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Evolution of Developmental Control Mechanisms

Replacement of mouse Sox10 by the *Drosophila* ortholog Sox100B provides evidence for co-option of SoxE proteins into vertebrate-specific gene-regulatory networks through altered expression

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ABSTRACT

Neural crest cells and oligodendrocytes as the myelinating glia of the central nervous system exist only in vertebrates. Their development is regulated by complex regulatory networks, of which the SoxE-type high-mobility-group domain transcription factors Sox8, Sox9 and Sox10 are essential components. Here we analyzed by in ovo electroporation in chicken and by gene replacement in the mouse whether the *Drosophila* ortholog Sox100B can functionally substitute for vertebrate SoxE proteins. Sox100B overexpression in the chicken neural tube led to the induction of neural crest cells as previously observed for vertebrate SoxE proteins. Furthermore, many aspects of neural crest and oligodendrocyte development were surprisingly normal in mice in which the *Sox10* coding information was replaced by *Sox100B* arguing that Sox100B integrates well into the gene-regulatory networks that drive these processes. Our results therefore provide strong evidence for a model in which SoxE proteins were co-opted to these gene-regulatory networks mainly through the acquisition of novel expression patterns. However, later developmental defects in several neural crest derived lineages in mice homozygous for the *Sox100B* replacement allele indicate that some degree of functional specialization and adaptation of SoxE protein properties have taken place in addition to the co-option event.

Introduction

Proteins of the Sox family of transcription factors are found in all metazoans (Guth and Wegner, 2008). Vertebrates and invertebrates share a highly conserved set of Sox proteins belonging to the SoxB, SoxC, SoxD, SoxE and SoxF subgroups, with fewer members of each subgroup usually present in invertebrates compared to vertebrates. The SoxE subgroup, for example, consists of single proteins in *Drosophila melanogaster* and *Caenorhabditis elegans*, whereas it contains three members (Sox8, Sox9 and Sox10) in all tetrapods and even more in teleosts due to an additional whole genome duplication.

The SoxE protein Sox10 has essential functions in early neural crest development, in the ensuing development of many cell types from the neural crest and in oligodendrocyte development (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Kim et al., 2003; Southard-Smith et al., 1998; Stolt et al., 2002). In Sox10-deficient mice, survival of early neural crest cells is decreased (Kapur, 1999; Kim et al., 2003). Additionally, neural crest-derived melanocytes of the skin and chromaffin cells of the adrenal medulla are not formed (Britsch et al., 2001; Reiprich et al., 2008; Southard-Smith et al., 1998). Many parts of the peripheral nervous system (PNS) are also affected. The nervous system of the gut (ENS) is completely missing, sympathetic ganglia are strongly reduced in size and no glial cells are formed in any part of the PNS (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Kim et al., 2003; Reiprich et al., 2008; Southard-Smith et al., 1998). As a consequence, Sox10-deficient mice die before or at birth.

Oligodendrocytes as the myelin-forming cells of the central nervous system (CNS) are one of the few non-neural crest-derived cell types that express Sox10. Although Sox10 has functions both in oligodendrocyte precursors and mature oligodendrocytes (Finzsch et al., 2008), generation of oligodendrocyte precursors is not affected in Sox10-deficient mice. Loss of Sox10, however, interferes with terminal oligodendrocyte differentiation and the production of myelin (Stolt et al., 2002).

Both the neural crest and oligodendrocytes have arisen only in vertebrate lineages during evolution and represent synapomorphies. The emergence of the neural crest is proposed to be a key factor

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during vertebrate evolution that facilitated the remodeling of the basal chordate body plan, in particular the head, in a manner that allowed a shift from filter feeding to an active predatory lifestyle (Gans and Northcutt, 1983). With their myelin sheath oligodendrocytes provide the structural basis for rapid saltatory conductance within the CNS which in turn represents an essential precondition for the evolution of complex brains (Zalc et al., 2008).

Currently it is unclear how these cell types arose during evolution (Baker, 2008). Studies on amphioxus, representing a basal chordate without neural crest, and agnathans, basal vertebrates with neural crest, indicate that the regulatory circuits that establish the neural plate border were already in place in chordates but that those responsible for neural crest induction are novel in vertebrates (Sauka-Spengler et al., 2007; Yu et al., 2008). Two models are proposed to explain how the components of the neural crest gene-regulatory network, the so-called neural-crest specifiers, were recruited. It may be envisaged that an existing regulatory protein first underwent structural mutations and changed its properties so that it became suitable for a novel role in neural crest specification. Alternatively, a regulatory protein may have been primarily recruited through changes in its expression pattern. In the second alternative, the regulatory protein may have been co-opted alone or as part of a whole regulatory circuit.

All three SoxE proteins including Sox10 are essential components of the vertebrate-specific regulatory networks that drive neural crest and oligodendrocyte development arguing that a common precursor of these SoxE proteins was recruited to the regulatory networks in early vertebrates. To explore how vertebrate SoxE proteins became part of these networks and to better understand their role in the evolution of neural crest cells and oligodendrocytes, we focused on the *Sox10* gene and compared its function with an invertebrate SoxE gene in overexpression studies and after gene replacement in the mouse. The chosen *Sox100B* gene from *D. melanogaster* is expressed in many tissues during fly development including hindgut, Malpighian tubules, and gonad (Hui Yong Loh and Russell, 2000). Gonadal expression is sexually dimorphic with Sox100B being specifically expressed in two male-specific cell populations, the male-specific somatic gonadal precursor cells and the pigment cell lineage (DeFalco et al., 2008, 2003). Sox100B-deficient flies have no discernible embryonic or larval phenotypes, but die as pharate adults. At this time, flies have severe abnormalities in their fat bodies and fail to develop normal testes (Nanda et al., 2009). Sox100B exhibits very few similarities in expression and function with vertebrate SoxE proteins, which are furthermore restricted to the developing male gonad (Guth and Wegner, 2008). Nevertheless, we show a surprising ability of invertebrate Sox100B to compensate for Sox10 loss during neural crest development and in oligodendrocyte differentiation. Co-option of SoxE proteins through changed expression may thus have played a decisive role in the appearance of neural crest cells and oligodendrocytes, before the co-opted SoxE protein was optimized for its new functions through structural mutations.

Materials and methods

Construction of targeting vectors, gene targeting, generation and genotyping of mouse mutants

Sequences corresponding to the open reading frame of *Drosophila Sox100B*, an IRES-EGFP and a neomycin resistance cassette with flanking loxP sites were placed between 5′ and 3′ *Sox10* genomic regions as homology arms (4.3 kb and 1.5 kb respectively) in the context of a pPNT vector backbone as described (Britsch et al., 2001; Kellerer et al., 2006; Ludwig et al., 2004b). The targeting vector thereby replaced the *Sox10* coding exons by a continuous *Sox100B* reading frame and a GFP coding sequence linked by an internal ribosomal entry site (Fig. 3A).

The construct was linearized with NotI and 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 wildtype allele and a 7.7 kb fragment in case of the $Sox10^{Sox100B}$ allele in genomic DNA digested with NcoI (Fig. 3B). 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 BamHI and ScaI. This probe hybridized to a 5.4 kb fragment in the $Sox10^{Sox100B}$ allele as opposed to a 4.6 kb fragment in the wildtype allele (Fig. 3B). Targeted ES cells were injected into C57Bl/6J blastocysts to generate chimeras. After germline transmission, heterozygous mice were backcrossed on a C3H background. Homozygous mutant embryos (F_2–F_5) were generated by heterozygote intercrosses. Sequencing of genomic DNA from the altered *Sox10* locus confirmed that no additional unintended mutations were introduced during cloning.

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′-CAGGTGGGCGT-TGGGCTCTT-3′) and two lower primers located 487 bp downstream of the start codon in intron 3 of the *Sox10* gene (5′-TCCCAGGCTAGCCC-TAGTG-3′) or 582 bp downstream of the start codon in the *Sox100B* open reading frame (5′-CGCTCGCCTCTGGCCATTGG-3′). A 568 bp fragment was indicative of the wildtype allele, a 663 bp fragment of the targeted allele (Fig. 3C).

Chicken in ovo electroporation

Fertilized chicken eggs were obtained from Lohmann (Cuxhaven, Germany) and incubated in a humidified incubator at 37.8 °C. Embryos were staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1953). pCAGGS-IRES-nls-GFP and pCAGGS-IRES-nls-GFP-based expression plasmids for *Drosophila* Sox100B and rat Sox10 were injected at a concentration of 2 µg/µl into the lumen of HH stage 10–11 neural tubes. Electrodes were placed at either side of the neural tube and electroporation was carried out using a BTX ECM830 electroporator delivering five 50 millisecond pulses of 30 V. Transfected embryos were allowed to develop for 48 h before dissection and analysis.

Tissue and RNA preparation from embryos, in situ hybridization, immunohistochemistry and quantitative RT-PCR

Mouse embryos were isolated at 10.5 dpc to 18.5 dpc from staged pregnancies and chicken embryos 48 h after electroporation. Subsequently, embryos were processed for RNA preparation, immunohistochemistry or in situ hybridization (Kellerer et al., 2006; Stolt et al., 2004, 2003).

Each immunohistochemistry and in situ hybridization was performed at least three times on at least three embryos per genotype. In situ hybridizations (except probe hybridization and final colorimetric detection) were performed automatically on a Biolane HTI (Hölle & Hüttner AG) using DIG-labelled antisense riboprobes for chicken *FoxD3* (a gift from M. Goulding, Salk Institute, La Jolla; Dottori et al., 2001) and for mouse *MBP*, *Mitf* and *Dct* (Britsch et al., 2001; Stolt et al., 2002).

Primary antibodies for immunohistochemistry included: anti-Sox8 guinea pig antiserum (1:1000 dilution, Stolt et al., 2005), anti-Sox10 guinea pig antiserum (1:2000 dilution, Maka et al., 2005), anti-Sox10 rabbit antiserum (1:100 dilution; Stolt et al., 2003), anti-Sox9 rabbit antiserum (1:1000 dilution, Stolt et al., 2003), anti-Sox100B rabbit antiserum (1:2000 dilution, Hui Yong Loh and Russell, 2000), anti-Phox2b rabbit antiserum (1:2000 dilution, a gift from C. Goridis, Ecole Normale Superieure, Paris), anti-Olig2 rabbit antiserum (1:10,000 dilution, a gift from D. Rowitch, UCSF, San Francisco), anti-B-FABP rabbit antiserum, (1:10,000 dilution, a gift from C. Birchmeier and T.

Müller, MDC, Berlin), anti-TH rabbit antiserum (1:1000 dilution, Biomol), anti-steroidogenic factor-1 (SF-1) rabbit antiserum (1:1000 dilution; a gift from K. Morohashi, National Institute for Basic Biology, Japan), anti-Krox20 rabbit antiserum (1:1000 dilution, Covance), anti-Oct6 rabbit antiserum (1:2000, Friedrich et al., 2005), anti-Sox5 rabbit serum (1:4000, Perez-Alcala et al., 2004), anti-Tubb3 mouse monoclonal (1:5,000 dilution; Covance), anti-NeuN mouse monoclonal (1:500 dilution, Chemicon), anti-laminin mouse monoclonal (1:3000, Developmental Studies Hybridoma Bank), anti-HNK1 mouse monoclonal (1:10,000, BD Pharmingen), anti-Cad7 (1:200 Developmental Studies Hybridoma Bank). Secondary antibodies conjugated to Cy3, Cy2 or Alexa fluor immunofluorescent dyes (Dianova and Molecular Probes) were used for detection. Dissected gastrointestinal tracts were also used for NADPH diaphorase staining (Scherer-Singler et al., 1983). Samples were analyzed and documented as described (Kellerer et al., 2006).

