ganglia but are very rare along the nerves (Fig. 4e,f). Thus, in *ErbB3* mutant mice, glial cells can be formed, but the numbers of such cells that line nerves are strongly reduced, reflecting the reduced numbers of neural crest cells along axons at E10.5. In the superior cervical ganglion, B-FABP-positive cells arise at E12.5 in control mice. In *Sox10* and *ErbB3* mutant mice, this most anterior sympathetic ganglion is small. B-FABP-positive satellite cells can be detected in *ErbB3* but not in *Sox10* mutants. Satellite cells were also absent in the nodose ganglion of *Sox10* mutants but not of *ErbB3<sup>-/-</sup>* mice (not shown).

*Sox10* is among those genes that are expressed in neural crest cells as well as in glia. In control embryos at E11.5, Sox10-positive cells are abundant in dorsal root ganglia and line the spinal nerves in great numbers (Fig. 4g). In *Sox10<sup>Dom</sup>/Sox10<sup>Dom</sup>* mutants at E11.5, reduced numbers of cells that express the mutant *Sox10* gene are



**Figure 4.** *Sox10* controls peripheral glial cell development. (a–f) Immunohistological analysis of wild-type (a,b), *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* (c,d), and *ErbB3<sup>-/-</sup>* (e,f) embryos at E11.5 on lumbar axial levels, using antibodies against B-FABP (red) and TuJ-1 (green) to visualize peripheral glial cells and neuronal cells, respectively. B-FABP antibody signals (a,c,e) and an overlay of B-FABP and TuJ-1 antibody signals (b,d,f) are shown. The arrowhead in d points towards the abnormally shaped dorsal root entry zones in *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* mutants; the arrows point towards B-FABP-positive Schwann cell precursors that line spinal nerves in control and *ErbB3<sup>-/-</sup>* mice. (g–l) In situ hybridization of wild-type (g,h), *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* (i,j), and *ErbB3<sup>-/-</sup>* (k,l) embryos at E11.5 with probes specific for *Sox10* (g,i,k) or *Notch-1* (h,j,l). Sections on lumbar axial levels are shown. Bars (a–f) 100  $\mu$ m; (g–l) 150  $\mu$ m.

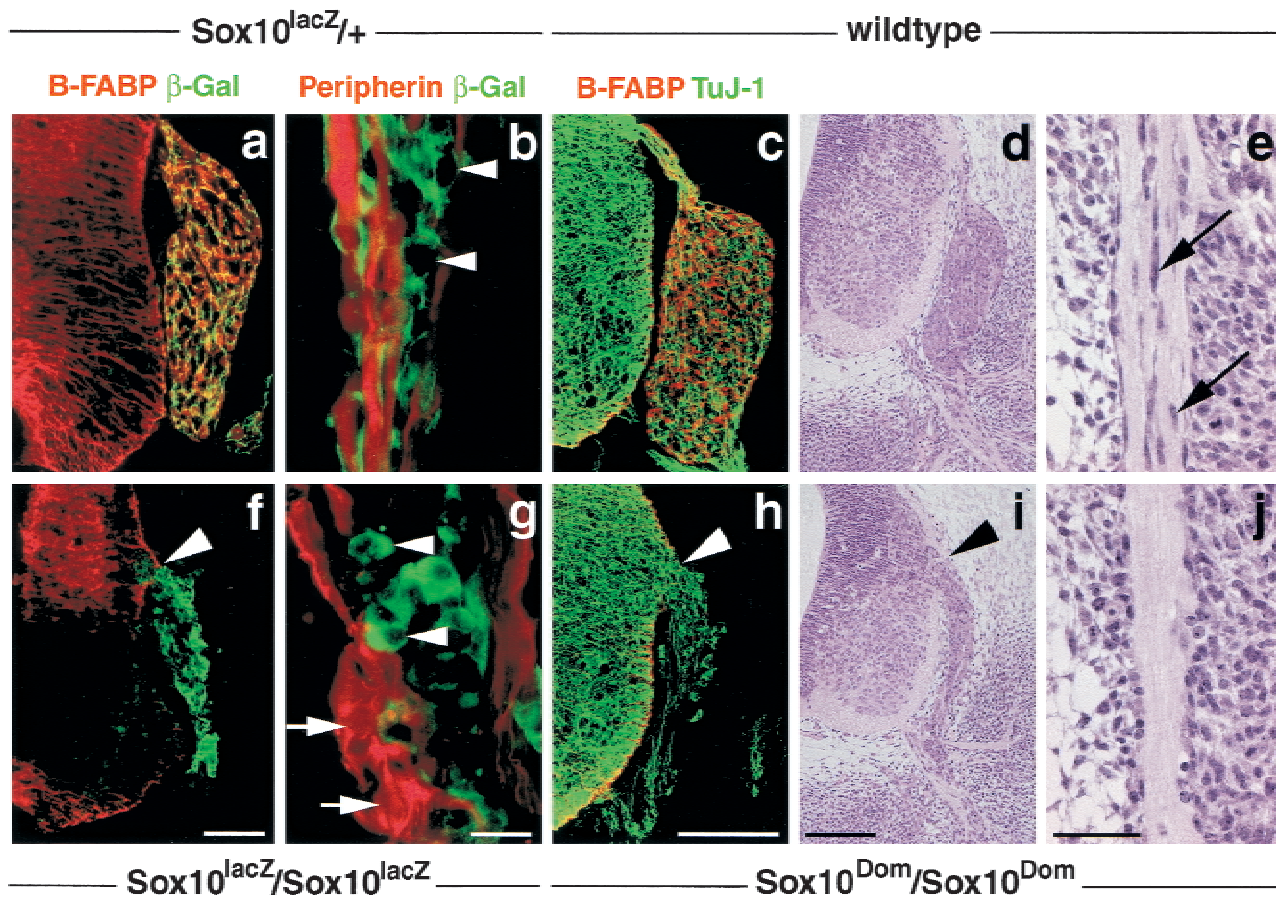
present in dorsal root ganglia and along the spinal nerves (Fig. 4i). Because differentiated glial cells that express B-FABP are not present, the few remaining *Sox10*-positive cells appear to correspond to undifferentiated neural crest cells. In *ErbB3<sup>-/-</sup>* mutants at E11.5, *Sox10*-positive cells are abundant in the ganglia but severely reduced along the spinal nerves, reflecting the distribution of B-FABP-positive cells (Fig. 4k). *Notch-1* is transiently expressed during early stages of glial differentiation; *Notch-1* signal controls the expression of *Hes-5* (Weinmaster et al. 1991; Kageyama and Nakanishi 1997). *Notch-1* or *Hes-5* transcripts are not detectable in dorsal root ganglia of *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* mice but are present in *ErbB3* mutants and control embryos (Fig. 4h,j,l; data not shown). Histological analysis shows that peripheral nerves lack Schwann cell nuclei in *Sox10* mutant mice at E12.5 (Fig. 5, cf. j and control in e). These nerves contain essentially only naked axons and are smaller in diameter than in control mice.

