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Replacement of the Sox10 transcription factor by Sox8 reveals incomplete functional equivalence

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Sox8 and Sox10 are two closely related transcription factors of the Sox protein family with overlapping expression patterns during development. They are believed to perform very similar functions because several developmental processes, including enteric nervous system development and oligodendrocyte differentiation, are regulated by both Sox proteins. To analyze the extent of functional equivalence between the two Sox proteins, we employed targeted mutagenesis to replace *Sox10* with *Sox8* in the mouse. In mice that expressed *Sox8* instead of *Sox10*, Sox10 deficiency was phenotypically rescued to different extents in affected tissues. Whereas development of glial cells and neurons in the sensory and sympathetic parts of the peripheral nervous system was almost normal when Sox10 was replaced by Sox8, melanocyte development was as defective as in Sox10-deficient mice. The ability of Sox8 to rescue the defects in enteric nervous system development and oligodendrocyte differentiation of Sox10-deficient mice was limited. We conclude that the extent of functional equivalence depends on the tissue and that, despite their relatedness, Sox8 and Sox10 have more unique functions than previously appreciated.

KEY WORDS: Sry, High-mobility-group, Redundancy, Glia, Oligodendrocyte, Neural crest

INTRODUCTION

The three vertebrate group E Sox proteins Sox8, Sox9 and Sox10 probably arose through gene duplication events from a single ancestral gene. These events must have occurred early during vertebrate evolution, as they are present in all vertebrates, whereas invertebrates contain a single SoxE gene (Bowles et al., 2000; Wegner, 1999). All three SoxE proteins are highly conserved in vertebrates (e.g. Aoki et al., 2003; Chiang et al., 2001; Takada and Koopman, 2003), exhibit overlapping expression patterns in the mouse (Chaboissier et al., 2004; Maka et al., 2005; Stolt et al., 2004; Stolt et al., 2005) and play important roles during embryonic development (Wegner and Stolt, 2005). Phenotypes of single and compound mouse mutants with deficiencies in SoxE genes suggest that, when co-expressed, SoxE proteins usually exert similar effects on the same developmental process. Nevertheless, the dependence of a developmental process on the co-expressed SoxE proteins may differ strongly. Sertoli cell differentiation in the male gonad, for example, is influenced by both Sox8 and Sox9 (Chaboissier et al., 2004). The influence of Sox9 is, however, much more pronounced than the influence of Sox8. In most analyzed cases, the lesser influence usually correlates with a lower level of expression. These observations are therefore compatible with a model in which SoxE proteins are functionally equivalent, but contribute differently to a specific developmental process according to their expression level. Among co-expressed SoxE proteins, Sox8 usually is expressed at the lowest level and has the least impact (Chaboissier et al., 2004; Maka et al., 2005; Stolt et al., 2004; Stolt et al., 2005).

In the past, we have studied the role of Sox10 during mouse embryonic development. Sox10 is strongly expressed in the neural crest where it is turned on shortly before neural crest cells start to migrate from the dorsal aspect of the neural tube (Kuhlbrodt et al., 1998; Southard-Smith et al., 1998). Sox10 is not only expressed in neural crest stem cells, but also in several neural crest derivatives where its presence is either transient or lasts through terminal differentiation into the mature state (Britsch et al., 2001; Southard-Smith et al., 1998). In accordance with its expression pattern, Sox10 has functions in neural crest stem cells where it is needed for maintenance of pluripotency and survival, and in peripheral glia, melanocytes and enteric neural crest, where it regulates specification and differentiation events (Britsch et al., 2001; Kim et al., 2003). *Sox10* gene defects lead to several neurocristopathies in mouse, man and zebrafish (Britsch et al., 2001; Dutton et al., 2001; Herbarth et al., 1998; Kapur, 1999; Pingault et al., 1998; Southard-Smith et al., 1998). These present as partial pigmentation abnormalities, colonic aganglionosis and in some instances as peripheral neuropathies in the heterozygous state. In homozygotes, melanocytes, peripheral glia and the enteric nervous system are entirely missing.

Outside the neural crest and its derivatives, Sox10 is found in the central nervous system (Kuhlbrodt et al., 1998) where it is restricted to the oligodendrocyte lineage. Oligodendrocyte development is affected by *Sox10* gene defects at the stage of terminal differentiation and myelination, although Sox10 is already expressed in these cells from the time of their specification (Inoue et al., 2004; Stolt et al., 2002).

Sox10 is expressed in an overlapping manner with both Sox9 and Sox8 (Cheung et al., 2005; Maka et al., 2005; Stolt et al., 2004; Stolt et al., 2003), and phenotypes are primarily observed at developmental stages where Sox10 exhibits singular or significantly higher expression than Sox9 or Sox8 in a particular cell type. For oligodendrocytes, this is the stage of terminal differentiation. At this point, Sox9 expression is extinguished and among the remaining SoxE proteins, Sox10 is expressed at a higher level than Sox8 (Stolt et al., 2004). Similarly, the enteric nervous system defect in Sox10-deficient mice arises in the vagal neural crest (Kapur, 1999), which predominantly expresses Sox10, no Sox9 and only low levels of Sox8 (Maka et al., 2005). In compound mutants, Sox10-dependent defects in the enteric nervous system and in oligodendrocyte

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differentiation are aggravated by additional loss of Sox8 (Maka et al., 2005; Stolt et al., 2004). These results are again compatible with functional equivalence among SoxE proteins and expression levels deciding whether functional redundancy among co-expressed SoxE proteins is partial or complete.

To study functional equivalence among SoxE proteins, we have generated a mouse in which we have replaced *Sox10* by *Sox8*. This mouse expresses Sox8 in all tissues where Sox10 is normally expressed, and in amounts that closely correspond to normal levels of Sox10 expression. Nevertheless, we find that Sox8 is not able to completely rescue the phenotype of Sox10-deficient mice. Instead, the level of phenotypic rescue varies between cell types from none to complete with no obvious correlation to Sox10 expression levels in the respective cell types. From these new data, we conclude that functional equivalence among SoxE proteins is only partial and that each SoxE protein has unique functions not shared by its relatives.

MATERIALS AND METHODS

Construction of targeting vector

Genomic sequence from the Sox10 locus of 129/Sv mice was obtained from a lambda phage library (Britsch et al., 2001). A 4.3 kb *Xba*I/*Sma*I fragment encompassing exons 1, 2 and part of exon 3 was used as 5′ homology region. Using a *Sma*I/*Nhe*I linker the fragment was extended enabling us to place the start codon of the *Sox8* open reading frame exactly over the start codon of the *Sox10* gene. The *Sox8* open reading frame was followed by an IRES-EGFP cassette. A 1.5 kb *Spe*I/*Eco*RI fragment downstream of the known untranslated region of exon 5 was used as 3′ homology region. Both the combination of 5′ homology region, Sox8 open reading frame and IRES-EGFP cassette as well as the 3′ homology region were inserted into pPNT (Tybulewicz et al., 1991) on either side of a neomycin resistance cassette with flanking loxP sites (Fig. 1A). The targeting vector thus replaced the complete open reading frame of *Sox10* by *Sox8* open reading frame and IRES-EGFP (Fig. 1A). The construct was sequenced to confirm that no mutation had been introduced into the Sox8-coding sequences during cloning and linearized with *Not*I before electroporation.

Gene targeting, generation and genotyping of mouse mutants

The linearized construct was electroporated into E14.1 ES cells which were then selected with G418 (400 μg per ml) and gancyclovir (2 μM). Selected ES cell clones were screened by Southern blotting with a 0.6 kb 5′ probe, which recognized a 6.6 kb fragment in case of the wild-type allele and a 6.0 kb fragment in case of the targeted allele in genomic DNA digested with *Nco*I (Fig. 1A,B). Appropriate integration of the 3′ end of the targeting construct was verified using a 0.6 kb 3′ probe on ES cell DNA digested with *Bam*HI and *Sca*I. This probe hybridized to a 5.4 kb fragment in the targeted allele as opposed to a 4.6 kb fragment in the wild-type allele (Fig. 1A,B). Two targeted ES cell lines were injected into C57Bl/6J blastocysts to generate chimeras. Chimeric males from two independent clones transmitted the targeted allele to their offspring. No differences were detected between mice derived from the two different ES cell lines. To remove the neomycin resistance cassette, heterozygous mutant mice were crossed with EIIa-Cre mice (Lakso et al., 1996). Homozygous mutant embryos were generated by heterozygote intercrosses. The final $Sox10^{Sox8ki}$ allele was sequenced to ensure that nucleotide changes had not accumulated in the Sox8-coding sequence.

Genotyping was routinely performed on DNA from tail tips, or in case of embryos from yolk sacs, by PCR analysis using a common upper primer located 81 bp upstream of the start codon (5′-CAGGTGGGCGTTGGGC-TCTT-3′) and two lower primers located 487 bp (5′-TCCCAGGCT-AGCCCTAGTG-3′) and 651 bp (5′-GCCCAGTTCAGTACCAGAGG-3′) downstream of the start codon in *Sox10* and *Sox8*, respectively. A 568 bp fragment was indicative of the wild-type allele, a 732 bp fragment of the targeted allele (Fig. 1E). Deletion of the neomycin resistance cassette was verified by PCR using primers located upstream (5′-AGCCATACCACAT-TTGTAGAGC-3′) and downstream (5′-CTAGATGCTGGCATAGGCGAT-3′) of the cassette. A 0.5 kb fragment was indicative of successful Cre-mediated deletion (Fig. 1D).

Quantitative RT-PCR analysis and Western blotting

Total RNA was extracted from whole embryos at 10.5 and 12.5 days post coitum (dpc) and from embryonic tissues at 13.5 and 18.5 dpc using the RNeasy kit (QIAGEN). Two micrograms of each RNA sample were reverse transcribed into 100 μl cDNA under standard conditions using Moloney murine leukaemia virus reverse transcriptase (NEB).