For quantitative RT-PCR, whole embryo RNA was reverse transcribed in a 20 µl reaction, before 1 µl of the obtained cDNA, diluted 1 to 5, was amplified by PCR on a Roche Lightcycler according to the manufacturer's instructions using the LightCycler-FastStart DNA Master SYBR Green kit. The following primer pairs were used: Primers 5′-ACAGCAGCAG-GAAGGCTTCT-3′ and 5′-TGTCCTCAGTGCGTCCTTAG-3′ for detection of *Sox10*, primers 5′-CTACTCCTACCCCGCAAACG-3′ and 5′-AGGGATTGA-CATAAGTGTAA-3′ for detection of *Sox100B*, primers 5′-TCAGTCTCG-GCTGTCCAGCC-3′ and 5′-GAAGAGCCCAACGCCACCT-3′ for detection of both *Sox10* and *Sox100B*, primers 5′-TGGAGTCTGGTGCCTATGCCTGT-3′ and 5′-GCCGAGCACTGCATCAGCTTTGT-3′ for detection of *Sox8* and primers 5′-GAACAGACTCACATCTCTCC-3′ and 5′-TGCTGCTTCGACATC-CACAC-3′ for detection of *Sox9*. *Rpl8* transcripts were used for normalization (Kellerer et al., 2006). The annealing temperature was 64 °C.

Cell culture, analysis of protein stability, gel retardation and luciferase assays

B16 melanoma, Neuro2a neuroblastoma and human embryonic kidney 293 cells were maintained in DMEM containing 10% FCS, and transfected using either polyethylenimine (PEI) or Superfect reagent (Qiagen, Hilden, Germany).

For analysis of protein stability, B16 melanoma cells were transfected with pCAGGS-IRES-nls-GFP based expression plasmids for myc-tagged Sox10 or myc-tagged Sox100B using PEI reagent and treated 48 h after transfection with 25 µg/ml cycloheximide for up to 24 h. Extracts were prepared after various times of cycloheximide treatment, and both ectopically expressed proteins were detected by western blot. Sox10- and Sox100B-specific bands on western blots were quantified using the NIH ImageJ software.

For gel retardation assays, 293 cells were transfected with pCMV5 based expression plasmids for shortened variants of Sox10 (amino acids 1–189) and Sox100B (amino acids 1–162) using PEI reagent. Cell extracts were prepared 48 h posttransfection and incubated separately or after mixing with ^{32}P-labelled oligonucleotides containing the prototypic dimeric site C/C′ or monomeric site B from the *Mpz* gene in the presence of poly(dGdC) as unspecific competitor before polyacrylamide gel electrophoresis and autoradiographic exposure of the dried gels (Peirano et al., 2000).

For luciferase assays, Neuro2a and B16 cells were transfected transiently in duplicates using Superfect reagent in 3.5-cm dishes with 500 ng of luciferase reporter and 500 ng of effector plasmids per dish. Luciferase reporters were used that contained the *Mitf* promoter (positions −1486 to +97, Bondurand et al., 2000), the *Dct* promoter (positions −685 to +443, Ludwig et al., 2004a) or the *Mpz* promoter (positions −435 to +48, Peirano et al., 2000). Effector plasmids corresponded to pCAGGS-IRES-nls-GFP based expression plasmids for Sox10 or Sox100B. Cells were harvested 48 h posttransfection, and extracts were assayed for luciferase activity. Luciferase activities obtained with the different effectors were normalized to luciferase activity observed with pCAGGS-IRES-nls-GFP alone. Bar graphs usually result from three duplicates and the S.E.M. is shown as error bar.

Results

Comparison of sequences and biochemical properties between mouse Sox10 and Sox100B from Drosophila

Mammalian SoxE proteins possess four different regions that have been functionally characterized. These include a region that immediately precedes the DNA-binding high-mobility-group (HMG) domain and has been defined as a dimerization domain (Peirano and Wegner, 2000; Schlierf et al., 2002), the HMG domain itself, a context-dependent transactivation domain K2 (Schepers et al., 2000; Schreiner et al., 2007) and a carboxy-terminal transactivation domain TA (Kuhlbrodt et al., 1998; Pusch et al., 1998; Südbeck et al., 1996) (Fig. 1A). The dimerization and DNA-binding domains are highly conserved at the amino acid level, whereas conserved amino acid residues are particularly prominent at the amino-terminal and carboxy-terminal ends of the K2 domain and the carboxy-terminal end of the TA domain.

To assess the degree of sequence conservation between Sox100B and the mammalian SoxE proteins, we performed an alignment of all four amino acid sequences using the TCoffee tool (Poirot et al., 2003) (Fig. 1B). Even after inclusion of Sox100B, the HMG domain remains very similar (71% amino acid identity, 91% amino acid similarity) and the dimerization domain retains a core of conserved amino acid residues. In contrast, the K2 and TA domains are not apparent in Sox100B, at least by sequence conservation.

To study the biochemical properties of Sox100B, we first determined its stability in transfected cycloheximide-treated B16 melanoma cells and found it comparable to Sox10, with half lives of approximately 2 h for both proteins (Fig. 1C). The DNA-binding ability of Sox100B was analyzed by using the well-characterized Sox10 recognition sites B and C/C′ from the *Mpz* gene as probes in gel retardation assays (Peirano et al., 2000). Both sites were efficiently bound by Sox100B (Fig. 1D). Complexes of Sox100B with site B and site C/C′ exhibited different mobilities which as in the case of Sox10 is indicative of monomeric binding to site B and dimeric binding to site C/C′ (Peirano et al., 2000). Joint incubation of site C/C′ with Sox10 and Sox100B furthermore led to the appearance of an additional complex with a mobility that was intermediate between the Sox10-specific and the Sox100B-specific complexes and corresponds to a heterodimer (Fig. 1D). Sox100B thus efficiently binds to both monomeric and dimeric recognition sites of vertebrate SoxE proteins arguing that the limited degree of Sox100B conservation to the dimerization domain of mammalian SoxE proteins is functionally relevant.

Considering the similar protein stabilities and DNA-binding properties, we asked whether Sox100B is able to activate promoters of mammalian SoxE target genes in cell culture. We focused on the Sox10 target genes *Mpz*, *Mitf* and *Dct* (Bondurand et al., 2000; Ludwig et al., 2004a; Peirano et al., 2000). In luciferase reporter gene assays all three promoters were efficiently activated by Sox100B independent of whether Neuro2a neuroblastoma was used or B16 melanoma cells (Figs. 1E–I). Sox100B is thus capable of activating mammalian SoxE target genes despite the low degree of sequence conservation in K2 and TA domains. However, it has to be kept in mind that luciferase reporter gene assays in transiently transfected cells are very basal assays that require verification in vivo.

Sox100B electroporation into the early neural tube of chicken

To gain an initial impression of Sox100B activity in vivo, we used in ovo electroporation to overexpress the *Drosophila* SoxE protein in the