Immunohistochemistry was used to determine the differentiation status and the fate of  $\beta$ -galactosidase-expressing cells in *Sox10<sup>lacZ</sup>* mice. In heterozygous embryos at E11.5, B-FABP and  $\beta$ -galactosidase staining did

overlap to a large extent, but colocalization of  $\beta$ -galactosidase and *Islet-1* or *peripherin* was not observed (Fig. 5a,b; data not shown). In *Sox10<sup>lacZ/Sox10<sup>lacZ</sup></sup>* mice, residual  $\beta$ -galactosidase-positive cells, which express neither B-FABP nor neuronal markers like *peripherin*, were present. Thus, despite their inability to differentiate into glial cells, the remaining  $\beta$ -galactosidase-positive cells had not adopted a neuronal fate in the *Sox10<sup>lacZ/Sox10<sup>lacZ</sup></sup>* mice. The further destiny of these cells was not followed, as an extensive and general apoptosis in dorsal root ganglia sets in at E11.5 in these mutants (see also below).

#### *Degeneration of sensory and motoneurons in Sox10 and ErbB3 mutant mice*

Dorsal root ganglia form in *Sox10* and *ErbB3* mutant mice and are of similar size as those of control embryos at E10.5 (Fig. 2a,c,e,g,m,o). However, the dorsal root ganglia form broad fusions with the lateral neural tube, which contain, inappropriately, neurons in *Sox10* mutants (arrowheads in Figs. 4d and 5h,i). Neuronal peri-