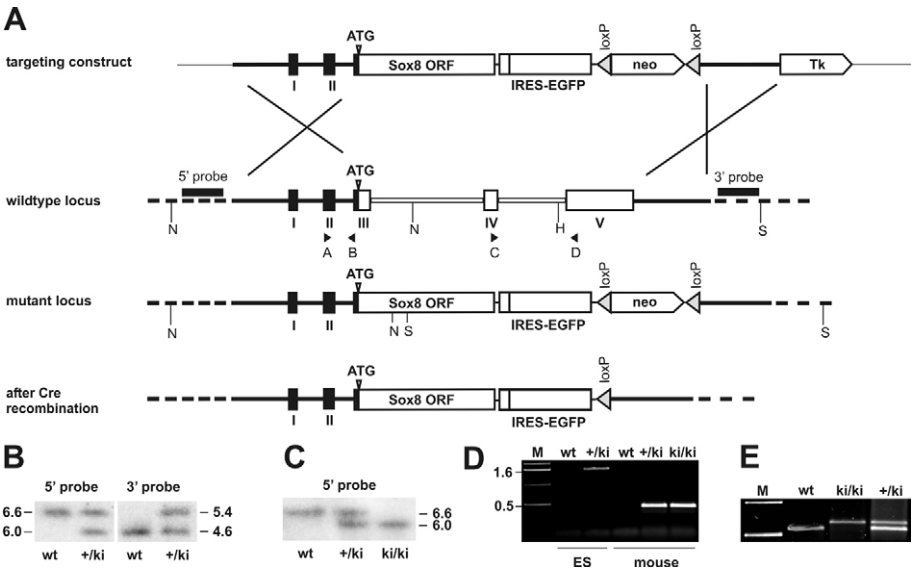

Fig. 1. Targeted replacement of *Sox10* by *Sox8* in mice. (**A**) Schematic representation from top to bottom of the targeting construct, the *Sox10* wild-type locus and the mutant locus before and after Cre recombination. The *Sox10* exons (I-V) and the *Sox8* open reading frame (ORF) are shown as boxes, 4.5 kb and 1.5 kb flanking regions as bars. Regions of homology between wild-type locus and targeting vector are depicted as black bars, introns 3 and 4 as open bars and surrounding genomic regions not contained in the targeting construct as stippled bars. Plasmid backbone sequences of the targeting construct are indicated by a thin line. Restriction sites for *Nco*I (N), *Bam*HI (H) and *Sca*I (S) are shown, as well as the localization of 5′ and 3′ probes and the start codon of the *Sox10* gene (ATG). The arrowheads indicate the localization of primers A-D used for quantitative RT-PCR. IRES-EGFP, EGFP open reading frame with preceding internal ribosomal entry site; neo, neomycin resistance cassette; loxP, recognition sites for Cre recombinase; Tk, Herpes simplex virus thymidine kinase gene cassette. (**B**) Southern blot analysis of DNA from wild-type (wt) and heterozygous (+/ki) ES cells digested with *Nco*I for use of the 5′ probe and *Bam*HI/*Sca*I for the 3′ probe. The size of bands corresponding to the wild-type (6.6 and 4.6 kb) and the targeted allele (6.0 and 5.4 kb) are indicated. (**C**) Southern blot analysis of DNA from wild-type (wt), heterozygous (+/ki) and homozygous (ki/ki) mice digested with *Nco*I for use of the 5′ probe. (**D**) PCR analysis of Cre-mediated deletion of the neomycin resistance cassette in mice carrying a $Sox10^{Sox8ki}$ allele (+/ki and ki/ki). ES cells with a $Sox10^{Sox8ki}$ allele (+/ki) still contained the neomycin resistance cassette and served as control. M, size marker. (**E**) PCR genotyping of wild-type (wt), heterozygous (+/ki) and homozygous (ki/ki) mice. DNA fragments in the size marker (M) are 1.0 kb and 0.5 kb.

The cDNA (1 μl) was amplified by polymerase chain reaction on a Roche Lightcycler according to the manufacturer's instructions using the LightCycler-FastStart DNA Master SYBR Green kit with the following primer pairs: primers A and B (5′-TCAGTCTCGGCTGTCCAGCC-3′ and 5′-GAAGAGCCCAACGCCACCT-3′) are located on exons II and III of *Sox10* and recognize transcripts from the wild-type and the mutant locus (Fig. 1A); primers C and D (5′-GTCAGATGGGAACCCAGAGCAC-3′ and 5′-CCCGTAGCCAGCTGCCGAG-3′) are located on exons IV and V of *Sox10* and recognize transcripts from the wild-type locus only (Fig. 1A); primers 5′-CCTGGGCATGGAGTCCTG-3′ and 5′-GGAGCAATGATCT-TGATCTTC-3′ amplify *β-actin* transcripts; primers 5′-GTTCGTGTAC-TGCGGCAAGA-3′ and 5′-ACAGGATTCATGGCCACACC-3′ amplify *rpl8* transcripts that were both used for normalization. For *β-actin* primers and primer pair A/B an annealing temperature of 60°C was used, 64°C for the others.

Brains from 18.5 dpc embryos were used to prepare nuclear extracts (Sock et al., 1996). Samples were loaded onto denaturing 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes (Sock et al., 1996). Polyclonal antisera directed against group E Sox proteins (1:3000 dilution) (Kuhlbrodt et al., 1998) or Sox8 (1:5000 dilution) (Stolt et al., 2005) and a monoclonal directed against RNA polymerase II (1:3000 dilution; Active Motif) served as primary antibodies, horseradish peroxidase-coupled protein A or goat anti-mouse antibody as secondary detection reagent in western blots using the ECL detection system (Sock et al., 1996).

Tissue preparation, immunohistochemistry and in situ hybridization

Embryos were isolated at 10.5 dpc to 18.5 dpc from staged pregnancies. After fixation in 4% paraformaldehyde, specimens were either dehydrated, bleached and rehydrated for whole-mount studies or cryoprotected by overnight incubation at 4°C in 30% sucrose, embedded in OCT compound at –80°C and sectioned at 10 μm or at 20 μm (Stolt et al., 2004; Stolt et al., 2003).

Immunohistochemistry was performed on 10 μm sections or on dissected gastrointestinal tracts. The following primary antibodies were used in various combinations: anti-Sox10 guinea pig antiserum (1:2000 dilution) (Stolt et al., 2004), anti-Sox8 guinea pig antiserum (1:1000 dilution) (Stolt et al., 2005), anti-Brn3.0 rabbit antiserum (1:100 dilution) (Fedtsova and Turner, 1995), anti-Oct6 rabbit antiserum (1:2000 dilution) (Sock et al., 1996), anti-Krox20 rabbit antiserum (1:500 dilution) (Sock et al., 1997), anti-PGP9.5 rabbit antiserum (1:400; Biotrend), anti-Phox2b rabbit antiserum (1:2000 dilution, gift of C. Goridis, Ecole Normale Superieure, Paris), anti-Olig2 rabbit antiserum (1:2000 dilution, gift of H. Takebayashi, Kyoto University), anti-B-FABP rabbit antiserum, (1:10,000 dilution, gift of C. Birchmeier and T. Müller, MDC, Berlin), anti-NF165 mouse monoclonal (1:200 dilution, Developmental Studies Hybridoma Bank), anti-NeuN mouse monoclonal (1:500 dilution, Chemicon), anti-MBP mouse monoclonal (1:500 dilution, Chemicon) and anti-MPZ mouse monoclonal (1:10000 dilution; gift of J. J. Archelos, Graz). Secondary antibodies conjugated to Cy2 and Cy3 immunofluorescent dyes (Dianova) were used for detection. Dissected gastrointestinal tracts were also used for NADPH diaphorase staining (Scherer-Singler et al., 1983).

In situ hybridization was performed on 20 μm sections with DIG-labelled antisense riboprobes for MBP and PLP or on whole embryos with probes for Kit, Mitf and Dct (Britsch et al., 2001; Stolt et al., 2002). All steps except probe hybridization and final colorimetric detection were performed automatically on a Biolane HTI (Hölle & Hüttner AG).

Samples were analyzed and documented either with a Leica (Bensheim, Germany) inverted microscope (DMIRB) equipped with a cooled MicroMax CCD camera (Princeton Instruments, Trenton, NJ), a Leica TCS SL confocal microscope or with a Leica MZFLIII stereomicroscope equipped with an Axiocam (Zeiss, Oberkochem, Germany).

RESULTS

Generation and characterization of mice in which Sox10 is replaced by Sox8

To delete *Sox10* and at the same time allow Sox8 expression in a Sox10-specific pattern, we removed all *Sox10*-coding sequences as well as introns 3 and 4, and simultaneously inserted *Sox8* open reading frame, internal ribosomal entry site, EGFP sequences and neomycin resistance cassette into the *Sox10* locus (Fig. 1A). The *Sox8* open reading frame was placed with its start codon exactly at the position of the *Sox10*-coding sequence, thus leaving 5′ untranslated region (UTR) and *Sox10* promoter sequences intact. Analogous insertion of a *lacZ* marker or *rtTA* sequences into the *Sox10* locus had previously shown that no essential regulatory elements were lost upon deletion of these *Sox10* sequences as β-galactosidase was detected and rtTA-responsive target genes were activated in the respective heterozygous embryos and early postnatal mice in all tissues that normally express Sox10 (Britsch et al., 2001; Ludwig et al., 2004b). Because of the internal ribosomal entry site, EGFP sequences should not only be present on the same transcript as *Sox8*-coding sequences, but should also be translated. EGFP occurrence in Sox10-expressing tissues is not harmful, as mice with Sox10 Cre-induced EGFP expression developed normally and remained healthy into adulthood (data not shown). The presence of flanking loxP sites should furthermore allow Cre-mediated removal of the neo selection cassette (Fig. 1A).

ES cell clones carrying these alterations at the *Sox10* locus were obtained by standard gene targeting techniques and injected into blastocysts (Fig. 1B). Chimeric mice from two independent ES cell clones transmitted the genetic alteration to their progeny (Fig. 1C,E). The neo cassette was deleted through Cre recombinase activity (Fig. 1D). Progeny from both ES cell clones had identical phenotypes. The resulting allele was sequenced to confirm that no mutations had occurred in the Sox8-coding sequences. It is henceforth referred to as $Sox10^{Sox8ki}$ or *Sox8* replacement allele.