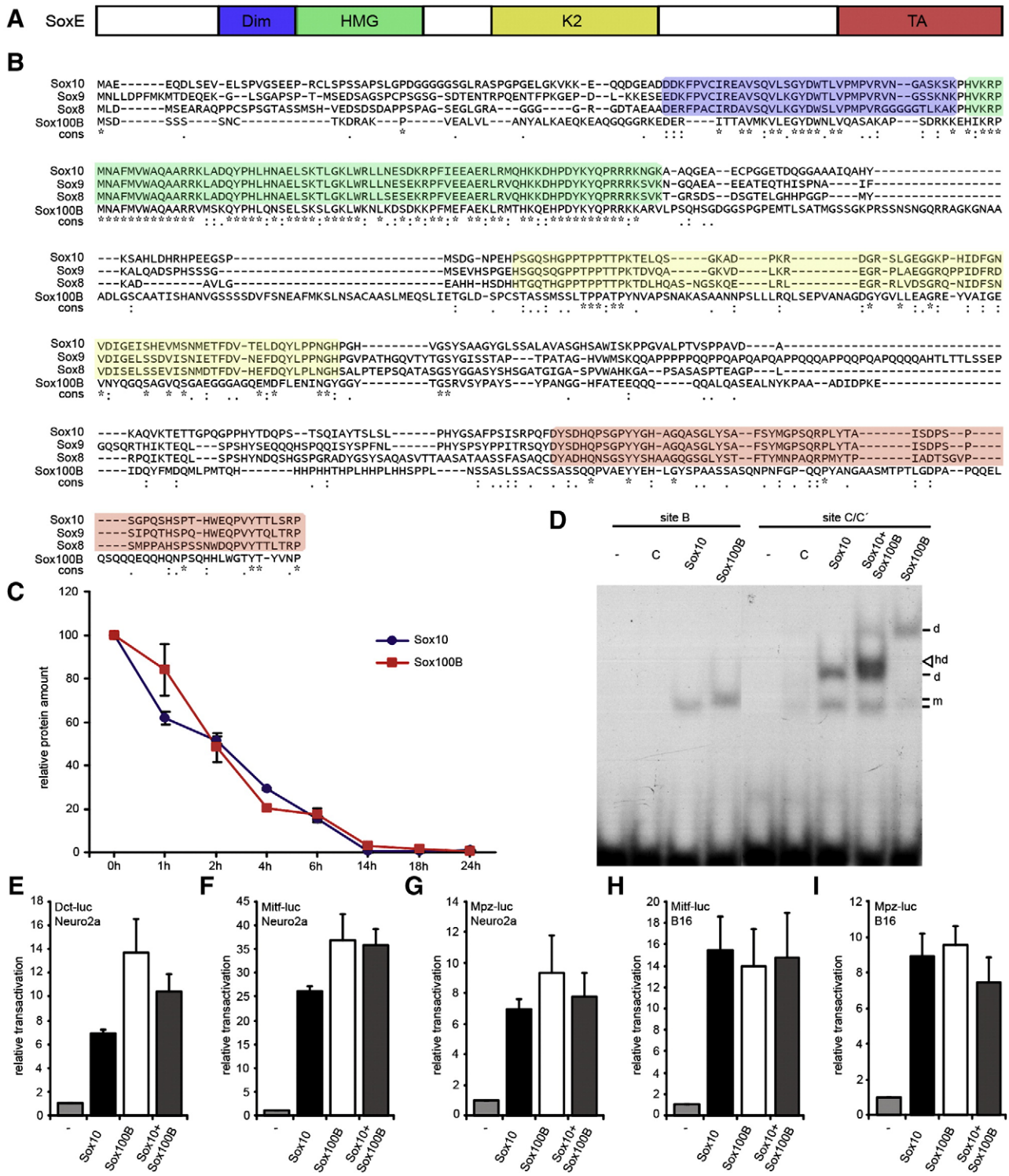

Fig. 1. Structural and biochemical comparison of Sox100B with mammalian SoxE proteins. (A) General structure of a mammalian SoxE protein with dimerization (Dim), HMG, K2 and carboxy-terminal transactivation (TA) domains as functional regions. (B) Sequence comparison between the mouse SoxE proteins Sox8, Sox9 and Sox10 and Sox100B from *Drosophila melanogaster*. Fully conserved amino acids are marked by an asterisk, closely related amino acids by two dots and biochemically similar ones by a single dot. (C) Protein stability of Sox10 (blue circle) and Sox100B (red square). B16 melanoma cells were transiently transfected with myc-tagged Sox10 or myc-tagged Sox100B and cultured for various times in the presence of cycloheximide as indicated. After extract preparation, ectopically expressed proteins were detected by western blots and relative amounts were quantified from band intensities with the respective amount in untreated cells set to 100%. (D) Comparison of DNA-binding by Sox10 and Sox100B. Cell extracts containing Sox10 (amino acids 1–189) or Sox100B (amino acids 1–162) were separately or jointly incubated with a radioactively labelled oligonucleotide containing the prototypic dimeric C/C′ site or monomeric B site from the *Mpz* gene (Peirano et al., 2000). Note that both Sox10 and Sox100B form a dimeric complex with C/C′ (d) whose mobility is lower than the respective monomeric complex with B (m) and that joint incubation of C/C′ with Sox10 and Sox100B yields a novel complex (hd, marked by an arrowhead). (E–I) Transactivation capacity of Sox10 and Sox100B. Transient transfections were performed in Neuro2a (E–G) and B16 (H,I) cells with luciferase reporters under the control of the *Dct* (E), the *Mitf* (F,H) or the *Mpz* (G,I) promoter. Luciferase reporters were transfected alone, in the presence of Sox10, Sox100B or a combination of both as effectors. Data are presented as fold induction, with the luciferase activity obtained for each construct in the absence of effector arbitrarily set to 1 ± S.E.M.

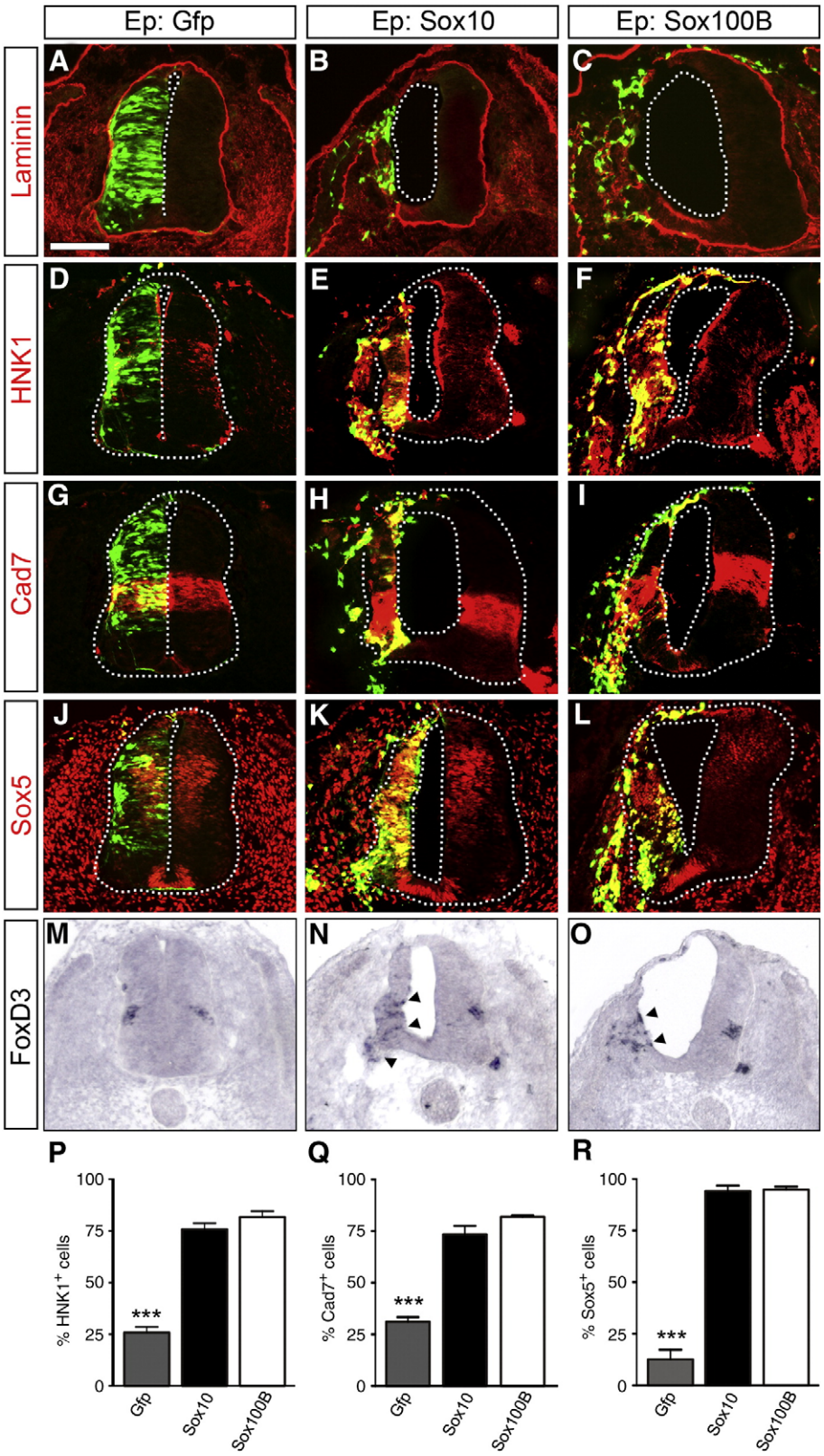

Fig. 2. Sox100B overexpression in early chicken spinal cord development. Chicken embryos were electroporated with pCAGGS-IRES-nls-GFP expression plasmids that contained no additional coding information (A,D,G,J,M) or open reading frames for *Sox10* (B,E,H,K,N) or *Sox100B* (C,F,I,L,O). Fixation was 48 h after the electroporation event. Shown are transverse sections with the electroporated side oriented to the left and marked by GFP autofluorescence (in green). Immunohistochemistry (in red) against laminin (A–C), HNK1 (D–F), cadherin-7 (Cad7) (G–I) and Sox5 (J–L) was performed and overlaid with the GFP autofluorescence signal. In situ hybridizations with a *FoxD3*-specific antisense riboprobe were additionally carried out (M–O). Arrowheads point to cells with induced *FoxD3* expression. Scale bar in A is valid for all panels and corresponds to 100 µm. (P–R) The percentage of electroporated cells expressing HNK1 (P), cadherin-7 (Q) or Sox5 (R) was determined for control (Gfp), Sox10 and Sox100B electroporations. Sections from five embryos were quantified for each electroporated construct and data are presented as mean ± SEM. The percentage of control-electroporated cells expressing HNK1, cadherin-7 or Sox5 was significantly lower than the percentages of SoxE-electroporated cells as determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test (***, $P<0.001$). No statistically significant difference was detected between Sox10 and Sox100B electroporations.

early chicken neural tube. In this assay, all mammalian SoxE proteins have previously been shown to induce neural crest characteristics in neural tube cells with only minor differences (Cheung and Briscoe, 2003; McKeown et al., 2005). By comparing control-electroporated with Sox10-electroporated cells, neural crest inducing activity becomes evident. Only Sox10-electroporated cells migrated out of the neural tube and disrupted the surrounding extracellular matrix (Perez-Alcala et al., 2004) (compare Figs. 2A,D,G,J to B,E,H,K). 73–94% of Sox10-electroporated cells furthermore expressed neural crest markers such as HNK1, cadherin-7 and Sox5 (Figs. 2E,H,K,P,Q,R) compared to 13–31% of control-electroporated cells (Figs. 2D,G,J,P,Q,R). When Sox100B was electroporated into the chicken neural tube, cells leaving the neural tube were detected and laminin staining revealed that the extracellular matrix surrounding the neural tube was no longer intact on the electroporated side (Fig. 2C). The percentage of Sox100B-electroporated cells expressing HNK1 (Figs. 2F,P), cadherin-7 (Figs. 2I,Q) and Sox5 (Figs. 2L,R) ranged between 82% and 94% arguing that Sox100B activated neural crest markers as efficiently as Sox10. In accord, *FoxD3* expression was induced comparably after electroporation of Sox100B and Sox10