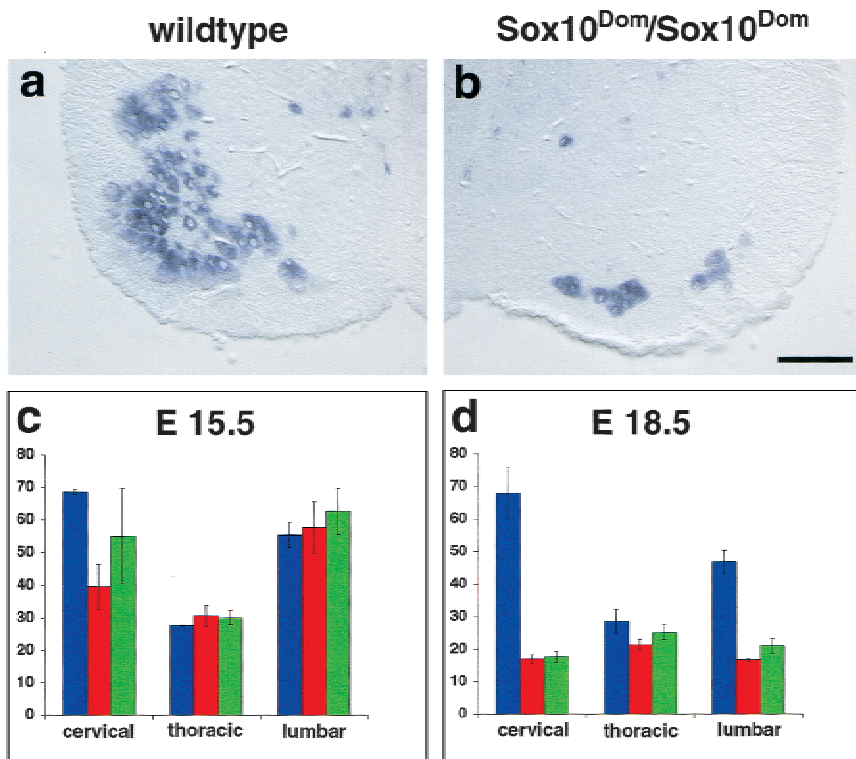


**Figure 5.** Appearance of dorsal root ganglia in homozygous *Sox10* mutant animals. Histological and immunohistological analyses of dorsal root ganglia and spinal nerves in *Sox10<sup>lacZ/+</sup>* (a,b), wild-type (c–e), *Sox10<sup>lacZ/Sox10<sup>lacZ</sup></sup>* (f,g), and *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* (h–j) animals at E11.5 (a,b,f,g) and E12.5 (c–e,h–j). (a,f) Immunohistological analysis using antibodies against B-FABP (red) and  $\beta$ -galactosidase (green). Note that B-FABP and  $\beta$ -galactosidase from the *Sox10*-lacZ reporter are coexpressed (yellow) in satellite glia of dorsal root ganglia of control mice. B-FABP is not expressed in *Sox10<sup>lacZ/Sox10<sup>lacZ</sup></sup>* mice, although  $\beta$ -galactosidase-positive cells persist. (b,g). High-magnification of ventral roots stained with antibodies against peripherin (red) and  $\beta$ -galactosidase (green). Arrowheads point towards cells that express  $\beta$ -galactosidase; arrows point towards neuronal cells expressing peripherin. Note that the two proteins are coexpressed in neither control nor homozygous mutant mice, indicating that the  $\beta$ -galactosidase-positive precursor cells have not adopted a neuronal fate in *Sox10<sup>lacZ/Sox10<sup>lacZ</sup></sup>* mice. (c,h) Immunohistological analysis with antibodies against TuJ-1 (green) and B-FABP (red), which visualize neuronal and glial cells, respectively. Histological appearance of dorsal root ganglia (d,i) and intercostal nerves (e,j) at E12.5. Note the marked reduction in size of the ganglion in the mutant mice. In control mice, nuclei associated with the intercostal nerve are abundant (arrows in e), which are not observed in the *Sox10* mutant. Note also the abnormal dorsal root entry zones in homozygous mutants, which are indicated by arrowheads in f, h, and i. Bars (a,f) 80  $\mu$ m; (b,g) 15  $\mu$ m; (c,d,h,i) 150  $\mu$ m; (e,j) 50  $\mu$ m.

karya were also detected in the ventral roots of spinal nerves of *Sox10* mutants (Fig. 5g). These changes were not present in *ErbB3<sup>-/-</sup>* mice. Even more conspicuous is the severe degeneration of neurons in dorsal root ganglia of *Sox10* mice, which is already detectable at E11.5 on lumbar axial levels and has affected a major proportion of the neurons at E12.5 (Fig. 5h,i). Degeneration of sensory neurons was also observed previously in *ErbB3<sup>-/-</sup>* mice, but first sets in around E12.5 on lumbar axial levels (Riethmacher et al. 1997; Woldeyesus et al. 1999). Not only is the onset of degeneration of sensory neurons earlier in *Sox10* mutants, but it is also more severe. At E18.5, very few cells are detectable in the remnants of lumbar dorsal root ganglia of *Sox10* mutants, whereas at this stage

about 20% of the sensory neurons can still be found in dorsal root ganglia of *ErbB3<sup>-/-</sup>* mutants (cf. Riethmacher et al. 1997; Woldeyesus et al. 1999).

Motoneurons are generated in *Sox10* mutants, but they subsequently degenerate. Motoneuron degeneration was also reported previously in *ErbB3<sup>-/-</sup>* mice (Riethmacher et al. 1997; Woldeyesus et al. 1999). We compared the numbers of motoneurons on different axial levels in *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>*, *ErbB3<sup>-/-</sup>* and control mice (Fig. 6). Compared with control mice at E15.5, numbers of motoneurons are somewhat reduced in *Sox10* and *ErbB3* mutant mice on cervical, but not on thoracic or lumbar, axial levels (Fig. 6c). At E18.5, a very significant loss of motoneurons is observed on cervical and lumbar axial



**Figure 6.** Loss of motoneurons in *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* and *ErbB3<sup>-/-</sup>* mutant mice. Sections of the cervical spinal cord of wild-type (a) and *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* (b) mutant animals at E18.5 after in situ hybridization with a VAcHT specific probe. (c,d) Motoneuron numbers in cervical, thoracic, and lumbar segments of spinal cords obtained from wild-type (blue columns), *Sox10<sup>Dom/Sox10<sup>Dom</sup></sup>* (red columns), and *ErbB3<sup>-/-</sup>* (green columns) mutants at E15.5 (c) and E18.5 (d). Mean numbers of motoneurons per section  $\pm$  S.D. are shown ( $n = 4$  embryos for each genotype and developmental stage). Counts were not corrected for neuron size, and the numbers of neurons at different developmental stages are therefore not strictly comparable. Bar (a,b) 100  $\mu$ m.

levels in *ErbB3* and *Sox10* mutants, and the extent of the motoneuron loss is identical. Motoneurons in thoracic segments are little affected (Fig. 6d).