All possible genotypes were obtained in the expected Mendelian ratios when mice heterozygous for the *Sox8* replacement allele ($Sox10^{+/Sox8ki}$) were crossed with each other and genotyping was performed on embryos. This was similarly observed for early embryonic stages (10.5-12.5 dpc) and for embryos between 14.5 and 18.5 dpc (Fig. 2A). No increased embryonic lethality occurred in $Sox10^{Sox8ki/Sox8ki}$ mice around 13.5 dpc, as previously observed for mice with inactive Sox10 ($Sox10^{Dom/Dom}$) (Britsch et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998). $Sox10^{Sox8ki/Sox8ki}$ mice also survived birth, in contrast to Sox10-deficient mice. Thus, Sox8 is able to rescue both the partial embryonic and the perinatal lethality of Sox10-deficient mice.

However, $Sox10^{Sox8ki/Sox8ki}$ mice failed to thrive, were severely growth retarded and invariably died during the first postnatal week so that no $Sox10^{Sox8ki/Sox8ki}$ mice were detected by genotyping at the time of weaning (data not shown). This already indicated that the phenotypic rescue was not complete.

To ensure that the incomplete phenotypic rescue was not due to reduced expression of the Sox8 replacement allele, total RNA was isolated from wild-type embryos and $Sox10^{Sox8ki/Sox8ki}$ littermates at either 10.5 dpc or 12.5 dpc. After reverse transcription, expression levels from wild-type allele and *Sox8* replacement allele were compared by quantitative RT-PCR with primers matching sequences in the 5′-UTR (primers A and B in Fig. 1A). The 5′-UTRs of transcripts for wild-type and replacement allele are identical so that the primers functioned equally well on both transcripts. At the same time the primers failed to detect the transcripts from the native *Sox8* gene. By quantitative RT-PCR, expression levels for the *Sox8* replacement allele were indistinguishable from the wild-type *Sox10* allele (Fig. 2B).

This approach only allowed us to compare expression levels of wild-type and Sox8 replacement allele at early times of development for whole embryos. To study expression levels of

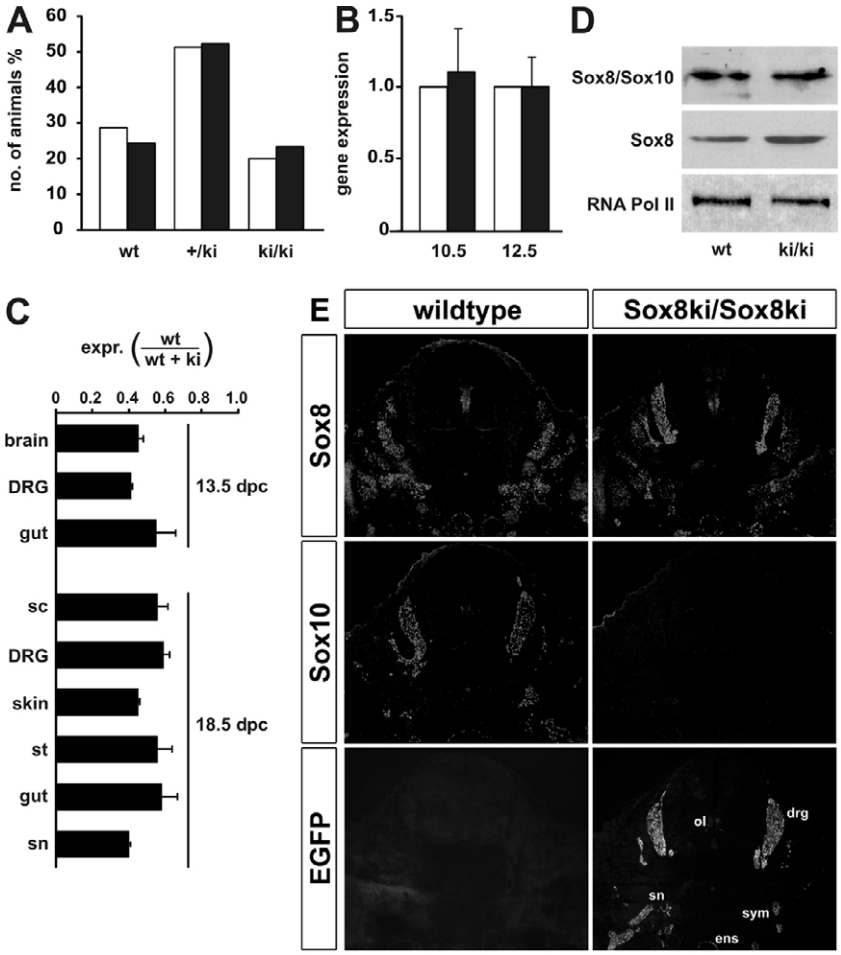

Fig. 2. Genotype distribution and SoxE gene expression in mouse embryos in which Sox10 is replaced by Sox8. (**A**) Embryos from crosses of $Sox10^{+/Sox8ki}$ males and females were sorted according to their genotype (wt, +/ki, ki/ki) as indicated below the bars. Embryos with an age between 10.5 and 12.5 dpc (white bars, *n*=150) were separately grouped from those between 14.5 and 18.5 dpc (black bars, *n*=111). (**B**) Comparison of expression levels for *Sox10* in wild-type embryos at 10.5 and 12.5 dpc (white bars) and for *Sox8* specifically expressed from the *Sox10* locus in age-matched $Sox10^{Sox8ki/Sox8ki}$ embryos (black bars) with primers recognizing a common sequence in both transcripts using quantitative Lightcycler-RT-PCR. Transcript levels in each sample were normalized to *β-actin*. After normalization, transcript levels in the wild type were arbitrarily set to 1. Experiments were repeated twice with material from two independently obtained embryos for each genotype and age. (**C**) Determination of the relative contribution of the wild-type allele to overall expression from the Sox10 locus in the indicated tissues of $Sox10^{+/Sox8ki}$ embryos at 13.5 dpc and 18.5 dpc. Values were obtained by comparing transcript levels from the wild-type allele to the ones from both alleles by quantitative Lightcycler-RT-PCR. Transcript levels were normalized to *rpl8* and to corresponding transcript levels in tissues from age-matched wild-type embryos. sc, spinal cord; sn, sciatic nerve; st, stomach. (**D**) Western blot of nuclear extracts prepared from brains of 18.5 dpc wild-type (wt) and $Sox10^{Sox8ki/Sox8ki}$ (ki/ki) embryos with antibodies directed against SoxE proteins (Sox8/Sox10), Sox8 and RNA polymerase II (RNA Pol II). RNA polymerase II served as loading control. (**E**) Immunohistological analysis of transverse sections from wild-type and $Sox10^{Sox8ki/Sox8ki}$ embryos at 12.5 dpc using antibodies against Sox8 and Sox10. Additionally, EGFP autofluorescence was documented in both genotypes. Sox8 expression in the $Sox10^{Sox8ki/Sox8ki}$ embryos is essentially a composite of Sox8 and Sox10 expression in the wild type.

both alleles in single tissues at later stages, we quantified overall expression from the *Sox10* locus in tissues from $Sox10^{+/Sox8ki}$ embryos at 13.5 dpc and 18.5 dpc with the already mentioned primers A and B, as well as expression of the wild-type allele with primers C and D (Fig. 1A). By comparing the obtained values to the ones for wild-type tissues, we were able to determine the contribution of the wild-type allele to overall expression from the *Sox10* locus in $Sox10^{+/Sox8ki}$ tissues as 40% to 59% (Fig. 2C). Considering the precision limits of the quantitative RT-PCR, wild-type and Sox8 replacement allele were thus expressed at approximately equal levels in the analyzed $Sox10^{+/Sox8ki}$ tissues. As there is furthermore no reason to assume that the wild-type allele should be expressed at different levels in $Sox10^{+/Sox8ki}$ and $Sox10^{+/+}$ tissues, we conclude that the Sox8 replacement allele is expressed at wild-type levels. Using nuclear extracts, we also compared protein levels in brains of 18.5-dpc old wild-type and $Sox10^{Sox8ki/Sox8ki}$ embryos. Relative molecular masses of Sox10 and Sox8 are so similar that both appear in a single band on western blots. Compared with wild-type brains, the combined amounts of Sox8 and Sox10 were unchanged in $Sox10^{Sox8ki/Sox8ki}$ brains, as evident from Western blots with anti-SoxE antibodies (Fig. 2D). At the same time, Sox8 levels were significantly increased in $Sox10^{Sox8ki/Sox8ki}$ brains, as assessed by Sox8-specific antibodies. In the central nervous system at least, the joint amount of Sox8 and Sox10 protein was thus not significantly altered upon replacement of *Sox10* by *Sox8* in our mouse model.

We next analyzed the spatiotemporal expression pattern of the *Sox8* replacement allele during mouse development from 10.5 dpc to 18.5 dpc using EGFP autofluorescence. EGFP was expressed from the *Sox10* locus at all analyzed stages in a pattern that strongly resembled Sox10 expression in the wild type (Fig. 2E and data not shown). At 12.5 dpc, EGFP was detected in many neural crest derivatives, including those in dorsal root ganglia, sympathetic ganglia, gut and along nerves (Fig. 2E). Expression also started in newly specified oligodendrocyte precursors at the border of the pMN domain. There was no Sox10-specific immunoreactivity in $Sox10^{Sox8ki/Sox8ki}$ embryos (Fig. 2E). When immunohistochemistry on $Sox10^{Sox8ki/Sox8ki}$ embryos was performed with antibodies against Sox8, a pattern was observed that corresponded to the composite pattern of Sox8 and Sox10 in the wild type (Fig. 2E). In 12.5 dpc $Sox10^{Sox8ki/Sox8ki}$ embryos, Sox8 in peripheral ganglia and along nerves almost exclusively stems from the replacement allele, whereas expression in myoblasts, chondrocytes and the ventricular zone of the spinal cord is caused by endogenous *Sox8* gene expression. We thus conclude, that Sox8 expression from the $Sox10^{Sox8ki}$ allele is comparable with Sox10 expression in the wild type with regards to expression levels and expression pattern.