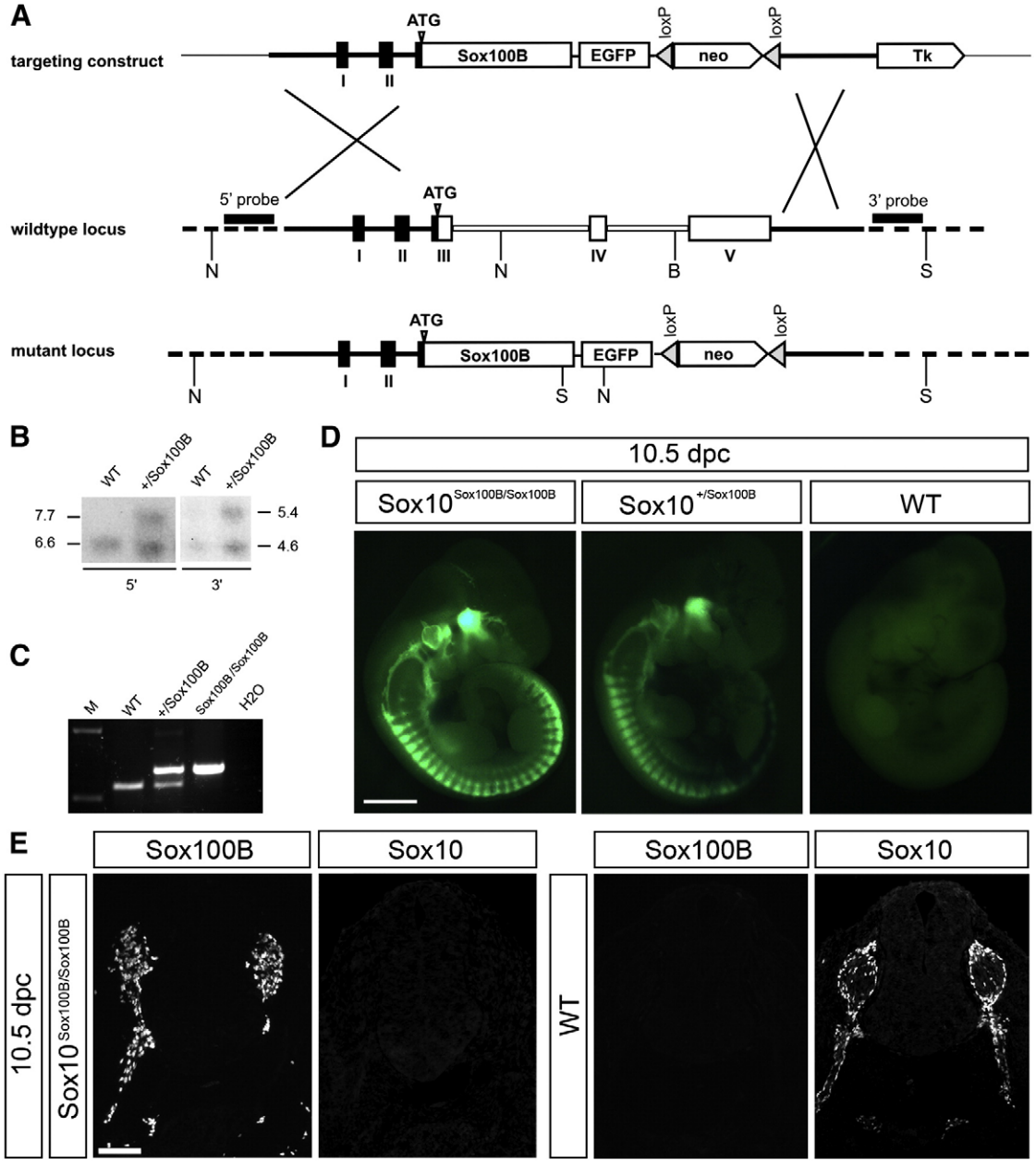

Fig. 3. Targeted replacement of *Sox10* by *Sox100B* sequences in mice. (A) Schematic representation from top to bottom of the targeting construct for the *Sox100B* replacement allele, the *Sox10* wildtype locus and the mutant locus. The *Sox10* exons (I–V) and the *Sox100B* open reading frame are shown as boxes, the 4.3 kb and 1.5 kb long flanking regions as bars. Regions of homology between wildtype 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 NcoI (N), BamHI (H) and ScaI (S) are shown as well as the localization of 5′ and 3′ probes and the start codon of the *Sox10* gene (ATG). 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 wildtype (wt) and heterozygous (+/Sox100B) ES cells digested with NcoI for use of the 5′ probe and BamHI/ScaI for the 3′ probe. The size of bands corresponding to the wildtype (6.6 kb for the 5′ probe and 4.6 kb for the 3′ probe) and the targeted alleles (7.7 kb for the 5′ probe and 5.4 kb for the 3′ probe) is indicated. (C) PCR genotyping of wildtype (WT), heterozygous (+/Sox100B) and homozygous (Sox100B/Sox100B) mouse embryos at 18.5 dpc. DNA fragments in the size marker (M) are 1.0 kb and 0.5 kb. (D) GFP autofluorescence of $Sox10^{Sox100B/Sox100B}$, $Sox10^{+/Sox100B}$ and wildtype (WT) embryos at 10.5 dpc. Scale bar corresponds to 1 mm. (E) Immunofluorescence on transverse sections of 10.5 dpc-old wildtype embryos (WT) and $Sox10^{Sox100B/Sox100B}$ littermates with antibodies directed against Sox100B (left) and Sox10 (right). Scale bar corresponds to 100 µm.

(Figs. 2M–O). We thus conclude that Sox100B functions similar to other SoxE proteins, including Sox10, regarding neural crest induction in this in vivo overexpression system.

Generation of Sox100B expressing mice

Sox100B protein levels are very high in electroporated cells and overexpression takes place in a background in which endogenous SoxE genes are present. Considering, that Sox9 can induce Sox10 expression (Cheung et al., 2005), it cannot be ruled out that Sox100B simply activates the expression of endogenous SoxE proteins which in turn may be responsible for inducing neural crest properties.

To analyze the functional properties under more physiological conditions, we chose to generate a mouse model in which we replaced Sox10 with Sox100B by exchanging the complete coding region of *Sox10* in exons 3–5 with a *Sox100B* cDNA (Fig. 3A). The *Sox100B* start codon was placed exactly at the position of the endogenous *Sox10* start codon in exon 3. This strategy has been previously employed to replace *Sox10* sequences with various marker genes, *Sox8*, or mutant *Sox10* sequences (Britsch et al., 2001; Kellerer et al., 2006; Ludwig et al., 2004b; Schreiner et al., 2007). Mutant *Sox10* sequences coded for proteins without dimerization domain (Sox10aa1) or without K2 domain (Sox10ΔK2), the very same domains that are only partially or very poorly conserved in Sox100B. These studies showed that expression levels of the inserted cDNA correspond closely to expression levels of the *Sox10* gene in the wildtype (Kellerer et al., 2006; Ludwig et al., 2004b; Schreiner et al., 2007). Re-application of the same strategy thus increased the likelihood that *Sox100B* expression levels would also be in a physiologically relevant range. It also ensured that phenotypic comparison of the novel with the previous mouse mutants would allow meaningful conclusions about the rescuing capacity of Sox100B relative to those of Sox8 and the various Sox10 mutants.

Using the targeting vector shown in Fig. 3A, ES cells were electroporated and clones were identified that had successfully undergone homologous recombination and carried a correctly recombined *Sox10* allele (Fig. 3B). After blastocyst injection, these ES cells generated chimeras that transmitted the targeted allele to their progeny (Fig. 3C). By studying expression of the GFP marker that was additionally introduced into the *Sox10* locus and expressed from the same transcript as Sox100B due to the presence of an internal ribosomal entry site (Fig. 3A), it is clear that the spatial and temporal expression pattern of the targeted allele is identical to the wildtype Sox10 expression pattern (Fig. 3D and data not shown) (Britsch et al., 2001). Immunohistochemistry with antibodies directed against Sox10 and Sox100B revealed that Sox10 protein was completely replaced by Sox100B in homozygous mutant embryos (Fig. 3E). Signal intensities for Sox10 and Sox100B were furthermore indistinguishable on the single cell level arguing for comparable amounts of both proteins per cell in the respective genotypes.

In support of this conclusion, *Sox100B* transcript levels in homozygous mutant embryos were similar to *Sox10* transcript levels in wildtype embryos at 11.5 dpc as determined by quantitative RT-PCR (Fig. 4A). Likewise, no statistically significant differences were detected between transcript levels in wildtype, heterozygous and homozygous mutant embryos when primers were used that equally recognized wildtype and mutant transcripts (Fig. 4B). Levels were also comparable between genotypes for *Sox8* and *Sox9* transcripts (Figs. 4C,D) and proteins. Immunohistochemistry on spinal nerves with antibodies directed against Sox8 or Sox9, for instance, yielded comparable numbers of positive cells (Figs. 4E,F) and similar staining intensities (Figs. 4G–J) in wildtype and homozygous mutant embryos at 18.5 dpc. Sox8 and Sox9 were not expressed in Sox10-positive cells of wildtype nerves nor in Sox100B-positive cells of mutant nerves (see inlays in Figs. 4G–J) arguing that Sox8 and Sox9 are not upregulated by Sox100B expression in Schwann cells. We conclude that Sox100B is expressed in the mutant at levels comparable to Sox10 in the wildtype and does not exert unforeseen influences on the expression of other SoxE family members.

Both heterozygous and homozygous embryos were obtained at all ages (Fig. 3C). GFP autofluorescence and Sox100B immunohistochemistry in homozygous embryos furthermore showed that neural crest cells were formed and migrated to their target tissues in a spatially and temporally normal manner in cranial, vagal, trunk, and sacral regions (Figs. 3D,E and data not shown). This confirms that Sox100B efficiently provides Sox10 functions in the early neural crest. However, homozygous newborns died immediately after birth suggesting that later development of some neural crest derivatives must be defective and that Sox100B and Sox10 are not fully equivalent with regard to their function. Analyses are therefore restricted to

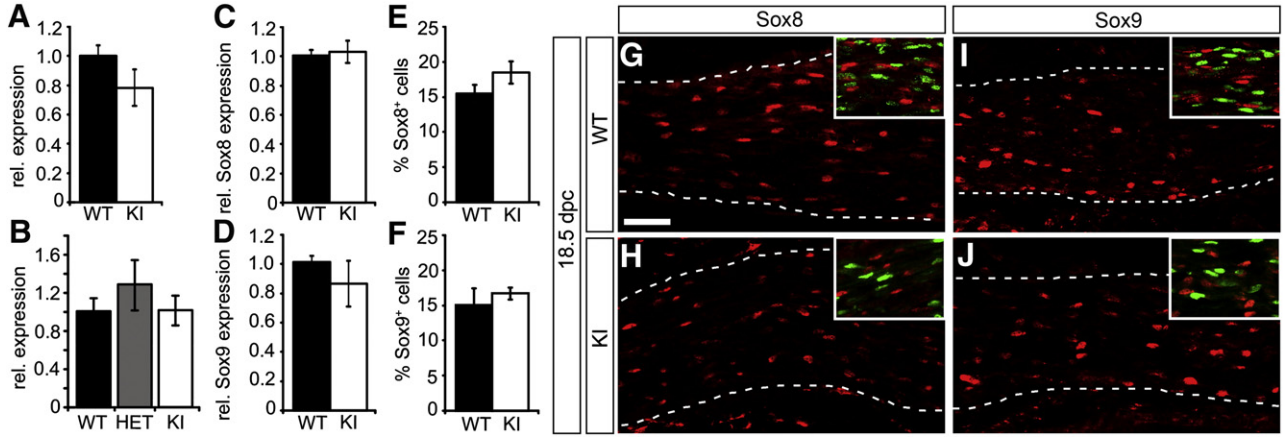

Fig. 4. Sox gene expression levels in Sox10$^{Sox100B/Sox100B}$ embryos. (A) *Sox10*-specific expression in wildtype embryos (WT, set to 1) was compared to *Sox100B*-specific expression in age-matched Sox10$^{Sox100B/Sox100B}$ embryos (KI) at 11.5 dpc following quantitative RT-PCR with gene-specific primers. (B) Total expression levels from the *Sox10* locus were determined in wildtype embryos (set to 1) as well as Sox10$^{+/Sox100B}$ (HET) and Sox10$^{Sox100B/Sox100B}$ littermates at 11.5 dpc by quantitative RT-PCR using primers that recognize both alleles equally. (C,D) Expression of Sox8 (C) and Sox9 (D) was quantified in Sox10$^{Sox100B/Sox100B}$ embryos at 11.5 dpc relative to age-matched wildtype embryos (set to 1) by gene-specific RT-PCR. Expression levels were normalized to *Rpl8* in all RT-PCR experiments. Experiments were repeated three times with material from two embryos for each genotype. (E,F) The percentage of Sox8-positive cells (E) and Sox9-positive cells (F) was determined along spinal nerves of wildtype and Sox10$^{Sox100B/Sox100B}$ embryos. Sections from three embryos were quantified per genotype and data are presented as mean ± SEM. No statistically significant differences were detected. (G–J) Immunofluorescence was performed on spinal nerves (demarcated by dotted lines) of wildtype (G,I) and Sox10$^{Sox100B/Sox100B}$ (H,J) embryos at 18.5 dpc with antibodies directed against Sox8 (G,H) and Sox9 (I,J). The inlays represent results from co-immunohistochemistry with antibodies against Sox10 (G,I) or Sox100B (H,J) (both in green) and show that expression of Sox8 and Sox9 is restricted to cells within the nerve that do not express Sox10 in the wildtype and Sox100B in the mutant. The scale bar in G corresponds to 50 μm and is valid for G–J.

embryonic stages and were carried out with embryos of the F_2–F_5 generation. Analyzed generations and genetic background were identical to those in previous studies where Sox10 was replaced by Sox8 or mutant Sox10 proteins (Kellerer et al., 2006; Schreiner et al., 2007). No evidence was obtained from the analysis of PNS and CNS development in mice with one *Sox100B* replacement allele and one *Sox10* null allele that Sox100B acts as a dominant-negative (data not shown).

