

# Hypomorphic *Sox10* alleles reveal novel protein functions and unravel developmental differences in glial lineages

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The transcription factor *Sox10* regulates early neural crest development, specification of neural crest-derived lineages and terminal differentiation of oligodendrocytes in the central nervous system. Here, we generated two novel hypomorphic *Sox10* alleles in the mouse. Mutant mice either expressed a *Sox10* protein with a triple alanine substitution in the dimerization domain, or a *Sox10* protein with a deletion in the central portion that we define as a cell-specific transactivation domain. Phenotypic analysis revealed important roles for a functional dimerization domain and the newly defined novel transactivation domain in melanocyte and enteric nervous system development, whereas early neural crest development and oligodendrocyte differentiation were surprisingly little disturbed in both mutants. Unique requirements were additionally detected for the novel transactivation domain in satellite glia differentiation and during Schwann cell myelination, whereas DNA-dependent dimerization was needed for immature Schwann cells to enter the promyelinating stage. These two hypomorphic alleles thus uncover novel functions of *Sox10* in satellite glia and Schwann cells during late developmental stages and reveal important developmental differences between these two types of peripheral glia and oligodendrocytes regarding their reliance on *Sox10*.

**KEY WORDS:** *Sry*, High mobility group, Glia, Oligodendrocyte, Neural crest, Schwann cell, Satellite glia

## INTRODUCTION

*Sox10*, like other transcription factors of the *Sox* family, is an important regulator of embryonic development (Hong and Saint-Jeannet, 2005; Kelsh, 2006; Wegner, 1999; Wegner and Stolt, 2005). Neural crest development, in particular, is heavily dependent on *Sox10* in all model vertebrate species analyzed so far (Aoki et al., 2003; Britsch et al., 2001; Cheung and Briscoe, 2003; Dutton et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998). Survival of neural crest stem cells and the maintenance of their multipotency require *Sox10* (Kim et al., 2003). In rodents, several lineage decisions of neural crest stem cells additionally depend on *Sox10* so that melanocytes, peripheral glia and cells of the enteric nervous system (ENS) are all missing in its absence (Britsch et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998). The continued presence of *Sox10* in peripheral glia furthermore suggests additional functions during later developmental stages and terminal differentiation of neural crest derivatives (Ito et al., 2006; Peirano et al., 2000).

Such a late function has already been verified in oligodendrocytes (Stolt et al., 2002). These myelin-forming cells of the central nervous system represent the second main site of *Sox10* expression and rely, for terminal differentiation and activation of several key myelin genes, on *Sox10* (Bondurand et al., 2001; Schlierf et al., 2006; Stolt et al., 2002).

*Sox10* structure-function studies have identified several domains that are characteristic of a transcription factor and necessary for its specific cellular actions. These include the DNA-binding high mobility group (HMG) domain (amino acids 101–180) and a transactivation domain at the extreme C-terminus (amino acids 400–466) (Kuhlbrodt et al., 1998b). These domains are highly similar to

the corresponding regions in *Sox8* and *Sox9*, to which *Sox10* is closely related and with which it forms subgroup E of the *Sox* protein family.

Further regions conserved among these three *SoxE* proteins include amino acids 61–101 and 233–306. Amino acids 61–101 have been defined as a DNA-dependent dimerization domain (Peirano and Wegner, 2000; Schlierf et al., 2002). Known *Sox10* target genes usually contain multiple response elements in their promoter, some of which bind one *Sox10* molecule, whereas others bind two *Sox10* molecules in a cooperative manner (for a review, see Wegner, 2005). This cooperative binding requires the dimerization domain. Although the dimerization domain is needed for full activation of target gene promoters in vitro (Schlierf et al., 2002), its physiological relevance in vivo remains to be proven.

No function has yet been attributed experimentally to the region between amino acids 233–306. This region, which we named the K2 domain, has been shown to possess transactivation potential in *Sox8* (Schepers et al., 2000). It might thus also be involved in mediating *Sox10*-dependent transcriptional activation. To analyze the function of the dimerization domain and the K2 domain in mice, we replaced the wild-type *Sox10* allele by mutant versions that either lacked the K2 domain or carried a triple alanine substitution in the dimerization domain that rendered it inactive in vitro.