Analysis of the cranial and vagal neural crest derivatives in Sox10^{Sox8ki} mice

Loss of Sox10 leads to severe defects in the cranial and vagal neural crest (Britsch et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998). In Sox10$^{Dom/Dom}$ mice, for example, cranial ganglia of predominantly neural crest origin are severely reduced, whereas those with significant placodal contributions are less affected (Herbarth et al., 1998). When cranial ganglia were analyzed by EGFP autofluorescence in Sox10$^{Sox8ki/Sox8ki}$ embryos at 10.5 dpc and compared with Sox10$^{+/Sox8ki}$ littermates, no obvious alterations were detected. All cranial ganglia were present in their regular shape (Fig. 3A,B,D,E). In particular, we did not observe the size reductions in the dorsal portions of the trigeminal and facial ganglia nor the thinning of the proximal glossopharyngeal and vagus parts, which are typical of Sox10-deficient mice (Herbarth et al., 1998). Whereas β-galactosidase staining in cranial ganglia of Sox10$^{lacZ/lacZ}$ embryos is strongly reduced relative to otic vesicle staining when compared with Sox10$^{+/lacZ}$ littermates (Britsch et al., 2001), no such differences were observed for the EGFP signal between Sox10$^{Sox8ki/Sox8ki}$ and Sox10$^{+/Sox8ki}$ embryos. By contrast, EGFP autofluorescence in the Sox10$^{Sox8ki/Sox8ki}$ embryos was uniformly increased throughout all expressing tissues when compared with Sox10$^{+/Sox8ki}$ littermates owing to higher EGFP expression from two instead of one allele (Fig. 3A,B,D,E). Age-matched wild-type embryos did not exhibit specific fluorescence (Fig. 3C,F). At least at this level of resolution, development of cranial ganglia is therefore normal in Sox10$^{Sox8ki/Sox8ki}$ mice.

Thus, we turned to the analysis of the enteric nervous system as the main derivative of the vagal neural crest. Sox10-deficient embryos lack an enteric nervous system throughout the whole gastrointestinal tract, including stomach and oesophagus (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998). Severe defects in the enteric nervous system were also detected in Sox10$^{Sox8ki/Sox8ki}$ embryos. At the end of embryogenesis, both small and large intestine were devoid of immunoreactivity for PGP9.5, a ubiquitin hydrolase expressed by all enteric neurons and neuronal precursors, and NADPH diaphorase staining, which specifically labels NOS-positive neurons (Fig. 4A,B). Similarly, there was no B-FABP immunoreactivity, which is specific for enteric glia (data not shown). EGFP autofluorescence confirmed that, fore- and hindgut were never colonized by enteric neural crest cells. Apoptotic cells were strongly increased in the area where vagal neural crest cells are normally found prior to gut colonization (data not shown). Oesophagus and stomach of Sox10$^{Sox8ki/Sox8ki}$ mice, by contrast, possessed normal looking enteric ganglia and plexus (Fig. 4A,B). In Sox10-deficient mice, these two areas also lack an enteric nervous system (Britsch et al., 2001; Southard-Smith et al., 1998). Although restricted to the anteriormost parts of the gastrointestinal tract, there is thus a certain degree of phenotypic rescue in Sox10$^{Sox8ki/Sox8ki}$ mice.

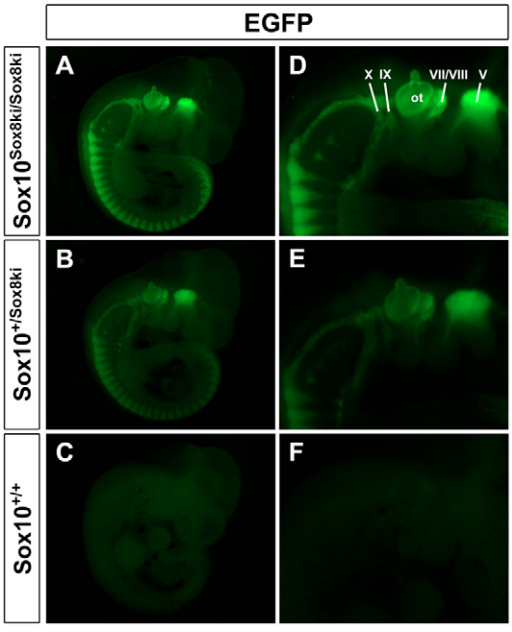

Fig. 3. Development of cranial ganglia in Sox10$^{Sox8ki/Sox8ki}$ embryos. Whole-mount EGFP autofluorescence of the cranial region of Sox10$^{Sox8ki/Sox8ki}$ embryos (**A**,**D**) and their Sox10$^{+/Sox8ki}$ (**B**,**E**) and wild-type Sox10$^{+/+}$ (**C**,**F**) littermates at 10.5 dpc. (D-F) Higher magnifications of A-C. V, trigeminal; VII/VIII, facial/acoustic; IX, glossopharyngeal; X, vagus; ot, otic vesicle.

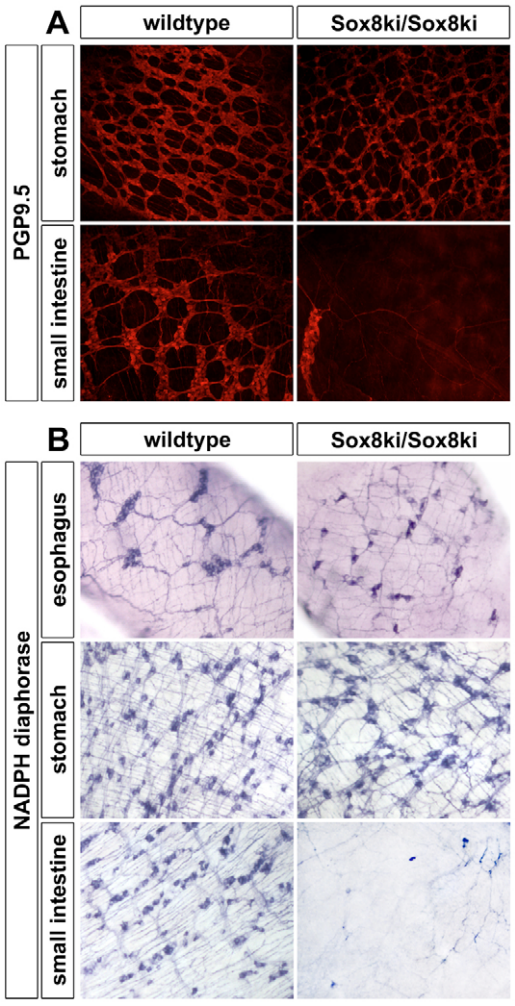

Fig. 4. Analysis of the enteric nervous system in Sox10$^{Sox8ki/Sox8ki}$ mice. Oesophagus, stomach and small intestine of wild-type embryos and Sox10$^{Sox8ki/Sox8ki}$ littermates were analyzed at 18.5 dpc by whole-mount immunohistochemistry with PGP9.5 antibodies (**A**) or by NADPH diaphorase staining (**B**). A region of the small intestine is shown that is immediately adjacent to the stomach. No immunohistochemical or NADPH diaphorase staining was observed in the gut distal to the stomach.

This conclusion is also supported by the absence of a megacolon in $Sox10^{+/Sox8ki}$ mice, which is regularly detected in $Sox10^{+/Dom}$ and $Sox10^{+/LacZ}$ mice (Britsch et al., 2001; Southard-Smith et al., 1998). $Sox10^{Sox8ki/Sox8ki}$ mice, however, suffered from severe gastrointestinal problems that are probably responsible for the observed postnatal lethality in this genotype.

Analysis of melanocyte development in $Sox10^{Sox8ki}$ mice

Melanocyte development is also extremely sensitive to loss of Sox10 (Britsch et al., 2001; Southard-Smith et al., 1998). Thus, it may not be surprising that melanocyte development was severely disturbed in $Sox10^{Sox8ki/Sox8ki}$ mice. $Sox10^{Sox8ki/Sox8ki}$ mice have a completely white coat (Fig. 5A). Melanin was absent from all hair follicles and hair shafts, as evident from whole skin preparations, indicating that melanocytes were absent or unable to produce melanin (Fig. 5B). To distinguish between these possibilities, we performed whole-mount in situ hybridization on 12.5 dpc embryos using several markers for early melanoblasts. With dopachrome tautomerase (Dct) as a marker, melanoblasts were not detected (Fig. 5C). Similarly, very few residual melanoblasts were spotted in $Sox10^{Sox8ki/Sox8ki}$ mice with a probe against the melanocyte-specific transcription factor Mitf. As both Dct and Mitf are direct target genes of Sox10 (Jiao et al., 2004; Ludwig et al., 2004a; Potterf et al., 2001), Kit was employed as a third marker. Although Kit additionally labels other cells than melanocytes such as mast cells, we observed a greater than 90% reduction of Kit positive cells in $Sox10^{Sox8ki/Sox8ki}$ mice (Fig. 5C). There is thus virtually no difference in the severity of the melanocyte defect compared with $Sox10^{LacZ/LacZ}$ mice (Britsch et al., 2001), arguing that Sox8 is unable to compensate for loss of Sox10 in the melanocyte lineage.