Ability of Sox100B to rescue melanocyte development in the absence of Sox10

Even in early generations, heterozygous mice were easily detected due to their white belly spot and a white medial stripe on the head, arguing that melanocytes are one of the affected neural crest derivatives. Sox10 is important for melanocyte development because it induces Mitf in melanoblasts. When Mitf expression was studied in mice homozygous for the *Sox100B* replacement allele, some *Mitf*-positive cells were found in the vicinity of the eye and the hindlimb region at 11.5 dpc (Fig. 5A). Few melanoblasts were also detected with *Dct* as an alternative melanoblast marker in the hindlimb region at 12.5 dpc (Fig. 5B), indicating that melanoblasts are specified from the neural crest when Sox10 is replaced by Sox100B. However, in mice homozygous for the *Sox100B* replacement allele, melanoblasts are strongly reduced, arguing that the *Drosophila* ortholog is a much less efficient inducer of melanocytes than Sox10 despite its ability to efficiently activate the promoters of the Sox10 target genes *Mitf* and *Dct* in cell culture assays. Comparable reductions in melanoblasts have previously been observed in mice where the *Sox10* gene was replaced by *Sox8* (Kellerer et al., 2006) and in mice expressing the Sox10aa1 mutant (Schreiner et al., 2007). Mice expressing Sox10ΔK2, in contrast, exhibited a slightly less severe reduction in melanoblasts (Table 1).

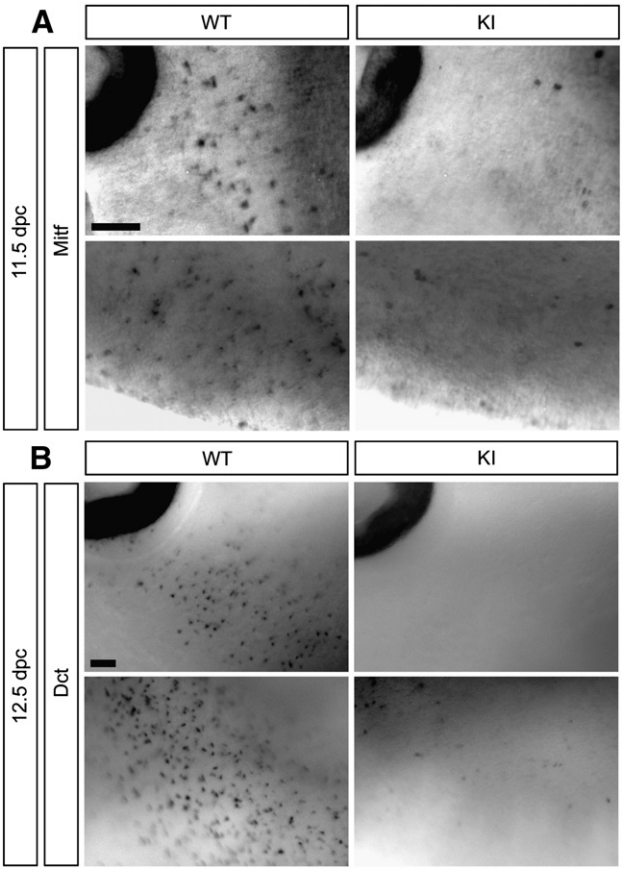

Fig. 5. Melanocyte development in $Sox10^{Sox100B/Sox100B}$ embryos. Whole mount in situ hybridizations were performed on wildtype (WT) and $Sox10^{Sox100B/Sox100B}$ (KI) embryos at 11.5 dpc using antisense riboprobes against *Mitf* at 11.5 dpc (A) and *Dct* at 12.5 dpc (B). Shown for each probe are representative in situ hybridizations from the eye and from the hindlimb region. Scale bars in A and B are valid for all respective four panels and correspond to 100 µm.

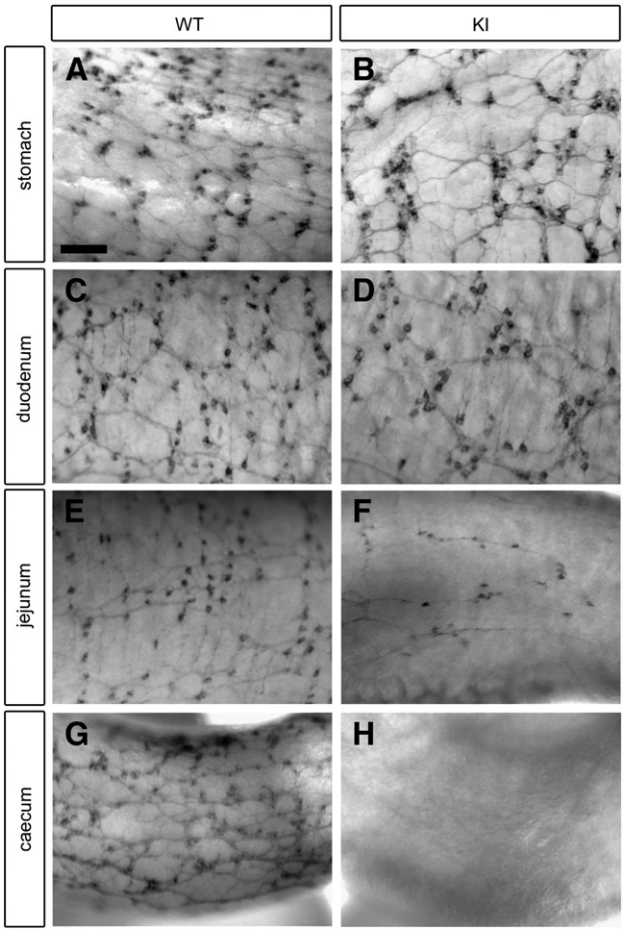

Fig. 6. Analysis of the ENS in $Sox10^{Sox100B/Sox100B}$ embryos. Stomach (A,B), duodenum (C,D), jejunum (E,F) and caecum (G,H) of wildtype (WT, panels A,C,E,G) and $Sox10^{Sox100B/Sox100B}$ (KI, panels B,D,F,H) embryos were analyzed at 18.5 dpc by NADPH diaphorase staining. The scale bar in A is valid for all panels and corresponds to 100 µm.

Ability of Sox100B to rescue enteric nervous system development in the absence of Sox10

Another neural crest derivative that was strongly affected in mice with hypomorphic *Sox10* alleles or a *Sox8* replacement allele is the enteric nervous system (ENS) (Kellerer et al., 2006; Schreiner et al., 2007). In Sox100B replacement mice, the ENS formed normally in the stomach (Figs. 6A,B). Even the duodenum was significantly colonized by enteric neural crest cells in the mutant, although there appeared to be slight differences in the localization of cell bodies and the overall organization of the plexus (Figs. 6C,D). At the level of the jejunum, differences compared to the wildtype were more prominent in the replacement mutant, since the ENS in this area is made up of only a few cells and a plexus is not formed (Figs. 6E,F). At caecal levels and beyond, ENS cells were completely missing (Figs. 6G,H).

Thus, enteric neural crest cells are specified when Sox10 is replaced by Sox100B. However, Sox100B cannot fully compensate for the loss of Sox10 during ENS development. It is intriguing that the defect in the Sox100B replacement mutant is much less severe than in the Sox8 replacement or in the Sox10aa1 mutant, and slightly less severe than in the Sox10ΔK2 mutant (Table 1), arguing that Sox100B

is a better substitute for Sox10 during ENS development than Sox8 or the hypomorphic Sox10 mutants (Kellerer et al., 2006; Schreiner et al., 2007).

Ability of Sox100B to rescue sympathetic nervous system and adrenal gland development in the absence of Sox10

Sympathetic nervous system defects in Sox10-deficient mice are most pronounced in caudal regions (Britsch et al., 2001; Reiprich et al., 2008). Thus, we focused on the caudal part of the sympathetic ganglion chain to analyze the ability of Sox100B to substitute for Sox10 in the sympathetic nervous system. Confirming the replacement, Sox10 was readily detected in wildtype sympathetic ganglia but not in the mutant (Figs. 7A,F,K,P). Instead, sympathetic ganglia in the mutant but not in the wildtype contained Sox100B-positive cells (Figs. 7B,G,L,Q). Immunohistochemistry revealed expression of panneuronal markers such as β3-tubulin (Tubb3, Figs. 7C,H,M,R), and also of adrenergic neuronal markers such as Phox2b (Figs. 7D,I,N,S) and tyrosine hydroxylase (TH, Figs. 7E,J,O,T) in sympathetic ganglia of both wildtype and the replacement mutant at 12.5 dpc and at 18.5 dpc. However, at 12.5 dpc, the number of Phox2b expressing cells was reduced in the mutant to 13 % of wildtype numbers (Fig. 7U). Even at 18.5 dpc, mutant ganglia contained only 65 % of Phox2b-positive cells in wildtype ganglia (Fig. 7V). Size of sympathetic ganglia (determined as the TH-positive area) was also significantly reduced in the replacement mutant with mutant ganglia occupying only 29 % of the area of wildtype ganglia at 12.5 dpc and 47 % at 18.5 dpc (Figs. 7W,X). These results argue that initial formation and early development of sympathetic ganglia is more impaired in mice homozygous for the *Sox100B* replacement allele than their later development and maintenance. Sox100B replacement mice resemble much more Sox8 replacement mice than Sox10ΔK2 and Sox10aa1 mutants which exhibit