#### Melanocyte development in *Sox10* mutant mice

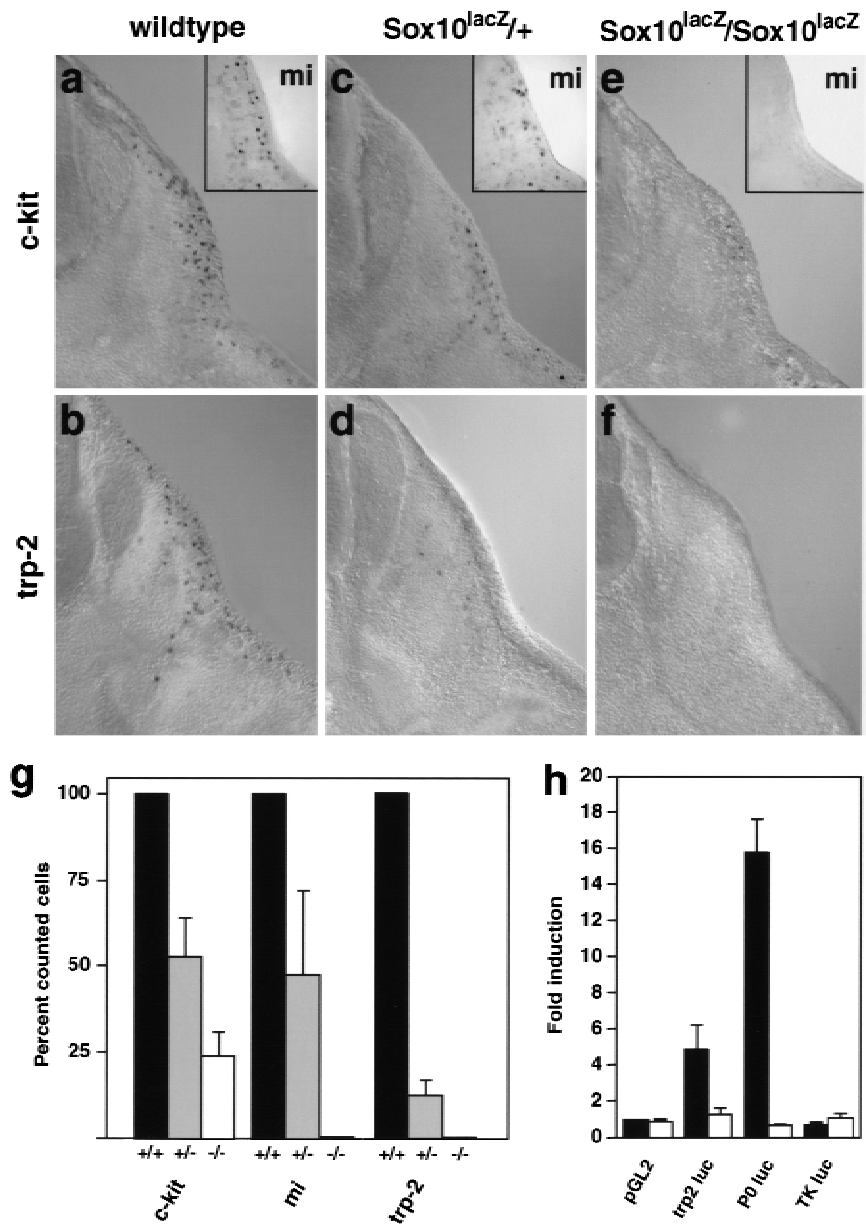
Melanocytes develop from neural crest cells that are generated late and migrate on the dorso-lateral pathway below the skin. These migrating cells begin to express genes typical for the melanocyte lineage, like *c-kit*, *trp-2*, or *mi* after they have moved a short distance from the neural tube. *Sox10* continues to be expressed in migrating melanocytes (arrow in Fig. 4g). In *Sox10* mutants, these cells are reduced in numbers (Figs. 4i and 7). Similarly, numbers of subcutaneous  $\beta$ -galactosidase-positive cells in *Sox10<sup>lacZ/Sox10<sup>lacZ</sup></sup>* mice are severely reduced (not shown).

Differentiation of melanocytes was analyzed in *Sox10* mutant mice, using markers for the early melanocyte lineage. The receptor tyrosine kinase *c-kit* and its specific ligand *steel*/stem cell factor play important roles in survival and migration of melanocytes (Geissler et al. 1988; Huang et al. 1990). In wild-type embryos, a stream of *c-kit*-positive melanocytes can be detected along the dorso-lateral pathway at E12.5 (Fig. 7a). We determined the number of such subcutaneous cells, using sections on the hindlimb level of E12.5 mice of different genotypes. Compared with wild-type mice, the numbers of *c-kit*-positive cells were reduced by half in mice with a heterozygous mutation in *Sox10* (Fig. 7c,g). In homozygous mice, the reduction was more pronounced. Mutants

contained only 24% of the cells compared with wild-type embryos (Fig. 7e,g).