Analysis of the peripheral nervous system in $Sox10^{Sox8ki}$ mice

The peripheral nervous system is also strongly affected in the absence of Sox10, with defects detectable in dorsal root ganglia, along spinal nerves and in sympathetic ganglia. This correlates with the fact that Sox10 is by far the predominant SoxE protein in the peripheral nervous system (Wegner and Stolt, 2005). At 12.5 dpc, dorsal root ganglia normally contain both sensory neurons and satellite glia, whereas in Sox10-deficient mice dorsal root ganglia are devoid of glia, start to lose neurons apoptotically and lack a continuous basement membrane (Britsch et al., 2001; Sonnenberg-Riethmacher et al., 2001; Southard-Smith et al., 1998). By contrast, satellite glia were easily detected at 12.5 dpc by B-FABP staining in dorsal root ganglia of $Sox10^{Sox8ki/Sox8ki}$ embryos (Fig. 6A). There was furthermore no obvious difference in the number of stained cells and the intensity of staining between $Sox10^{Sox8ki/Sox8ki}$ embryos and wild-type littermates. Similar results were obtained for the sensory neurons of dorsal root ganglia with several independent markers, including NeuN (Neuna60 – Mouse Genome Informatics), Brn3.0 (Pou4f1 – Mouse Genome Informatics) and islet 1 (Fig. 6A and data not shown). Therefore, both gliogenesis and neurogenesis had occurred normally in the dorsal root ganglia of $Sox10^{Sox8ki/Sox8ki}$ embryos despite the fact that *Sox10* had been replaced by *Sox8* (Fig. 6A). The basement membrane around the dorsal root ganglia was also completely intact in $Sox10^{Sox8ki/Sox8ki}$

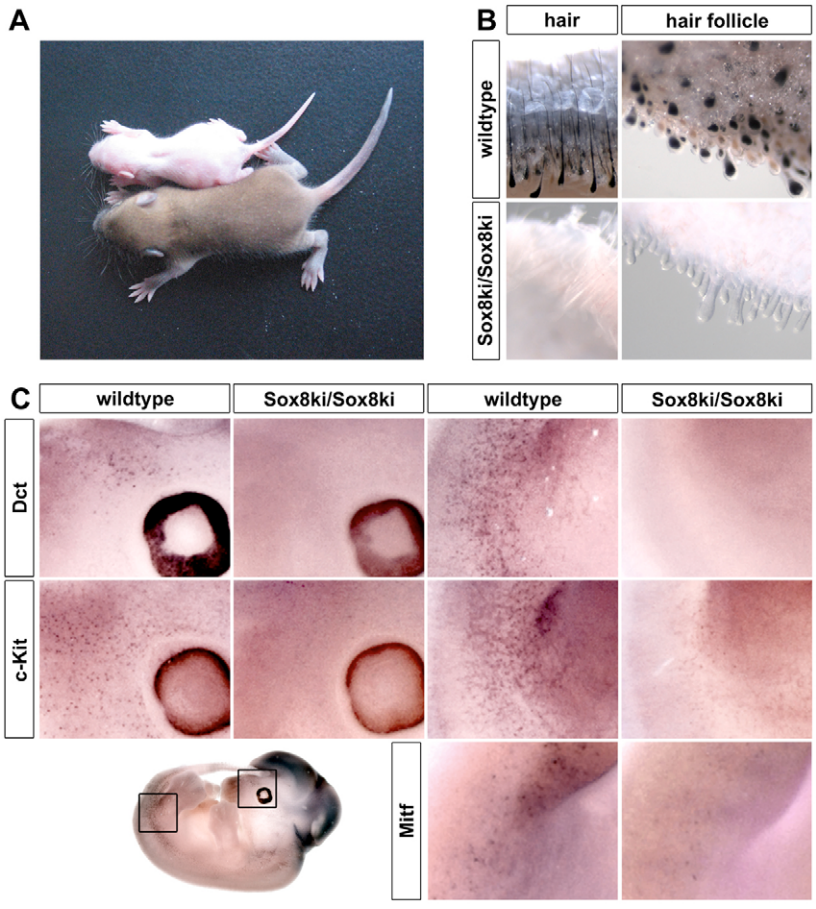

Fig. 5. Melanocyte development in $Sox10^{Sox8ki/Sox8ki}$ mice. (**A**) Comparison of 7-day old $Sox10^{Sox8ki/Sox8ki}$ mice (top) and wild-type littermates (bottom). $Sox10^{Sox8ki/Sox8ki}$ mice are white and smaller than wild type. (**B**) Skin preparations from 7-day-old wild-type mice and $Sox10^{Sox8ki/Sox8ki}$ littermates shown from the side and from below to visualize hair and hair follicle. (**C**) Whole-mount in situ hybridization was performed on wild-type and $Sox10^{Sox8ki/Sox8ki}$ embryos at 12.5 dpc using antisense riboprobes against Dct, Kit and Mitf. The regions from which pictures were taken are boxed in the embryo in the lower left corner.

mice (data not shown). Dorsal root ganglia still had a normal shape at 18.5 dpc and contained normal numbers of neurons and glia (Fig. 6B). This is in strong contrast to Sox10-deficient mice where at this age dorsal root ganglia are rudimentary and have lost nearly all neurons in the absence of trophic support from satellite glia (Britsch et al., 2001).

Sox10-deficient embryos at this age also have thinned and defasciculated peripheral nerves that lack all Schwann cells (Britsch et al., 2001; Herbarth et al., 1998; Sonnenberg-Riethmacher et al., 2001; Southard-Smith et al., 1998). When peripheral nerves of $Sox10^{Sox8ki/Sox8ki}$ were stained with neurofilament 165 (NF-165), no obvious thinning was observed. Instead neurofilament staining resembled that of wild-type nerves (Fig. 7A,B). Additionally, cells were present along the peripheral nerves of $Sox10^{Sox8ki/Sox8ki}$ embryos that exhibited EGFP autofluorescence and, by morphology and position, probably correspond to the Schwann cells that, in the wild type, instead possessed nuclear Sox10 staining (Fig. 7G-J). At this stage, many Schwann cells in the wild type had entered the promyelinating stage marked by expression of the transcription factors Oct6 and Krox20 (Bermingham et al., 1996; Jaegle et al., 1996; Topilko et al., 1994; Zorick et al., 1996). Immunohistochemical analysis of $Sox10^{Sox8ki/Sox8ki}$ embryos with these markers not only confirmed the Schwann cell identity of the cells detected along the peripheral nerves, but also proved that these cells had progressed normally through lineage development into the promyelinating stage (Fig. 7C-F). Normal development of myelinating Schwann cells was further confirmed when peripheral nerves were studied at postnatal day 3. At this age, sheath-like structures in the nerve had formed and started to accumulate significant amounts of the myelin basic protein (MBP) and the myelin protein zero (MPZ), indicating the onset of myelination (Fig. 7K-N). Again, no difference could be detected with any of the markers between the wild-type and $Sox10^{Sox8ki/Sox8ki}$ mice, arguing that Schwann cell development proceeds on schedule when Sox10 is replaced by Sox8. The normal development of dorsal root ganglia and peripheral nerves also explains why $Sox10^{Sox8ki/Sox8ki}$ mice survive birth and are able to breathe, in contrast to $Sox10^{LacZ/LacZ}$ mice.

Sympathetic ganglia also formed normally in $Sox10^{Sox8ki/Sox8ki}$ mice (Fig. 8 and data not shown). At 12.5 dpc, there was no significant difference in the number of neural crest precursors and glia, which are marked by Sox10 in the wild type and instead by Sox8 in $Sox10^{Sox8ki/Sox8ki}$ littermates (Fig. 8A-D). The majority of cells in the sympathetic ganglia at this time were positive for Phox2b, a marker for autonomic noradrenergic neurons, and expressed NF-165 abundantly (Fig. 8E-H). This was similarly the

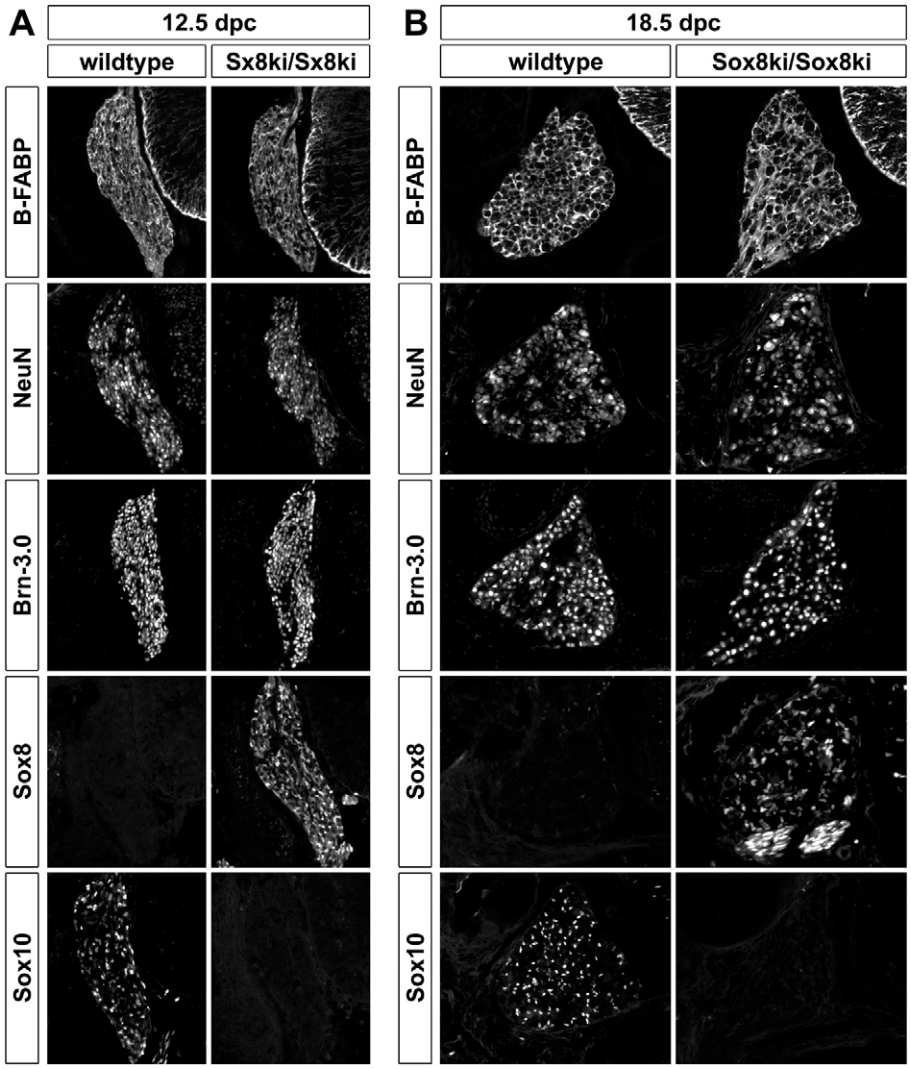

Fig. 6. Analysis of dorsal root ganglia in $Sox10^{Sox8ki/Sox8ki}$ mice.
Immunohistochemistry was carried out on transverse sections through dorsal root ganglia of wild-type and $Sox10^{Sox8ki/Sox8ki}$ embryos at 12.5 dpc (**A**) and 18.5 dpc (**B**) using antibodies against the glial marker B-FABP and the neuronal markers NeuN and Brn3.0. Occurrence of Sox8 and Sox10 was additionally documented in both genotypes with specific antibodies.

case for the wild-type and for Sox10$^{Sox8ki/Sox8ki}$ embryos, indicating that neurogenesis had taken place on schedule. On average, sympathetic ganglia in Sox10$^{Sox8ki/Sox8ki}$ embryos appeared slightly smaller in size and contained fewer Phox2b-positive cells than their wild-type counterparts (Fig. 8G,H). So far, this constitutes the only evidence for altered peripheral nervous system development in Sox10$^{Sox8ki/Sox8ki}$ mice outside the enteric nervous system. However, these changes are too minor to cause the noradrenergic deficit at mid-embryogenesis that has been discussed as the molecular basis for the increased embryonic lethality of Sox10-deficient mice at 13.5 dpc (Britsch et al., 2001).