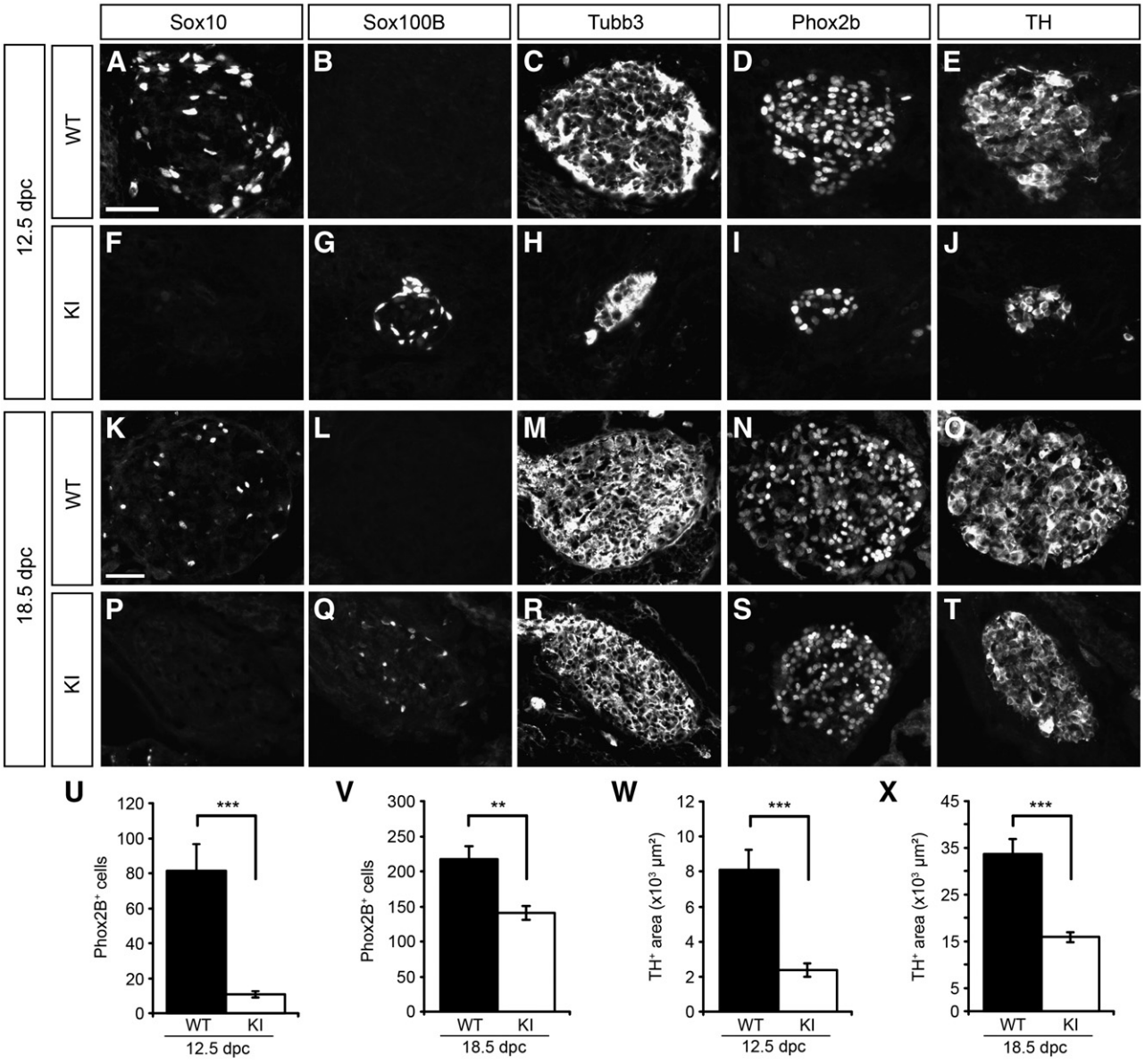

Fig. 7. Development of sympathetic ganglia in $Sox10^{Sox100B/Sox100B}$ embryos. Immunohistochemical staining of sympathetic ganglia was performed on wildtype (WT, panels A–E,K–O) and $Sox10^{Sox100B/Sox100B}$ (KI, panels F–J,P–T) embryos at 12.5 dpc (A–J) and 18.5 dpc (K–T) in caudal parts of the sympathetic chain using antibodies against Sox10 (A,F,K,P), Sox100B (B,G,L,Q), Tubb3 (C,H,M,R), Phox2b (D,I,N,S) and TH (E,J,O,T). The scale bar in A is valid for A–J and the one in K for K–T. Both correspond to 50 µm. (U–X) Expression of Phox2b (U,V) and TH (W,X) was quantified in wildtype (WT) and $Sox10^{Sox100B/Sox100B}$ (KI) sympathetic ganglia at 12.5 dpc (U,W) and 18.5 dpc. (V,X) Immunoreactive cells were counted for Phox2b, whereas the immunoreactive area was determined in $\mu m^2 \times 10^3$ for TH as described (Reiprich et al., 2008). Data are presented as mean ± SEM. Statistically significant differences of $P<0.01$ (**) and $P<0.001$ (***) are shown above each bar as determined by the Student's *t* test.

a more drastic phenotype (Britsch et al., 2001; Reiprich et al., 2008) (Table 1).

Defects were also detected in the developing adrenal medulla which like the sympathetic nervous system develops from a sympathoadrenal precursor cell population. At 12.5 dpc, the location of the adrenal gland is marked by SF-1 expressing cells of the future adrenal cortex (Fig. 8A). At this age, we already observed a slight decrease in TH-positive precursor cells (Fig. 8A). By 18.5 dpc, TH-positive cells in the mutant adrenal medulla remained reduced in number (Fig. 8B). In Sox100B replacement mice, TH-positive cells make up only 9% of the area of the adrenal gland in contrast to the wildtype where TH-positive cells cover 14.5% of the gland (Fig. 8C). Taking into account, however, that an adrenal medulla is completely lacking in Sox10-deficient mice and very strongly reduced in the Sox10ΔK2 and Sox10aa1 mutants, the ability of Sox100B to rescue this defect is substantial and at least as good as the mammalian Sox8 protein (Table 1) (Reiprich et al., 2008).

Ability of Sox100B to rescue dorsal root ganglion development in the absence of Sox10

An additional defect in Sox10-deficient mice is apparent in the dorsal root ganglia (DRG). These PNS structures originally develop in the absence of Sox10, but already start to degenerate at 12.5 dpc (Britsch et al., 2001). Satellite glia are completely absent from DRG, and neurogenesis is also impaired in Sox10-deficient mice. In the Sox100B replacement mutant satellite glia were present in ganglia throughout development. At both 12.5 dpc and at 18.5 dpc, glial cells were marked by Sox100B which replaced Sox10 expression in wildtype glia (Figs. 9A,B,E,F,I,J,M,N). In addition, glial cells in the replacement mutant and in the wildtype were both positive for B-FABP which represents the earliest available glial marker in the PNS (Figs. 9C,G,K,O). Neurons were also present in the DRG of the Sox100B replacement mutant as evident from NeuN staining (Figs. 9D,H,L,P). Again this shows that Sox100B is at least partially capable of substituting for Sox10 during DRG development. Quantification revealed that the number of glial cells was only slightly reduced at 12.5 dpc to 78% of wildtype numbers (Fig. 9Q) and indistinguishable from the wildtype at 18.5 dpc (Fig. 9R). In contrast, neuron numbers were reduced to approximately 40% in the Sox100B replacement mutant both at 12.5 dpc and 18.5 dpc (Figs. 9S,T) and are therefore predominantly responsible for the reduced overall size of the ganglia. It thus appears that Sox100B expressing satellite glia in the replacement mutant cannot support DRG neurons as efficiently as Sox10 expressing glia in the wildtype. In contrast to the Sox8 replacement mutant (Kellerer et al., 2006), DRG development is thus not completely rescued in the Sox100B replacement mutant (Table 1). The Sox100B replacement phenotype is also stronger than observed in Sox10aa1 mutants where only mild alterations in DRG are visible at late stages of embryonic development (Schreiner et al., 2007). In terms of severity, the DRG defect in Sox100B replacement mice is most similar to that observed in the Sox10ΔK2 mutant (Schreiner et al., 2007). Sox100B, however, remains expressed in DRG, whereas DRG degeneration in the Sox10ΔK2 mutant is preceded by disappearance of the mutant Sox10 protein from the ganglia.

Ability of Sox100B to rescue Schwann cell development in the absence of Sox10

Glial development throughout the PNS requires Sox10. This not only includes satellite glia in the DRG but also Schwann cells along the nerves (Britsch et al., 2001). Analysis of the Sox100B replacement mutant reveals that Schwann cells develop normally (Fig. 10 and data not shown). At 18.5 dpc, for example, there are as many Sox100B-positive cells along the peripheral nerve in the Sox100B replacement mutant as there are Sox10-positive cells in the wildtype (Figs. 10A,B,C, D,K). A significant percentage of these Schwann cells show Oct6 expression which is indicative of their progression from an immature Schwann cell to a promyelinating Schwann cell (Figs. 10E,F). The number of Oct6-positive cells is comparable between wildtype and mutant (Fig. 10L). Also comparable is the fraction of Schwann cells expressing Krox-20 at 18.5 dpc, indicating that Schwann cells have initiated the final phase of differentiation and myelination on schedule in the Sox100B replacement mutant (Figs. 10G,H). In agreement, myelin markers such as *MBP* are expressed in the mutant in a pattern and intensity that is comparable to the wildtype (Figs. 10I,J). Similar to Sox8 (Kellerer et al., 2006), Sox100B can effectively rescue the Schwann cell defect in Sox10-deficient mice (Table 1). Sox100B is thus much more effective in driving Schwann cell development than

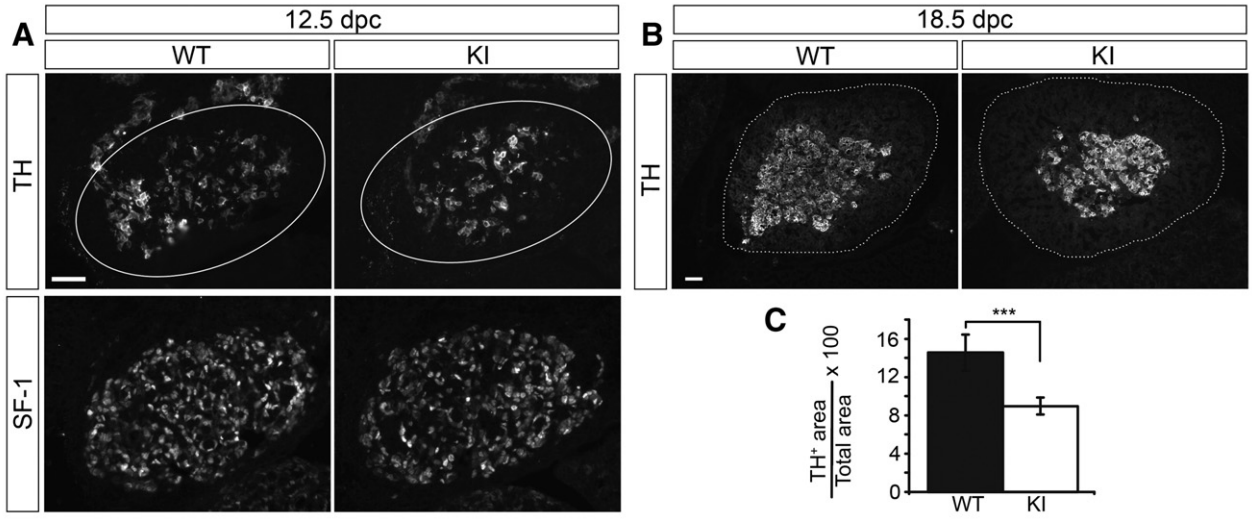

Fig. 8. Development of the adrenal gland in $Sox10^{Sox100B/Sox100B}$ embryos. Immunohistochemical staining of the adrenal gland was performed on wildtype (WT) and $Sox10^{Sox100B/Sox100B}$ (KI) embryos at 12.5 dpc (A) and 18.5 dpc (B) using antibodies against TH and SF-1. (C) TH staining at 18.5 dpc was also used to calculate the relative contribution of the medulla to the adrenal gland in both genotypes. Three adrenal glands were used per genotype for quantifications. Data are presented as mean ± SEM. The difference between the two genotypes is statistically significant (***, $P<0.001$) as determined by the Student's *t* test. Scale bars in A and B correspond to 50 µm. Both are valid for all panels.