Melanocyte development requires also the bHLH transcription factor *mi* that is encoded by the *microphthalmia* locus (for recent review, see Goding 2000). In wild-type mice at E12.5, the number of *mi*-positive cells was comparable to the number of *c-kit*-positive cells (Fig. 7c). In mice heterozygous for the *Sox10* mutation, *mi*-positive cells were reduced by half (Fig. 7c,g). We were unable to detect any *mi*-positive cells in homozygous *Sox10* mutant embryos (Fig. 7e,g). The tyrosinase-related protein 2 (*trp-2*) is encoded by the *slaty* locus and is expressed early in development of the melanocytic lineage (Jackson et al. 1992). The number of *trp-2*-positive cells in wild-type embryos at E12.5 was comparable to the number of *c-kit*- or *mi*-positive cells, consistent with the three markers labeling the same population of cells in the skin at this stage. In heterozygous mice, we detected only 12% of the *trp-2*-positive cells counted in wild-type mice (Fig. 7d,g). Thus, the reduction in numbers of *trp-2*-positive cells is more pronounced than the reduction in *mi*- or *c-kit*-positive cells. Moreover, those *trp-2*-positive cells present in heterozygous mice appeared to stain less intensely. In homozygous *Sox10* mutants, *trp-2* expressing cells were not detected (Fig. 7f,g). The absence of *trp-2*-positive cells in homozygous *Sox10* mutants also was apparent in other regions of the embryo and at E11.5 (not shown). Thus, a quantification of melanoblast numbers demonstrates that a severe and early loss of melanoblasts occurs in *Sox10* mutant mice. In contrast, development of melanocytes in the retinal pigment epithelium was not affected.





**Figure 7.** *Sox10* controls development of melanoblasts and expression of the *trp-2* gene. (a–f) Transverse sections of wild-type (a,b), heterozygous (c,d), and homozygous (e,f) *Sox10<sup>lacZ</sup>* mice at E12.5 on corresponding hindlimb levels. Embryos were hybridized with probes specific for c-kit (a,c,e; main panels), mi (a,c,e; insets), and *trp-2* (b,d,f). (g) Melanoblasts positive for c-kit, mi, or *trp-2* in wild-type (+/+), heterozygous (+/-), and homozygous (-/-) *Sox10*-deficient mice at E12.5. The numbers of positive cells in wild-type mice identified by each marker were arbitrarily set to 100%. (h) Luciferase reporter assay determining the interaction of *Sox10* and the *trp-2* gene. Reporter genes were transfected into *tet-on* N2A cells. Luciferase was expressed under the control of the 3.7-kb *trp-2* promoter (*trp2luc*), the P<sub>0</sub> promoter (P0luc), or the thymidine kinase promoter (TKluc) or were lacking a specific promoter (pGL2). Luciferase activity was measured before and after doxycycline treatment. *Sox10*-dependent promoter activation was expressed as the ratio between these two values. Bars represent the result from three independent transfections +/- S.E.M.

*Trp-2 is a direct target gene regulated by Sox10*

The effect of the heterozygous *Sox10* mutation on *trp-2* expression levels prompted us to investigate whether a direct regulator–target gene relationship exists between *Sox10* and *trp-2*. We analyzed expression of a luciferase reporter gene controlled by the mouse *trp-2* promoter, using a 3.7-kb *trp-2* fragment (-3290 to +443 relative to the transcriptional start site) that suffices to drive transgene expression in melanocytes (Budd and Jackson 1995). This reporter was transiently transfected into N2A cells that express *Sox10* in a doxycycline-dependent manner. We observed a fivefold activation of the *trp-2* promoter in the presence of *Sox10* (Fig. 7h). In parallel transfections, the P<sub>0</sub> promoter was stimulated 16-fold. *Sox10*-dependent activation of the *trp-2* promoter was specific, as the thymidine kinase promoter was not ac-

tivated upon *Sox10* induction; moreover, no increase in *trp-2* promoter activity was observed in control N2A cells treated with doxycycline (Fig. 7h).

**Discussion**

Spontaneous mutations of the *Sox10* gene were identified in mice and man that, in a heterozygous state, can affect pigmentation and the development of enteric ganglia. These spontaneous mutant alleles can encode truncated proteins, which might act in a dominant-negative manner (Kuhlbrodt et al. 1998b; Pingault et al. 1998; Southard-Smith et al. 1999). We generated a novel, targeted allele of the mouse *Sox10* gene that deletes the entire reading frame. The analysis of these *Sox10<sup>lacZ</sup>* mutant mice demonstrates that haploinsufficiency can ex-

plain the pigmentation and megacolon phenotypes observed in heterozygous *Sox10<sup>Dom</sup>* mice and in patients with Waardenburg-Hirschsprung disease. Thus, although spontaneous mutations might have also dominant-negative effects, such mechanisms are not necessary for the manifestation of an overt phenotype. The expressivity of pigmentation defects in heterozygous *Sox<sup>lacZ</sup>* mice depends on the genetic background, as found previously in *Sox10<sup>Dom</sup>* mutants. Modifier loci affect thus the penetrance of certain phenotypes caused by haploinsufficiency.