Analysis of oligodendrocyte development in Sox10^{Sox8ki} mice

Finally, we analyzed oligodendrocyte development in spinal cords of Sox10$^{Sox8ki/Sox8ki}$ mice. As oligodendrocyte development is normal in Sox10-deficient mice until the onset of terminal differentiation (Stolt et al., 2002), we did not expect a phenotype in oligodendrocyte precursors of Sox10$^{Sox8ki/Sox8ki}$ mice up until 18.5 dpc. This was indeed the case. The number of EGFP-positive cells in Sox10$^{Sox8ki/Sox8ki}$ spinal cords corresponded well to the number of Sox10-positive cells in the wild-type spinal cord at all stages analyzed (Fig. 9A-C,J-L). No changes were observed in the Sox8 expression pattern (Fig. 9D-I). Immunohistochemistry with Olig2 as an independent marker of the oligodendrocyte lineage confirmed that oligodendrocyte precursors were indistinguishable in numbers and distribution between the wild type and the Sox10$^{Sox8ki/Sox8ki}$ mutant from 12.5 dpc until 18.5 dpc (Fig. 9P-U).

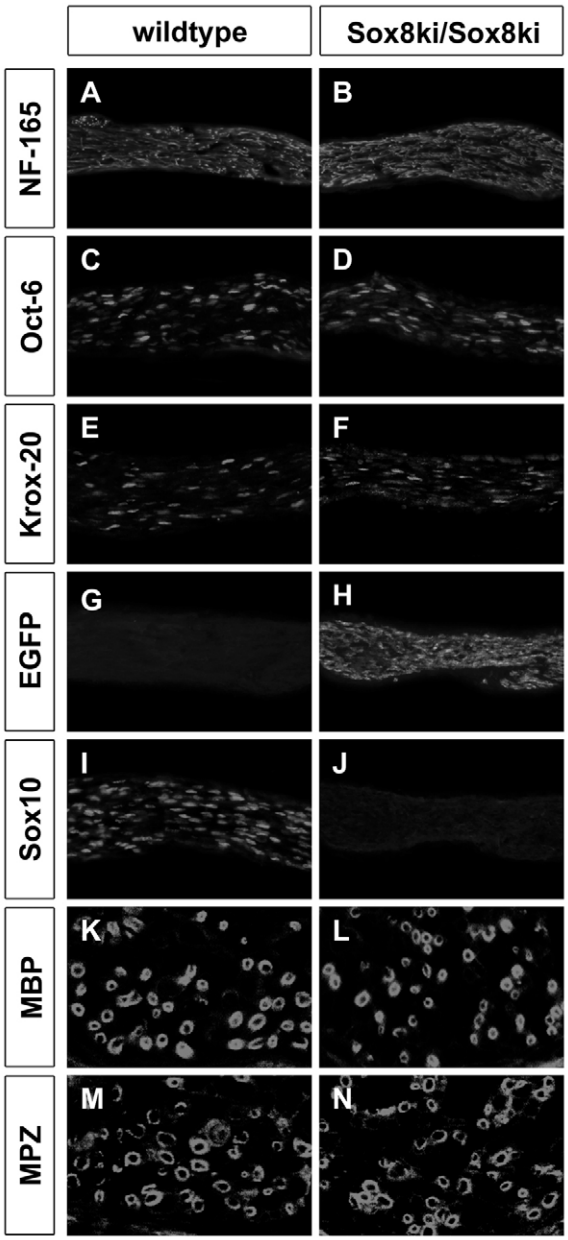

Fig. 7. Peripheral nerve development in Sox10$^{Sox8ki/Sox8ki}$ mice. (**A-J**) Immunohistological analysis of spinal nerves from wild-type (A,C,E,G,I) and Sox10$^{Sox8ki/Sox8ki}$ (B,D,F,H,J) embryos at 18.5 dpc was carried out using antibodies against NF-165 (A,B), Oct6 (C,D) and Krox20 (E,F). EGFP autofluorescence (G,H) and occurrence of Sox10 (I,J) was additionally visualized in both genotypes. (**K-N**) Immunohistochemistry on transverse sections of peripheral nerves from wild-type (K,M) and Sox10$^{Sox8ki/Sox8ki}$ (L,N) mice at postnatal day 3 using antibodies against MBP (K,L) and MPZ (M,N).

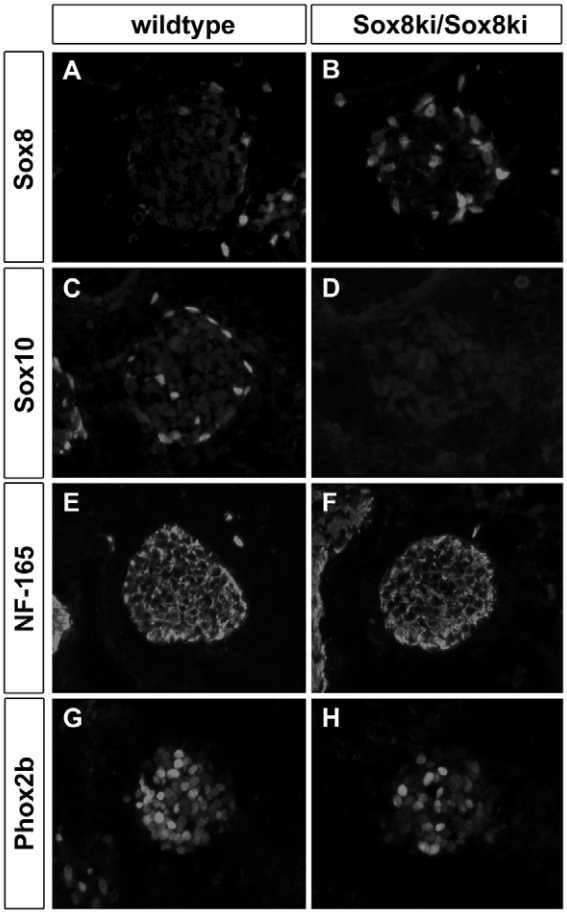

Fig. 8. Development of sympathetic ganglia in Sox10$^{Sox8ki/Sox8ki}$ mice. Immunohistochemical staining of sympathetic ganglia was performed on wild-type (A,C,E,G) and Sox10$^{Sox8ki/Sox8ki}$ (B,D,F,H) embryos at 12.5 dpc using antibodies against Sox8 (**A**,**B**), Sox10 (**C**,**D**), NF-165 (**E**,**F**) and Phox2b (**G**,**H**). Sympathetic ganglia in Sox10$^{Sox8ki/Sox8ki}$ embryos are slightly smaller and contain fewer Phox2b-positive cells.

Therefore, we turned to the analysis of terminal differentiation. In situ hybridization with probes for MBP and proteolipid protein (PLP) revealed substantial numbers of maturing oligodendrocytes in the future white matter of wild-type spinal cords at 18.5 dpc (Fig. 10A,C). Their number steeply increased in the wild type during the first postnatal week (Fig. 10E,G). When spinal cords of $Sox10^{Sox8ki/Sox8ki}$ littermates were analyzed, there were only very few MBP- and PLP-expressing cells at 18.5 dpc (Fig. 10B,D). Similarly at postnatal day 7, the number of myelinating oligodendrocytes was significantly lower than in the wild type (Fig. 10F,H), and in comparison with previously published data, even significantly lower than in $Sox10^{+/lacZ}$ spinal cords (Stolt et al., 2004). Sox8, therefore cannot effectively replace Sox10 during terminal oligodendrocyte differentiation. There is, however, a very small amount of Sox8-specific rescue in the $Sox10^{Sox8ki/Sox8ki}$ mutant, because Sox10-deficient spinal cords are completely devoid of myelinating oligodendrocytes at 18.5 dpc (Stolt et al., 2002).

DISCUSSION

Generation of paralogues through gene duplication is a key mechanism for increasing gene number in an organism. Pairs or groups of paralogues also exist for many transcription factors, with Sox proteins being just one example. A single Sox protein in invertebrates usually corresponds to several closely related vertebrate Sox proteins. In case of Sox100B, the SoxE protein from *Drosophila melanogaster*, these are Sox8, Sox9 and Sox10 in all vertebrates so far analyzed (Bowles et al., 2000; Wegner, 1999).