## MATERIALS AND METHODS

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

The K2 domain mutation was generated by overlap-PCR, deleting bp 1279–1500 in the rat *Sox10* cDNA (accession NM019193), corresponding to amino acids 233–306 in the *Sox10* protein. The *Sox10* aa1 mutant carries a substitution of amino acids Cys71, Ile72 and Arg73 by alanines (Schlierf et al., 2002). Sequences corresponding to the open reading frame for one of the mutant *Sox10* proteins 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 with a continuous *Sox10* reading frame carrying the

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deletion of the K2 domain or the aa1 mutation (Fig. 2A,B). Sequencing confirmed that no additional unintended mutation had been introduced during cloning.

Both constructs were linearized with *Xba*I and electroporated into E14.1 ES cells, which were then selected with G418 (400  $\mu$ g/ml) and gancyclovir (2  $\mu$ M). Selected ES cell clones were screened by Southern blotting with a 0.6 kb 5' probe, which recognized a 6.6 kb fragment of the wild-type allele, a 5.9 kb fragment of the *Sox10<sup>aa1</sup>* allele and a 10.9 kb fragment of the *Sox10<sup>ΔK2</sup>* allele in genomic DNA digested with *Nco*I (Fig. 2A-C). 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 10.4 kb fragment in the *Sox10<sup>aa1</sup>* allele and to a 10.2 kb allele in the *Sox10<sup>ΔK2</sup>* allele, as opposed to a 4.6 kb fragment in the wild-type allele (Fig. 2A-C). Two targeted ES cell lines for each mutant 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. The neomycin resistance cassette was removed by Cre-mediated recombination, and removal was verified as described (Kellerer et al., 2006). Heterozygous mice were backcrossed on a C3H background. Homozygous mutant embryos (F2-F5) were generated by heterozygote intercrosses.

Genotyping was routinely performed on DNA from tail tips or, in the case of embryos from yolk sacs, by PCR analysis using a common primer located 81 bp upstream of the start codon (5'-CAGGTGGG-CGTTGGGCTCTT-3') and two primers located 487 bp downstream of the start codon in intron 3 of the *Sox10* gene (5'-TCCCAGGCTAG-CCCTAGTG-3') or 617 bp downstream of the start codon in mutant *Sox10* reading frames (5'-GTGAGCCTGGATAGCAGCAG-3'). Fragments of 568 and 698 bp were indicative of the wild-type and targeted alleles, respectively (Fig. 2D).

#### Transfections, luciferase assays, immunocytochemistry and western blots

Expression plasmids for Sox10, Sox10  $\Delta$ K2, and Sox10 Q377X were based on pCMV5 (Kuhlbrodt et al., 1998a; Ludwig et al., 2004a). Luciferase reporter carried the 1 kb *Mpz* promoter (P0-luc), 3.7 kb of the

dopachrome tautomerase promoter (3.7 Trp2-luc) and 0.7 kb of the *Mbp* promoter (0.7 Mbp-luc) (Ludwig et al., 2004a; Peirano et al., 2000; Stolt et al., 2004).

For luciferase assays Neuro2a neuroblastoma cells were cultivated in 24 well plates, transfected with 0.5  $\mu$ g reporter and 0.5  $\mu$ g effector plasmids in the case of P0-luc and 3.7 Trp2-luc or with 0.025  $\mu$ g reporter and 0.5  $\mu$ g effector in the case of 0.7 Mbp-luc using Superfect reagent (Qiagen). Cells were transfected in triplicate and harvested 48 hours after transfection.