#### *Sox10, ErbB3, and glial development*

In homozygous *Sox10* mutant mice, the entire peripheral nervous system is defective and, in addition, particular motoneurons are absent. Our analysis reveals that two distinct mechanisms cause these changes. Early phenotypes arise during the development of neural crest cells and are in contrast to the late phenotypes, the degeneration of already formed ganglia or nerves. *Sox10* controls, among other genes, the expression of *ErbB3*, which encodes a Neuregulin receptor (Adlkofer and Lai 2000; Garratt et al. 2000). In the absence of ErbB3-mediated signals, migration and survival of particular neural crest cell types are impaired, which account for many early phenotypes in *Sox10* mutants. The degeneration of neurons in *Sox10* mutant mice can be attributed to the lack of glial cells.

The lack of glial cells in *Sox10* mutants is caused by a differentiation defect. Differentiated glial cells were absent in those portions of the peripheral nervous system that form initially in *Sox10* mutants. Schwann cell precursors along the projections of sensory and motoneurons did not form, nor did we detect satellite cells in sensory ganglia or in the superior cervical ganglion of the sympathetic nervous system. It is of interest to note that *Sox10* was reported previously also to control directly expression of the myelin-associated gene *P<sub>0</sub>* (Peirano et al. 2000). A key role of *Sox10* in the development of peripheral glial cells and in the control of genes that determine glial cell identity is thus emerging. It is of interest to note recent reports on some patients with *Sox10* mutations, who display neurological symptoms reminiscent of Charcot-Marie-Tooth disease type 1, a disease typically associated with Schwann cell deficits (Inoue et al. 1999; Pingault et al. 2000; Touraine et al. 2000).

Homozygous *Sox10* and *ErbB3* mutant mice both display severe deficits in glial development, but the extent of these differs. The entire peripheral glial cell population, that is, Schwann cells and satellite cells, is missing in *Sox10* mutants. In contrast, in *ErbB3* mutants only the Schwann cell lineage is affected; precursors are reduced at early stages, and later, Schwann cells are absent. The underlying mechanisms that cause these changes also differ. In *Sox10* mutants, the differentiation of peripheral glial cells is arrested in general at a very early stage. In contrast, the ErbB3/ErbB2 receptors and their ligand Neuregulin-1 control migration of Schwann cell

precursors along axon tracts as well as survival and proliferation of such precursors (Adlkofer and Lai 2000; Garratt et al. 2000). We carefully assessed the presence of neural crest cells in dorsal root ganglia and along the spinal nerves before the onset of glial differentiation in *Sox10* mutants, using *Sox10*,  $\beta$ -galactosidase encoded by the *Sox10<sup>lacZ</sup>* allele, or *p75<sup>NTR</sup>* as markers. At E10.5, a day prior to glial differentiation, neural crest cells are still abundant in the dorsal root ganglia, and the ganglia appear normal in size. Thus, initially, the mutation does not interfere with the maintenance of neural crest cells in the newly formed ganglia. At other sites, for instance along the spinal nerves, a severe reduction in numbers of neural crest cells is notable in *Sox10* mutants. A mere reduction in precursor numbers along the spinal nerves does however not interfere with glial differentiation: In *ErbB3* mutant mice, numbers are reduced, but the few remaining cells can nevertheless differentiate.

What is the fate of undifferentiated neural crest cells that do not generate glia in *Sox10* mutants? In vivo, we have not obtained evidence that these cells form neurons or other, non-glial cell types that derive from the neural crest. Our data indicate that the precursors arrest their differentiation and are then eliminated by cell death. A more detailed analysis of this is hampered by the general cell death in the dorsal root ganglia of *Sox10* mutants, which sets in around the time the first differentiated glial cells are observed in control mice. This apoptosis is not restricted to neural crest cells but also affects neurons.

It should be noted that *Sox10* is expressed broadly in neural crest cells. However, only a subpopulation of these cells will take a glial cell fate and maintain *Sox10* expression. Expression of *Sox10* does not therefore suffice to generate glia, and additional signals are required. The function of *Sox10* might be modified in cells that will form glia, for instance by a phosphorylation event controlled by signals that induce glial differentiation. Alternatively, *Sox10* might cooperate with additional factor(s) regulated by such signals. The transcription factors *Pax3*, *Krox20*, and *SCIP/Tst-1/Oct6* cooperate with *Sox10*, but mutations of mouse *Pax3*, *Krox20* or *SCIP/Tst-1/Oct6* do not appear to affect the differentiation of neural crest cells into glia (Epstein et al. 1991; Franz and Kothary 1993; Topilko et al. 1994; Jaegle et al. 1996; Kuhlbrodt et al. 1998a). Our finding that *Sox10* is essential for differentiation of neural crest cells into glia might suggest an experimental basis for the future elucidation of molecular mechanism(s) that specify the peripheral glial lineage. It is noteworthy that a transient Notch activation accelerates glial differentiation and suffices to suppress a neurogenic fate in cultured neural crest cells (Morrison et al. 2000; Wakamatsu et al. 2000). *Notch-1* is expressed at low levels in neural crest cells and is transiently up-regulated during early glial development. Interestingly, *Notch-1* expression is not detected in sensory ganglia of *Sox10* mutant mice, as is the expression of *Hes-5*, which is controlled by Notch signals. The effect on *Notch-1* expression might cause or contribute to the general deficit in glial differentiation.