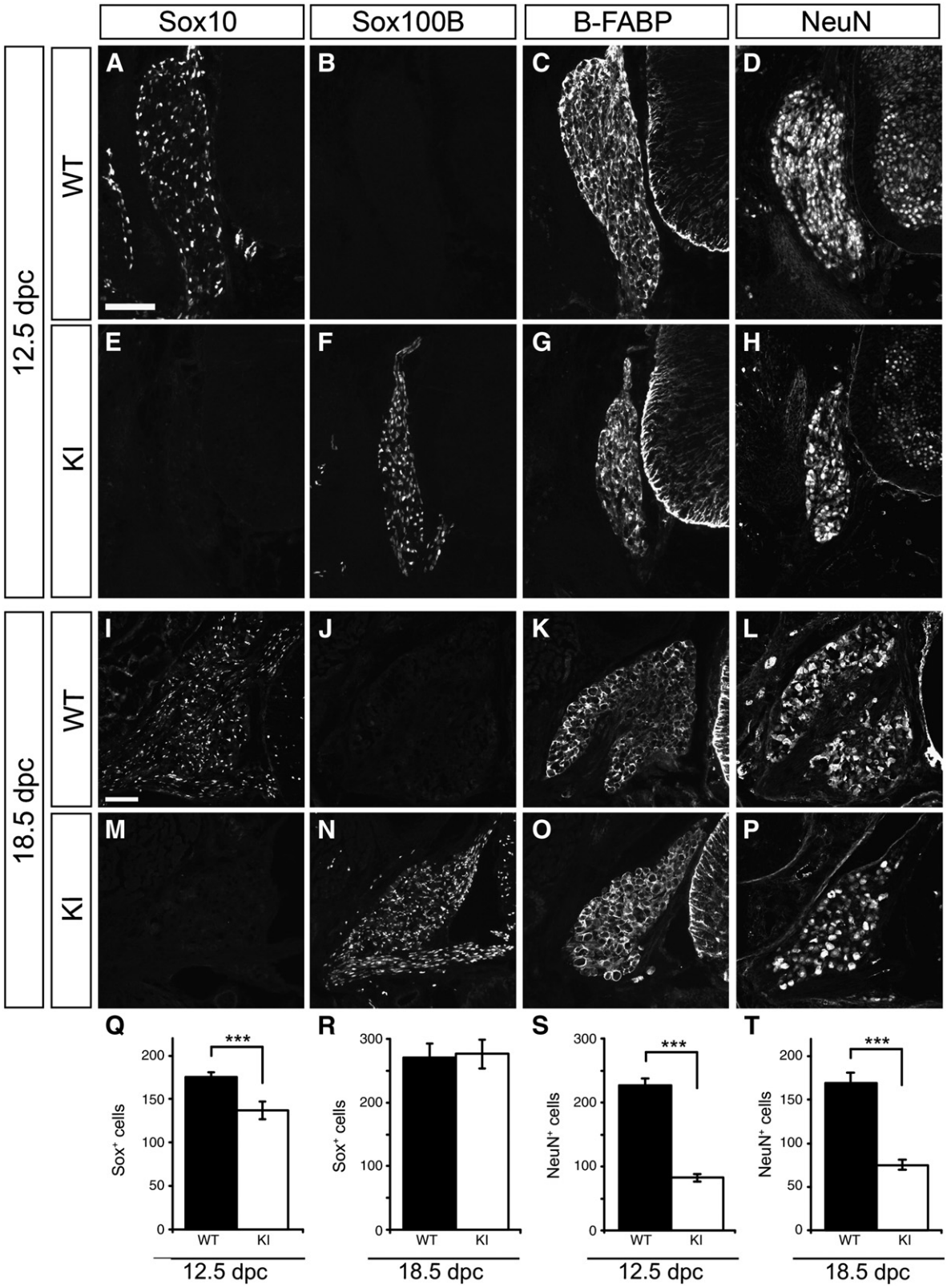

Fig. 9. Analysis of DRG in $Sox10^{Sox100B/Sox100B}$ embryos. Immunohistochemistry was carried out on transverse sections through DRG of wildtype (WT, panels A–D,I–L) and $Sox10^{Sox100B/Sox100B}$ (KI, panels E–H,M–P) embryos at 12.5 dpc (A–H) and 18.5 dpc (I–P) in thoracic regions using antibodies against Sox10 (A,E,I,M), Sox100B (B,F,J,N), the glial marker B-FABP (C,G,K,O) and the neuronal marker NeuN (D,H,L,P). The spinal cord is to the right of the DRG. The scale bar in A is valid for A–H and the one in I for I–P. Both correspond to 100 μm. (Q–T) Expression of Sox10/Sox100B (Q,R) and NeuN (S,T) was quantified in wildtype (WT) and $Sox10^{Sox100B/Sox100B}$ (KI) DRG at 12.5 dpc (Q,S) and 18.5 dpc (R,T) by counting the immunoreactive cells. Data are presented as mean ± SEM. Statistically significant differences of $P<0.001$ (***) are shown above each bar as determined by the Student's *t* test.

the Sox10 mutants that are dimerization-deficient or lack the K2 domain (Schreiner et al., 2007).

Ability of Sox100B to rescue oligodendrocyte development in the absence of Sox10

In the developing CNS, Sox10 is restricted to the myelin-forming oligodendrocytes and their precursors, and terminal differentiation of oligodendrocytes is impaired in Sox10-deficient mice (Stolt et al., 2002). In the Sox100B replacement mutant, Sox100B was first detected in the ventral spinal cord around 12.5 dpc in the vicinity of the ventricular zone of the pMN domain. This corresponds both in timing and pattern to the normal onset of Sox10 expression in the wildtype (Fig. 11A). Similar to Sox10 in the wildtype, Sox100B remained expressed in the oligodendrocyte precursors throughout embryonic development until 18.5 dpc (Fig. 11B). Furthermore, the number of cells belonging to the oligodendrocyte lineage was comparable between wildtype and Sox100B replacement mutant throughout embryogenesis as evident from quantifications of Olig2 stainings, thus demonstrating that Sox100B expression does not interfere with oligodendrocyte development (Figs. 11D,E). Importantly, the number of terminally differentiating (i.e. *MBP*- and *PLP*-expressing) oligodendrocytes in mutant spinal cord at 18.5 dpc equalled those in the wildtype (Fig. 11C and data not shown). As these cells are completely missing in spinal cords of Sox10-deficient mice (Stolt et al., 2002), we conclude that Sox100B efficiently rescues the oligodendrocyte terminal differentiation defect observed in the absence of Sox10. A similarly efficient rescue was previously observed in the Sox10aa1 mutant and, slightly less efficiently, in the Sox10ΔK2 mutant (Schreiner et al., 2007). Intriguingly, however, such a rescue was not observed in the Sox8 replacement mutant (Kellerer et al., 2006) indicating that Sox100B is a much better substitute for Sox10 during oligodendrocyte development than the mammalian SoxE protein Sox8.

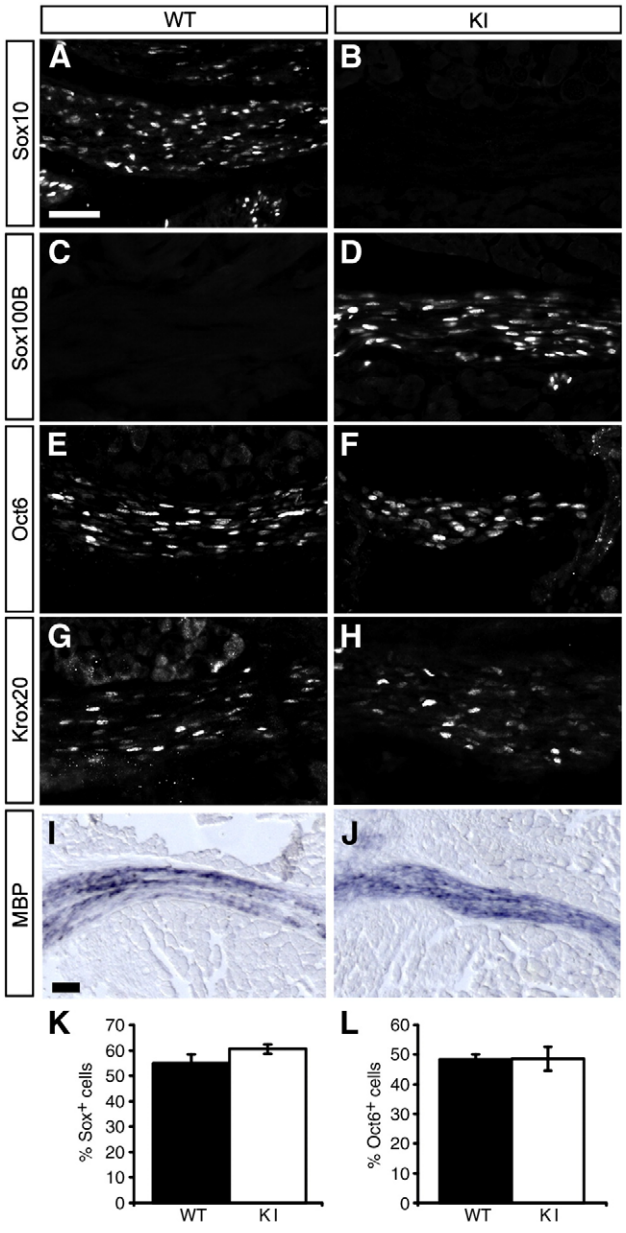

Fig. 10. Peripheral nerve development in $Sox10^{Sox100B/Sox100B}$ embryos. Immunohistological analysis of spinal nerves from wildtype (WT, panels A,C,E,G,I) and $Sox10^{Sox100B/Sox100B}$ (KI, panels B,D,F,H,J) embryos at 18.5 dpc was carried out using antibodies against Sox10 (A,B), Sox100B (C,D), Oct6 (E,F) and Krox20 (G,H). *MBP* expression was additionally visualized in both genotypes by in situ hybridization (I,J). The scale bar in A is also valid for B–H, whereas the scale bar in I is also valid for J. Both correspond to 50 μm. (K,L) The number of cells immunoreactive for Sox10/Sox100B (K) and Oct6 (L) was quantified in wildtype (WT) and $Sox10^{Sox100B/Sox100B}$ (KI) spinal nerves at 18.5 dpc. Data are presented as mean ± SEM. Differences between genotypes were not statistically significant as determined by the Student's *t* test.

Table 1
Summary of the rescuing capacity of different mutant *Sox10* alleles.

Development of…	Rescue by allele			
	$Sox10^{Sox100B}$	$Sox10^{Sox8}$	$Sox10^{aa1}$	$Sox10^{\Delta K2}$
Early neural crest	+	+	+	+
Melanocytes	−	−	−	±/−
ENS	±	−	−	±/−
Sympathetic ganglia (caudal)	±	±	−	−
Adrenal medulla	±	±	−	−
DRG	±	+	+/±	±
Schwann cells	+	+	±	±
Oligodendrocytes	+	−	+	±

+, complete rescue; ±, substantial partial rescue; −, minor to no rescue.
Data for the $Sox10^{Sox8}$, $Sox10^{aa1}$ and the $Sox10^{\Delta K2}$ alleles are compiled from Kellerer et al. (2006), Reiprich et al. (2008) and Schreiner et al. (2007).

Discussion

We have studied the ability of the *Drosophila* SoxE protein, Sox100B, to perform the functions of the vertebrate SoxE protein Sox10 in cell lineages that do not exist in *Drosophila*. Both overexpression studies in the chicken and gene replacement in the mouse clearly demonstrate that Sox100B integrates well into the gene-regulatory networks that drive early neural crest development. Oligodendrocyte development is also indistinguishable in wildtype and *Sox100B* replacement homozygotes until birth. We therefore conclude that Sox100B and Sox10 are functionally similar.