Paralogous transcription factors often exhibit overlapping expression patterns. They, furthermore, share many structural features and exhibit significant sequence homologies, raising the issue of their extent of functional equivalence. In the mouse, few examples exist where functional equivalence among paralogous transcription factors has been addressed by gene replacement strategies. The results of these studies have been diverse (Acampora et al., 1999; Bouchard et al., 2000; Coppola et al., 2005; Friedrich et al., 2005; Hanks et al., 1995). The Engrailed proteins En1 and En2, for example, were found to function interchangeably and therefore equivalently as were the Pax proteins Pax2 and Pax5 (Bouchard et

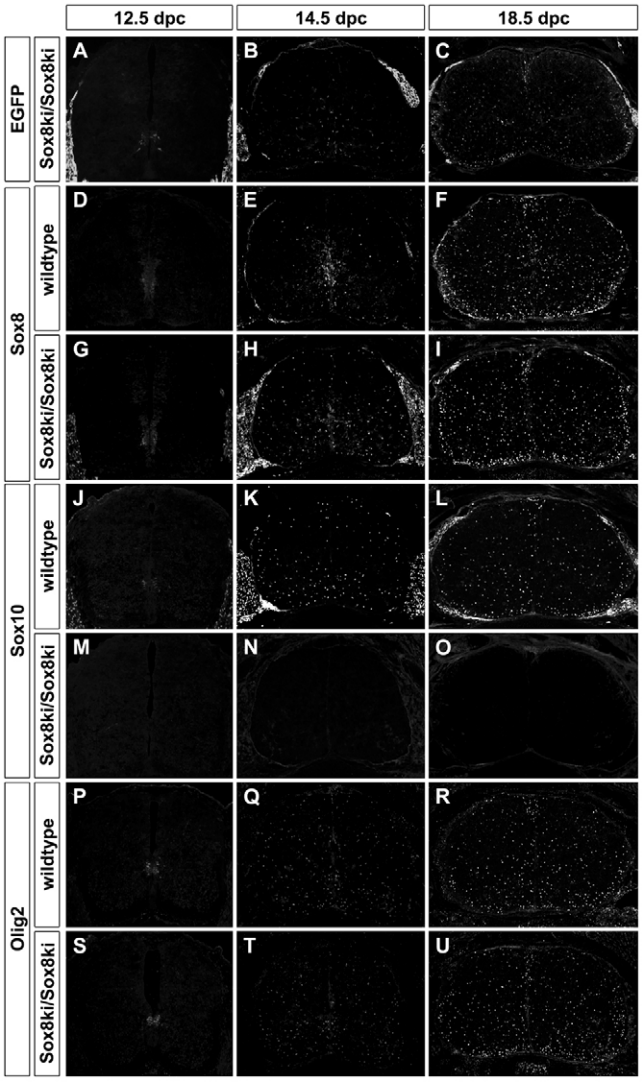

Fig. 9. Development of oligodendrocyte precursors in $Sox10^{Sox8ki/Sox8ki}$ mice. Specification and subsequent distribution of oligodendrocyte precursors throughout the spinal cord of $Sox10^{Sox8ki/Sox8ki}$ embryos (**A-C**,**G-I**,**M-O**,**S-U**) and wild-type littermates (**D-F**,**J-L**,**P-R**) was analyzed on transverse sections from the forelimb region at 12.5 dpc (A,D,G,J,M,P,S), 14.5 dpc (B,E,H,K,N,Q,T) and 18.5 dpc (C,F,I,L,O,R,U) using EGFP autofluorescence (A-C) and immunohistochemistry with antibodies directed against Sox8 (D-I), Sox10 (J-O) and Olig2 (P-U).

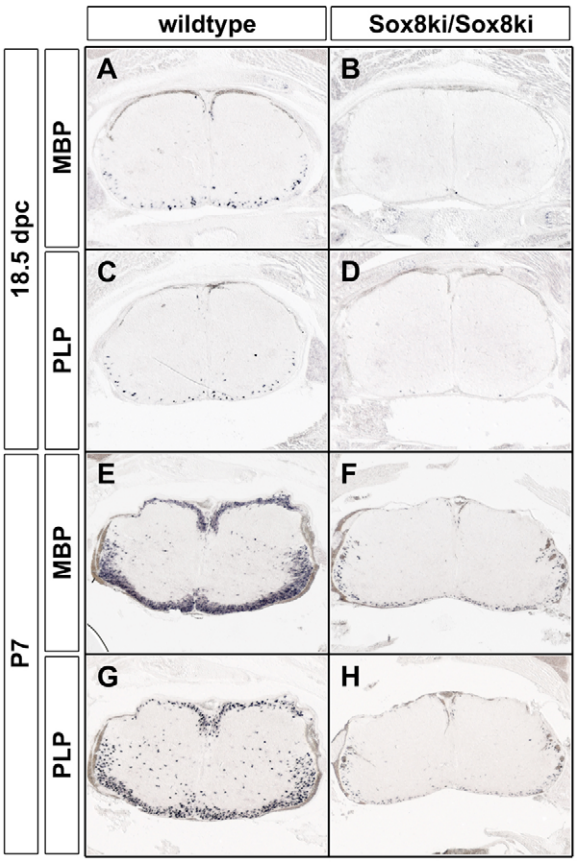

Fig. 10. Terminal differentiation of oligodendrocytes in $Sox10^{Sox8ki/Sox8ki}$ mice. In situ hybridization with probes specific for MBP (A,B,E,F) and PLP (C,D,G,H) were performed on transverse spinal cord sections from the forelimb region at 18.5 dpc (**A-D**) and postnatal day 7 (**E-H**). (A,C,E,G) Wild-type spinal cords; (B,D,F,H) $Sox10^{Sox8ki/Sox8ki}$ spinal cords.

al., 2000; Hanks et al., 1995). The homeodomain proteins Phox2a and Phox2b, however, were only partially equivalent and both exhibited unique functions during neural differentiation (Coppola et al., 2005). Our study on mice in which *Sox10* was replaced by *Sox8* support the notion that the paralogous members of Sox group E are also only partially equivalent.

To validate our mouse model, we had to ensure that Sox8 is expressed in $Sox10^{Sox8ki/Sox8ki}$ embryos in the same spatiotemporal pattern and in identical amounts as Sox10 in the wild type. To achieve this goal, we removed the complete open reading frame of *Sox10* and embedded the *Sox8* open reading frame in such a way into the *Sox10* locus that gene regulatory elements, transcriptional start site, 5′ non-coding exons and translational start site were all left untouched. The major difference between the wild-type *Sox10* locus and the replacement allele is that the *Sox8* open reading frame is present on a single exon, whereas the *Sox10* open reading frame in the wild type is split between three exons. The *Sox8* replacement allele is nevertheless transcribed as an intron-containing message that is spliced prior to translation.

We checked expression levels of the *Sox8* replacement allele and compared it with the wild type. There was no significant difference in transcript levels between the wild-type and the *Sox8* replacement allele, both at the level of the complete $Sox10^{Sox8ki/Sox8ki}$ embryo at early times of development and in several tissues of $Sox10^{+/Sox8ki}$ embryos at later times. Importantly, expression of wild-type and the Sox8 replacement allele was also comparable in those tissues in which there was no complete rescue by the Sox8 replacement allele, including the melanocyte containing skin, the gut and the CNS. Additionally, the amount of Sox8 protein in the brain of $Sox10^{Sox8ki/Sox8ki}$ was very similar to the combined amounts of Sox8 and Sox10 protein in the wild type. Although we might have missed differences in expression levels in a minor population of normally Sox10-expressing cells, the most likely conclusion from our study is that overall amounts for SoxE transcripts and proteins are not significantly altered in our mouse mutant. Therefore, we believe that the $Sox10^{Sox8ki/Sox8ki}$ mouse is indeed a suitable model with which to study the functional equivalence of Sox8 and Sox10.

The limited phenotypic rescue of the Sox10 deficiency in the $Sox10^{Sox8ki/Sox8ki}$ mouse was unexpected and the two SoxE proteins are far less exchangeable than predicted from previous studies on Sox8/Sox10 compound mutants (Maka et al., 2005; Stolt et al., 2004). The phenotypic rescue furthermore varied strongly between tissues, so that it was nearly complete in the peripheral nervous system, limited in enteric nervous system and oligodendrocytes, and non-existent in the melanocyte lineage. Interestingly, the effectiveness with which Sox8 was able to rescue did not correlate with its natural occurrence in the tissue or cell type. Thus, there was strong rescue in the peripheral nervous system, but none in melanocytes, although neither tissue expresses Sox8 endogenously. Severe developmental defects were furthermore observed in the developing enteric nervous system and in differentiating oligodendrocytes of $Sox10^{Sox8ki/Sox8ki}$ mice, despite the naturally occurring co-expression of Sox8 and Sox10 in these tissues.

Recent overexpression studies had pointed to significant functional redundancy between closely related Sox proteins (Bylund et al., 2003; Cheung and Briscoe, 2003; Graham et al., 2003; Taylor and LaBonne, 2005; Zhang et al., 2005). Studies on SoxE proteins had indicated that all three SoxE proteins function alike during early neural crest development by expanding the neural crest at the expense of the neural tube (Cheung and Briscoe, 2003; Taylor and LaBonne, 2005). These studies do not necessarily contradict our results, as early neural crest development (apart from the vagal neural crest) was also fairly normal in our $Sox10^{Sox8ki/Sox8ki}$ mice (data not shown). In our mouse model, functional differences between the two SoxE proteins became obvious at later developmental processes which are easier to study in gene replacement studies than in conventional electroporation or microinjection studies. Similar observations were also made in the Phox2 replacements (Coppola et al., 2005).

Our gene replacement approach also guarantees a tight control on spatiotemporal expression. In chick electroporation or *Xenopus* microinjection experiments, overexpressed proteins are usually difficult to control in their expression levels and equally difficult to confine regionally and temporally. High expression levels, in turn, may mask functional differences that exist between Sox proteins at lower physiological concentrations. Furthermore, overexpression studies on SoxE proteins were performed in wild-type embryos that still possessed all three SoxE genes. Thus, overexpression of one SoxE protein could have led to increased expression of the other ones, making it difficult to attribute effects solely to the overexpressed Sox protein. At least in the case of Sox9, ectopic expression has been reported to induce endogenous Sox10 expression (Cheung and Briscoe, 2003).

Our results require a reinterpretation of previously obtained data. Although there is no doubt, that Sox8 contributes to enteric nervous system development and oligodendrocyte differentiation at times when Sox10 is very important for these processes (Maka et al., 2005; Stolt et al., 2004), it appears unlikely now that the different contributions of Sox8 and Sox10 to these developmental processes can solely be attributed to their different level of expression. As evident from our analysis of enteric nervous system development and oligodendrocyte differentiation in $Sox10^{Sox8ki/Sox8ki}$ mice, there have to be functions that are unique to Sox10 and cannot be taken over by Sox8. The spatiotemporal regulation of endothelin receptor-B expression by Sox10 in enteric neural crest precursors may be such a case, as Sox8 apparently failed to compensate the loss of Sox10 (Zhu et al., 2004). The reciprocal existence of unique functions for Sox8 appears likely, but has not formally been proven in our experiments. If existent, however, they must be non-essential, as both enteric nervous system development and oligodendrocyte differentiation are eventually completed in Sox8-deficient mice (Sock et al., 2001).