### Degeneration of sensory, sympathetic, and motor neurons in *Sox10* mutant mice

In *Sox10* mutant mice, motoneurons form, but degenerate subsequently. The degeneration of motoneurons displays distinct kinetics and affects particular subclasses of motoneurons. Indirect effects account for the motoneuron death in *Sox10* mutants, as at no time in development did we detect *Sox10* transcripts in motoneurons or their precursors. In *ErbB3* mutant mice, motoneurons also degenerate. Degeneration is identical in extent, kinetics, and affected axial levels in *ErbB3* and *Sox10* mutant mice. It is noteworthy that *Sox10* is not expressed in muscle, and *ErbB3* expression in muscle is not changed in *Sox10* mutants. The common feature of the two mutants is the severe deficit in development of Schwann cells that accompany motor nerves. The comparison of the *Sox10* and *ErbB3* mutants demonstrates, unambiguously, that motoneuron degeneration is caused by a lack of Schwann cells and that Schwann cells have an essential role in the maintenance of distinct motoneuron subclasses.

Sensory neurons in dorsal root ganglia form and subsequently degenerate in *Sox10* mutants. Also, the superior cervical ganglion is formed, albeit at reduced size, and it degenerates. We propose that the absence of peripheral glial cells causes the degeneration of sensory and sympathetic neurons in *Sox10* mutants, and that both glial cell types, satellite cells as well as Schwann cells, provide essential trophic support. In *ErbB3* mutants, sensory neurons also degenerate, but the degeneration is less pronounced and sets in later than in *Sox10* mutants. Satellite cells that form in *ErbB3*<sup>-/-</sup> mice might thus provide trophic support, which allows a partial rescue of the sensory neurons.

### The role of *Sox10* in melanocyte development

A late-emerging population of neural crest cells, which migrate along a dorso-lateral path below the skin, generate melanoblasts, the nonpigmented precursor cells of melanocytes. These cells start to express genes typical for the melanocyte lineage, like *c-kit*, *mi*, and *trp-2*, after they have moved a short distance away from the neural tube. *Sox10* is expressed in the migrating melanoblasts. The development of the melanocyte lineage depends on *Sox10*, and, moreover, is even dosage dependent. A significant reduction in melanoblast numbers was noted in the heterozygous *Sox10*<sup>lacZ</sup> mice, accounting for pigmentation defects present in these animals. Analysis of this phenotype on a C3H background may have augmented the effect, as this genetic background accentuates the melanocyte defect in heterozygous *Sox10*<sup>Dom</sup> mice (Southard-Smith et al. 1999). Depending on the marker employed for the detection of melanoblasts, the decrease in numbers in heterozygous mice ranged from 50%–80%. Given the minor pigmentation defects in adult mice, it appears that the loss of melanoblasts is at least in part compensated during further development.

In the homozygous *Sox10* mutants, melanoblasts are

very severely affected. We did not detect *mi*, *trp-2*, or *Sox10*-positive cells located subcutaneously, whereas few *c-kit*-positive cells remained. Hematopoietic precursor cells also express *c-kit*, and it is possible that the remaining *c-kit*-positive cells are not melanoblasts but of a different ontogenetic origin. Alternatively, a residual population of melanoblasts that expresses *c-kit* might exist, that is detected by none of the other markers employed here, that is, *mi*, *trp-2*, or *Sox10*. The presence of such melanoblasts would indicate that expression of *mi*, *trp-2*, and *Sox10* are controlled directly or indirectly by *Sox10* in the melanoblast lineage. Additional experimental evidence indicates that *mi* and *trp-2* are *Sox10* target genes. The human MITF promoter is strongly activated by *Sox10* in transient transfection experiments and contains multiple binding sites for *Sox10* (Bondurand et al. 2000). Regulation of *mi* by *Sox10* can account for many aspects of the melanocyte defect seen in *Sox10* mutant mice, as *mi*/MITF is a key regulator of melanocyte development (Goding 2000). We provide here evidence that *trp-2* expression critically depends on *Sox10* and that the *trp-2* gene is a *Sox10* target. First, *trp-2*-positive cells are reduced in numbers in heterozygous *Sox10*<sup>lacZ</sup> mice, and *trp-2* mRNA levels are sensitive to *Sox10* gene dosage. Second, transient transfection of the *trp-2* promoter driving a reporter gene shows that the promoter is activated by *Sox10*. The setup of the transfection assay favors a model in which the regulation of *trp-2* expression by *Sox10* is direct. However, this remains to be proven by a further characterization of binding sites for *Sox10* in the *trp-2* promoter.