In evolutionary terms this argues that an existing SoxE protein could have been easily integrated into the novel regulatory networks that drive neural crest and oligodendrocyte development. As the SoxE protein would have been functional without major structural alterations, a newly acquired expression domain in the neural crest or in oligodendrocytes would per se have been sufficient to endow the SoxE protein with a pivotal regulatory role in these cell types. In agreement with such a model, the appearance of SoxE proteins at the neural plate border correlates with the emergence of a neural crest. SoxE proteins are not expressed at the neural plate border in amphioxus, a basal chordate, but are found there in the basal agnathan vertebrates lamprey and hagfish (McCauley and Bronner-Fraser, 2006; Sauka-Spengler et al., 2007; Yu et al., 2008).

Also in agreement with this model, the SoxE proteins expressed in the neural crest of agnathans differ from the SoxE proteins in gnathostomes (McCauley and Bronner-Fraser, 2006; Ota et al.,

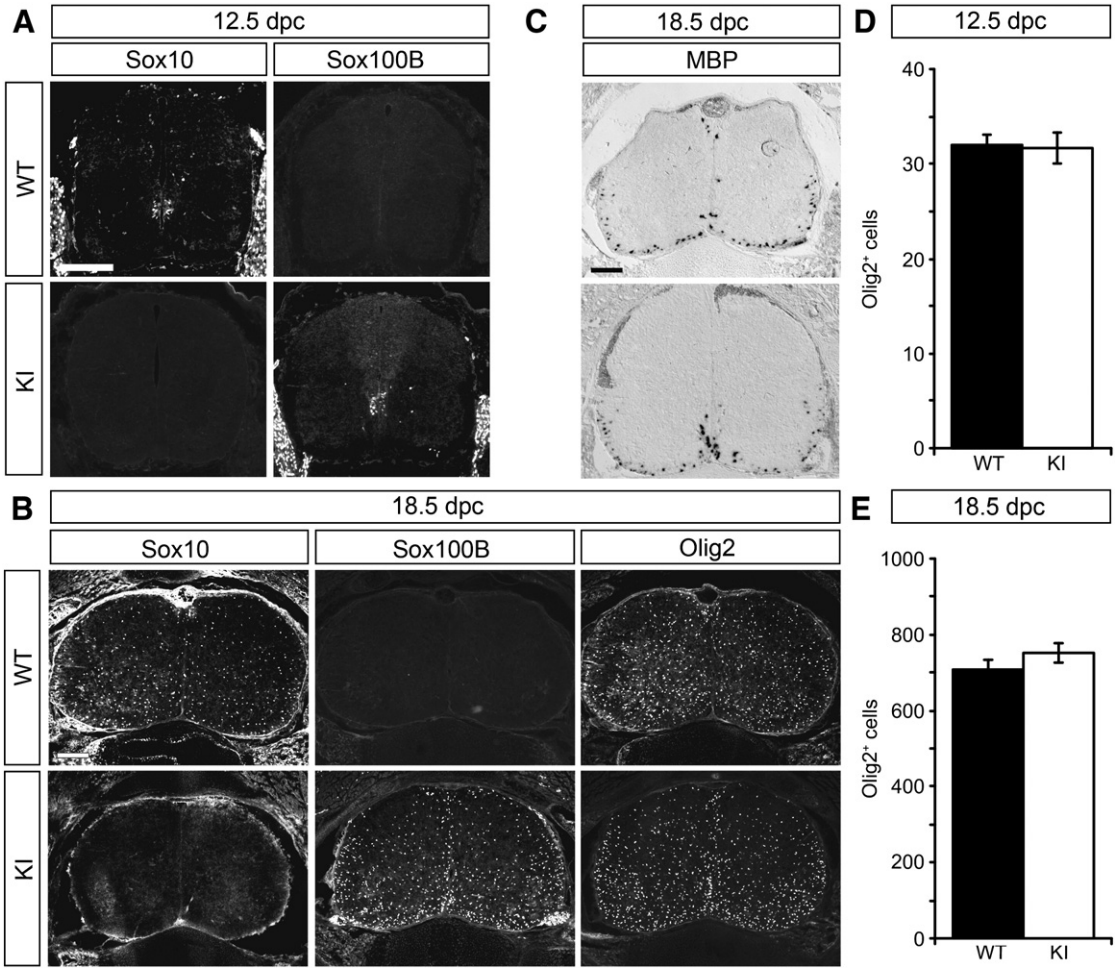

Fig. 11. Oligodendrocyte development in $Sox10^{Sox100B/Sox100B}$ embryos. Specification (A), subsequent distribution of oligodendrocyte precursors (B) and terminal differentiation (C) were analyzed on transverse spinal cord sections from the forelimb region of wildtype (WT) and $Sox10^{Sox100B/Sox100B}$ (KI) embryos at 12.5 dpc (A) and 18.5 dpc (B,C) by immunohistochemistry with antibodies directed against Sox10, Sox100B and Olig2 (A,B) and by in situ hybridization with an *MBP*-specific probe (C). Scale bars in A, B and C are valid for all respective panels. All correspond to 200 μm. (D,E) The number of Olig2-positive cells was quantified in wildtype (WT) and $Sox10^{Sox100B/Sox100B}$ (KI) spinal cords at 12.5 dpc (D) and 18.5 dpc (E). Data are presented as mean ± SEM. Differences between genotypes were not statistically significant as determined by the Student's *t* test.

2007). There is limited homology between lamprey SoxE3 and Sox9 from tetrapods or teleosts outside the HMG domain, and virtually no homology between lamprey SoxE1 and SoxE2 proteins and the Sox8 and Sox10 proteins (McCauley and Bronner-Fraser, 2006). Nevertheless, these agnathan SoxE proteins function in neural crest development as well as their counterparts in gnathostomes (McCauley and Bronner-Fraser, 2006; Ota et al., 2007).

Even in gnathostomes there is some variation in the usage of SoxE proteins with Sox8 being a major regulator of neural crest development only in amphibians (O'Donnell et al., 2006). Taking into account that all SoxE proteins tested so far are equally capable of driving early neural crest development under widely varying experimental conditions (Cheung and Briscoe, 2003; Kellerer et al., 2006; Taylor and LaBonne, 2005) there is overwhelming evidence that SoxE proteins were co-opted to the neural crest gene-regulatory network by changes in their expression without an immediate need for altered biochemical properties. The recent identification of multiple enhancers in the vicinity of the mouse Sox10 gene may be instrumental in determining how this novel expression was acquired (Antonellis et al., 2008; Werner et al., 2007).

Our data also suggest that SoxE proteins may have been similarly co-opted to the gene-regulatory network that drives oligodendrocyte development. Considering how little is known about the evolution of oligodendrocytes and the components of the oligodendrocyte gene-regulatory network, our data provide one of the first insights into this interesting topic.

In mice homozygous for the *Sox100B* replacement allele, neural crest cells are capable of giving rise to all types of derivatives that normally require Sox10 including melanoblasts, cells of the enteric and sympathetic nervous systems, cells of the adrenal medulla, and peripheral glia (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Reiprich et al., 2008; Southard-Smith et al., 1998). The phenotypic rescue is, however, not complete since defects are observed during later stages of neural crest development (Table 1). Development of the melanocyte lineage was most severely impaired and substantial defects were also evident in the enteric and sympathetic nervous systems, whereas other parts of the PNS, including DRG, were less strongly affected. In contrast, Schwann cell development is comparatively normal with terminally differentiating myelinating Schwann cells detected at birth in homozygous Sox100B replacement mutants. This argues that Sox10 and its *Drosophila* ortholog are similar but not identical in their properties and functions. They may, for instance, interact with different partner proteins or be differentially receptive to signal-dependent posttranslational modifications. Again this finding can be interpreted in evolutionary terms and suggests that once co-opted to a neural crest-specific gene-regulatory network, vertebrate SoxE proteins have consecutively undergone alterations in their biochemical properties, interaction capacities, and signal responsiveness to better suit their

roles in the neural crest. In particular, the increasing diversification of the neural crest into many different types of derivatives required adaptive changes in SoxE protein structure. This scenario would also allow co-expressed and originally identical SoxE proteins to diversify structurally and functionally with Sox9 becoming essential for the development of ectomesenchymal cell lineages in the cranial neural crest and Sox10 playing numerous decisive roles in the various non-ectomesenchymal neural crest lineages. These increasing adaptations, sub- and neofunctionalizations could contribute to the eventual lockdown of the regulatory network (Erwin and Davidson, 2009).

In this respect, it is also interesting to compare Sox100B and the previously studied mammalian Sox8 protein in their ability to provide Sox10-specific functions (Kellerer et al., 2006). In general, both proteins are fairly good substitutes for Sox10. However, there are also clear differences between the phenotypes observed with each protein. Sox8 can rescue development of DRG and sympathetic ganglia more efficiently than Sox100B, whereas Sox100B is clearly a better substitute for Sox10 during ENS development. Additionally, Sox100B can induce terminal differentiation of oligodendrocytes as efficiently as Sox10, whereas Sox8 is severely impaired in this function. Taking into account that Sox8 is co-expressed with Sox10 in developing ENS and oligodendrocytes and that mild defects that occur in these cell lineages in $Sox10^{+/lacZ}$ mice can be substantially aggravated by additional loss of Sox8 (Maka et al., 2005; Stolt et al., 2004), it is intriguing and surprising that Sox100B is the better substitute. It may point to the fact, however, that Sox8 and Sox10 have undergone some degree of subfunctionalization during ENS and oligodendrocyte development. If this is the case, it suggests that all the essential functions have segregated with Sox10 and the less important functions with Sox8. The resulting divergent adaptations in protein structure may now, however, prevent Sox8 from fully replacing Sox10. Sox100B, in contrast, lacks these structural specializations and might therefore be a better, although not perfect substitute. If this is the case, we expect that Sox100B will be a better substitute for Sox9 during cranial neural crest development than Sox10.

The fact that the invertebrate SoxE protein is as efficient in replacing Sox10 as the mammalian Sox8 is also intriguing with respect to structural considerations. There is clearly much greater overall sequence similarity between Sox10 and Sox8 than between Sox10 and Sox100B, in particular within the dimerization, K2, and carboxy-terminal transactivation domains. *Sox10* alleles with an inactivated dimerization domain or deleted K2 domain have furthermore shown that these domains are required for proper function (Schreiner et al., 2007). With exception of the melanocyte defect in the ΔK2 mutant, the corresponding hypomorphic mouse mutants usually have developmental defects that are at least as severe as and often more severe than those in mice homozygous for the *Sox100B* replacement allele. This clearly argues that the functions of the mammalian dimerization and K2 domains must at least in part be conserved in Sox100B. Our biochemical studies indeed confirmed that Sox100B is capable of DNA-dependent dimer formation and possesses transactivation capacity. A definitive biochemical test for K2 domain activity is currently not available and neither sequence alignments nor structural prediction algorithms have so far revealed a K2-like domain in Sox100B. Yet, a functional equivalent must be present. Our findings may thus inspire further comparative biochemical studies in which functionally important residues and conformational determinants of SoxE proteins are defined. More importantly, however, they shed light on the evolution of gene-regulatory networks and provide strong evidence that the role of SoxE proteins in neural crest and oligodendrocyte development is ancestral in vertebrates and was acquired through altered expression and co-option to novel gene-regulatory networks.

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