At present, it is unclear what exactly these unique functions are. Nor is it clear what the structural basis for these functional differences is. Biochemical studies have so far failed to reveal dramatic differences in DNA binding activity or transactivation potential among SoxE proteins (Schepers et al., 2003; Stolt et al., 2004) indicating that the differences may be subtle or temporary. It is tempting to speculate that the functional differences are mediated by the non-conserved regions, which in the case of Sox10 are the first 60 amino acids and regions from its central part. Additionally, at least part of the functional differences between SoxE proteins might be based on differential patterns of posttranslational modifications. These would have largely escaped previous analyses, as these modifications are usually unstable and not faithfully preserved in biochemical experiments unless special precautions are taken. Examples for such differences in posttranslational modifications are indeed emerging. It has been shown recently, that Sox9 and Sox10 proteins are sumoylated (Girard and Goossens, 2006; Taylor and LaBonne, 2005). Whereas the C-terminal sumoylation sites are conserved in Sox8, the N-terminal site, corresponding to lysine 55 in Sox10 is not. The sumoylation site that is not conserved between Sox10 and Sox8 could therefore uniquely influence Sox10 activity. Additionally, two phosphorylation sites for

protein kinase A with clear functional importance are present in Sox9, but absent from Sox8 and Sox10 (Huang et al., 2000). It will be important to determine the various post-translational modifications in the future and see how they affect and diversify the function of the three SoxE proteins.

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References

Acampora, D., Avantaggiato, V., Tuorto, F., Barone, P., Perera, M., Choo, D., Wu, D., Corte, G. and Simeone, A. (1999). Differential transcriptional control as the major molecular event in generating Otx1$^{-/-}$ and Otx2$^{-/-}$ divergent phenotypes. *Development* **126**, 1417-1426.

Aoki, Y., Saint-Germain, N., Gyda, M., Magner-Fink, E., Lee, Y. H., Credidio, C. and Saint-Jeannet, J. P. (2003). Sox10 regulates the development of neural crest-derived melanocytes in Xenopus. *Dev. Biol.* **259**, 19-33.

Bermingham, J. R., Scherer, S. S., O'Connell, S., Arroyo, E., Kalla, K. A., Powell, F. L. and Rosenfeld, M. G. (1996). Tst-1/Oct-6/SCIP regulates a unique step in peripheral myelination and is required for normal respiration. *Genes Dev.* **10**, 1751-1762.

Bouchard, M., Pfeffer, P. and Busslinger, M. (2000). Functional equivalence of the transcription factors Pax2 and Pax5 in mouse development. *Development* **127**, 3703-3713.

Bowles, J., Schepers, G. and Koopman, P. (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev. Biol.* **227**, 239-255.

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.

Bylund, M., Andersson, E., Novitch, B. G. and Muhr, J. (2003). Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat. Neurosci.* **6**, 1162-1168.

Chaboissier, M.-C., Kobayashi, A., Vidal, V. I. P., Lützkendorf, S., van de Kant, H. J. G., Wegner, M., de Rooij, D. G., Behringer, R. R. and Schedl, A. (2004). Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development* **131**, 1891-1901.

Cheung, M. and Briscoe, J. (2003). Neural crest development is regulated by the transcription factor Sox9. *Development* **130**, 5681-5693.

Cheung, M., Chaboissier, M.-C., Mynett, A., Hirst, E., Schedl, A. and Briscoe, J. (2005). The transcriptional control of trunk neural crest induction, survival, and delamination. *Dev. Cell* **8**, 179-192.

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.

Coppola, E., Pattyn, A., Guthrie, S. C., Goridis, C. and Studer, M. (2005). Reciprocal gene replacements reveal unique functions for Phox2 genes during neural differentiation. *EMBO J.* **24**, 4392-4403.

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

Fedtsova, N. and Turner, E. E. (1995). Brn-3.0 expression identifies early post-mitotic CNS neurons and sensory neural precursors. *Mech. Dev.* **53**, 291-304.

Friedrich, R., Schlierf, B., Tamm, E. R., Bösl, M. R. and Wegner, M. (2005). The class III POU domain protein Brn-1 can fully replace the related Oct-6 during Schwann cell development and myelination. *Mol. Cell. Biol.* **25**, 1821-1829.

Girard, M. and Goossens, M. (2006). Sumoylation of the SOX10 transcription factor regulates its transcriptional activity. *FEBS Lett.* **580**, 1635-1641.

Graham, V., Khudyakov, J., Ellis, P. and Pevny, L. (2003). SOX2 functions to maintain neural progenitor identity. *Neuron* **39**, 749-765.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B. and Joyner, A. L. (1995). Rescue of the En-1 mutant phenotype by replacement of En-1 with En-2. *Science* **269**, 679-682.

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.

Huang, W., Zhou, X., Lefebvre, V. and de Crombrugghe, B. (2000). Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer. *Mol. Cell. Biol.* **20**, 4149-4158.

Inoue, K., Khajavi, M., Ohyama, T., Hirabayashi, S.-I., Wilson, J., Reggin, J. D., Mancias, P., Butler, I. J., Wilkinson, M. F., Wegner, M. et al. (2004). Molecular mechanism for distinct neurological phenotypes conveyed by allelic truncating mutations. *Nat. Genet.* **36**, 361-369.

Jaegle, M., Mandemakers, W., Broos, L., Zwart, R., Karis, A., Visser, P., Grosveld, F. and Meijer, D. (1996). The POU factor Oct-6 and Schwann cell differentiation. *Science* **273**, 507-510.

Jiao, Z., Mollaaghababa, R., Pavan, W. J., Antonellis, A., Green, E. D. and Hornyak, T. J. (2004). Direct interaction of Sox10 with the promoter of murine Dopachrome Tautomerase (Dct) and synergistic activation of Dct expression with Mitf. *Pigment Cell Res.* **17**, 352-362.

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.

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.

Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M. (1998). Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.* **18**, 237-250.

Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W. and Westphal, H. (1996). Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. *Proc. Natl. Acad. Sci. USA* **93**, 5860-5865.

Ludwig, A., Rehberg, S. and Wegner, M. (2004a). Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. *FEBS Lett.* **556**, 236-244.

Ludwig, A., Schlierf, B., Schardt, A., Nave, K. A. and Wegner, M. (2004b). A Sox10 rtTA mouse line for tetracycline-inducible expression of transgenes in neural crest cells and oligodendrocytes. *Genesis* **40**, 171-175.

Maka, M., Stolt, C. C. and Wegner, M. (2005). Identification of Sox8 as a modifier gene in a mouse model of Hirschsprung disease reveals underlying molecular defect. *Dev. Biol.* **277**, 155-169.

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.

Potterf, S. B., Mollaaghababa, R., Hou, L., Southard-Smith, E. M., Hornyak, T. J., Arnheiter, H. and Pavan, W. J. (2001). Analysis of SOX10 function in neural crest-derived melanocyte development: SOX10-dependent transcriptional control of dopachrome tautomerase. *Dev. Biol.* **237**, 245-257.

Schepers, G., Wilson, M., Wilhelm, D. and Koopman, P. (2003). Sox8 is expressed during testis differentiation in mice and synergizes with SF1 to activate the AMH promoter in vitro. *J. Biol. Chem.* **278**, 28101-28108.

Scherer-Singler, U., Vincent, S. R., Kimura, H. and McGeer, E. G. (1983). Demonstration of a unique population of neurons with NADPH-diaphorase histochemistry. *J. Neurosci. Methods* **9**, 229-234.

Sock, E., Enderich, J., Rosenfeld, M. G. and Wegner, M. (1996). Identification of the nuclear localization signal of the POU domain protein Tst-1/Oct6. *J. Biol. Chem.* **271**, 17512-17518.

Sock, E., Leger, H., Kuhlbrodt, K., Schreiber, J., Enderich, J., Richter-Landsberg, C. and Wegner, M. (1997). Expression of Krox proteins during differentiation of the O2-A progenitor cell line CG-4. *J. Neurochem.* **68**, 1911-1919.

Sock, E., Schmidt, K., Hermanns-Borgmeyer, I., Bösl, M. R. and Wegner, M. (2001). Idiopathic weight reduction in mice deficient in the high-mobility-group transcription factor Sox8. *Mol. Cell. Biol.* **21**, 6951-6959.

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.

Stolt, C. C., Rehberg, S., Ader, M., Lommes, P., Riethmacher, D., Schachner, M., Bartsch, U. and Wegner, M. (2002). Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. *Genes Dev.* **16**, 165-170.

Stolt, C. C., Lommes, P., Sock, E., Chaboissier, M.-C., Schedl, A. and Wegner, M. (2003). The Sox9 transcription factor determines glial fate choice in the developing spinal cord. *Genes Dev.* **17**, 1677-1689.

Stolt, C. C., Lommes, P., Friedrich, R. P. and Wegner, M. (2004). Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. *Development* **131**, 2349-2358.

Stolt, C. C., Schmitt, S., Lommes, P., Sock, E. and Wegner, M. (2005). Impact of transcription factor Sox8 on oligodendrocyte specification in the mouse embryonic spinal cord. *Dev. Biol.* **281**, 323-331.

Takada, S. and Koopman, P. (2003). Origin and possible roles of the SOX8 transcription factor gene during sexual development. *Cytogenet. Genome Res.* **101**, 212-218.

Taylor, K. M. and LaBonne, C. (2005). SoxE factors function equivalently during neural crest and inner ear development and their activity is regulated by SUMOylation. *Dev. Cell* **9**, 593-603.

Topilko, P., Schneider, M. S., Levi, G., Baron, V. E. A., Chennoufi, A. B.,

Seitanidou, T., Babinet, C. and Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. *Nature* **371**, 796-799.
Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T. and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* **65**, 1153-1163.
Wegner, M. (1999). From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res.* **27**, 1409-1420.
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.
Zhang, C., Basta, T. and Klymkowsky, M. W. (2005). Sox7 and Sox18 are essential for cardiogenesis in Xenopus. *Dev. Dyn.* **234**, 878-891.
Zhu, L., Lee, H.-O., Jordan, C. R. S., Cantrell, V. A., Southard-Smith, E. M. and Shin, M. K. (2004). Spatiotemporal regulation of endothelin receptor-B by Sox10 in neural crest-derived enteric neuron precursors. *Nat. Genet.* **36**, 732-737.
Zorick, T. S., Syroid, D. E., Arroyo, E., Scherer, S. S. and Lemke, G. (1996). The transcription factors SCIP and Krox-20 mark distinct stages and cell fates in Schwann cell differentiation. *Mol. Cell. Neurosci.* **8**, 129-145.