### Materials and methods

#### *Sox10*<sup>lacZ</sup> and *Sox10*<sup>Dom</sup> mutant mice

The *Sox10* locus was isolated from a 129/Sv genomic λ library. The targeting vector (Fig. 1a) was constructed by standard techniques. The linearized targeting vector was electroporated into 129/Sv R1 ES cells, which were selected with G418 and gancyclovir. Selected ES cell clones were screened for homologous recombination by PCR, which was confirmed by Southern hybridization using probes located 5' and 3' of the targeting vector (Fig. 1a,b; data not shown). Two independently targeted ES cell clones were used for injection into C57Bl/6J blastocysts to generate chimeras and mutant mouse strains. No phenotypic differences were detected in mice derived from the two ES cell lines. Genotyping of mutant mice was performed by PCR.

The *Sox10*<sup>Dom</sup> allele spontaneously arose on a C57Bl/6J × C3HeB/FeJ hybrid background at the Jackson Laboratories (Lane and Liu 1984). Animals used here had a C57Bl/6J × C3HeB/FeJ mixed genetic background. The mice were genotyped by PCR using primers that amplify the *D15Mit71* microsatellite, which generates a 14-bp polymorphism between C57Bl/6J and C3HeB/FeJ. The *Sox10*<sup>Dom</sup> allele segregated with an 118-bp amplification product identical to that obtained with C57Bl/6J genomic DNA.

#### *β*-galactosidase staining, *in situ* hybridization, immunohistology, and histology

Detection of *β*-galactosidase activity followed standard procedures: After fixation in 4% paraformaldehyde (2 h), embryos

were incubated at 37°C in 1% X-gal (1–6 h). Whole-mount in situ hybridization was performed as described (Britsch et al. 1998). For generation of riboprobes the following plasmids were used: *ErbB3*, pDR3: *EcoRI*, ~1.4-kb probe; *Sox10*, pZL1/SX10: *NcoI*, ~1.3-kb probe; *Notch-1*, pMN7: *XbaI*, ~4-kb probe; *VACHT*, pVAT2B: *BamHI*, ~1.3-kb probe; *c-kit*, pGEM-ckit: *EcoRI*, ~3.7-kb probe; *trp-2*, p5A7: *HindIII*, ~1.2-kb probe; *mi*, pGEM-mi: *NdeI*, ~1.2-kb probe. For vibratome sectioning, embryos were embedded into 20% gelatine and fixed in 4% PFA. For immunohistological analysis, embryos were prefixed in 2% PFA and cryoprotected with 20% sucrose. Cryosections (10 µm) were postfixed for 5–10 min with 2% PFA and blocked with 20% serum. The following antibodies were used: rabbit polyclonal anti-peripherin (Chemicon; 1:200), rabbit polyclonal anti-B-FABP (Kurtz et al. 1994; 1:5000), mouse monoclonal anti-β-tubulin (clone TuJ-1; Covance; 1:500), mouse monoclonal anti-islet-1 (DSHB; clone 40.2 D6; 1:100), mouse monoclonal anti-β-galactosidase (Roche Biochemicals; 1:75), Cy2-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG (Dianova; 1:200). Sections were examined with a confocal microscope. For histological analysis, mouse embryos were fixed in 4% PFA at 4°C, dehydrated and embedded in Technovit 7100 (Kulzer); 6 µm sections were stained with haematoxylin/eosin. Motoneuron counts were performed as described previously (Woldeyesus et al. 1999).

#### Cell culture, RNA preparation, reverse transcription, PCR, and Northern blot

Stable transfectants of N2A neuroblastoma cells, which express rat Sox10, rat Sox11, or a mutant version of Sox10 under a doxycycline-inducible promoter were maintained as described (Kuhlbrodt et al. 1998b; Peirano et al. 2000). Sox gene expression was induced by the addition of 2.5 µg/mL doxycycline (Sigma) to the medium. Total RNA was isolated from N2A transfectants 10 h after the addition of doxycycline and reverse transcribed into cDNA as described (Peirano et al. 2000). For the quantification of *ErbB3*, cDNA was amplified with primer pairs specific for *ErbB3*, *Sox10*, *Sox11*, and *GAPDH*. The primer pair used for *ErbB3* was 5'-GCGTTGCCAGTTGTCCCATATAA-3' and 5'-AGCGTCTCATAGCCCTTTTGTGTT-3'; the other primers and polymerase chain reactions conditions have been described (Peirano et al. 2000). Northern blotting was performed on 2 µg of poly(A)<sup>+</sup> RNA per sample using a 1150-bp mouse *ErbB3* cDNA fragment.

#### Quantification of melanoblasts and *trp-2-luciferase* reporter assay

Numbers of melanoblasts were determined on sections of wild-type, heterozygous, and homozygous *Sox10<sup>lacZ</sup>* embryos at E12.5 after in situ hybridization. Only cells in melanoblast-typical positions were counted. A total of 12 sections from 3 different embryos were counted for each genotype. Luciferase reporter genes under the control of the 3.7-kb *trp-2* promoter (*trp2luc*), the P<sub>0</sub> promoter (P0luc), or the thymidine kinase promoter (TKluc) as well as a reporter construct lacking a specific promoter (pGL2) were transfected into *tet-on* Neuro2A cells. Luciferase activity was measured before and after doxycycline treatment. Sox10-dependent promoter activation was expressed as the ratio between these two values.

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