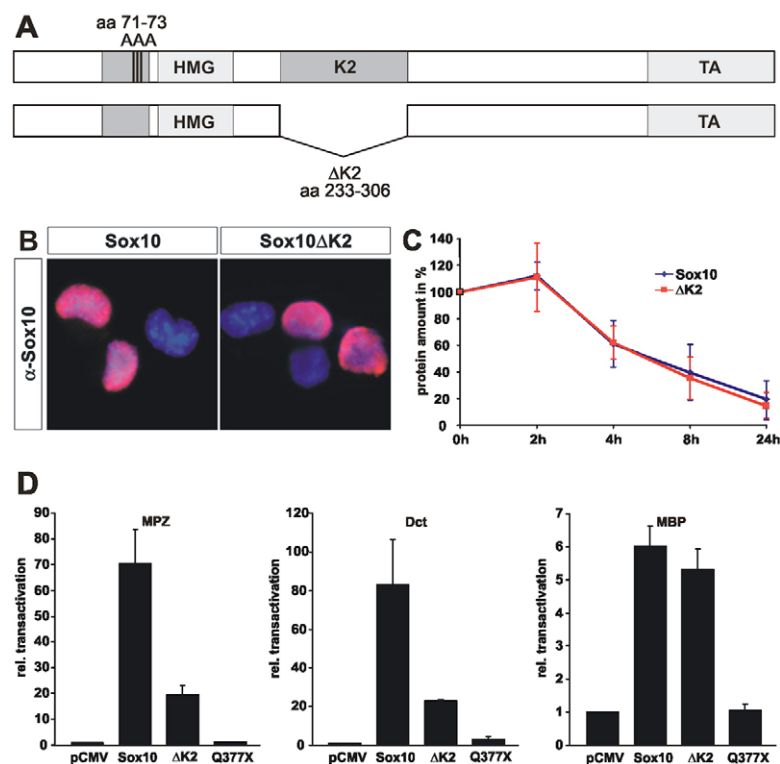
Immunocytochemistry was performed on Neuro2a cells seeded on coverslips in 3-cm plates and transfected with 2  $\mu$ g pCMV5-Sox10 or pCMV5-Sox10  $\Delta$ K2. After treatment with 4% paraformaldehyde (PFA) for 30 minutes, coverslips were incubated successively with anti-Sox10 rabbit antiserum (1:4000) (Stolt et al., 2003) and goat anti-rabbit Cy3 (1:200, Dianova).

For analysis of protein stability, Neuro2a cells were transfected with wild-type and mutant Sox10 constructs using calcium phosphate precipitates and treated 48 hours after transfection with 25  $\mu$ g/ml cycloheximide for up to 24 hours. Extracts were prepared after various times of cycloheximide treatment, and Sox10 detected by western blot as described (Stolt et al., 2006). Sox10-specific bands on western blots were quantified using the NIH ImageJ software.

#### Tissue, RNA and protein preparation from embryos, immunoprecipitations and quantitative RT-PCR

Embryos were isolated at 10.5-18.5 dpc from staged pregnancies and processed for extract preparation, RNA isolation, immunohistochemistry or in situ hybridization (Kellerer et al., 2006; Stolt et al., 2004; Stolt et al., 2003).

Immunoprecipitations were performed on whole embryo extracts using the anti-Sox10 rabbit antiserum (Wißmüller et al., 2006). After reverse transcription of 2  $\mu$ g whole embryo RNA in a 20  $\mu$ l reaction, 1  $\mu$ l of the obtained cDNA was amplified by PCR 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 1 and 2, 5'-TCAGTCTCGGCTGTCCAGCC-3' and 5'-GAAGAGCCCAACGC-CACCT-3'; primers 3 and 4, 5'-TCTACAGCCCATCTCTGAC-3' and 5'-CAGCCTCCTCCACTGCCA-3' (Fig. 2A). *Rpl8* transcripts were used for normalization (Kellerer et al., 2006). Annealing was at 64°C.



**Fig. 1. The Sox10 mutations.** (A) Schematic of the aa1 and  $\Delta$ K2 mutations. (B) Subcellular localization of wild-type Sox10 and the  $\Delta$ K2 mutant in transiently transfected Neuro2a cells was determined by immunocytochemistry with a Sox10-specific antibody. Nuclei were counterstained by DAPI. (C) Stability of wild-type Sox10 (blue circle) and the  $\Delta$ K2 mutant (red square) were compared in transiently transfected Neuro2a cells cultured for various times in the presence of cycloheximide as indicated. Extracts were prepared and Sox10 proteins detected by western blot. Relative amounts were quantified from band intensities with the amount in untreated cells set to 100%. (D) The transactivation capacity of the  $\Delta$ K2 mutant was analyzed in luciferase reporter gene assays. Transient transfections were performed in Neuro2a cells with luciferase reporters under the control of the *Mpz* promoter (positions -915 to +48), the *Dct* promoter (positions -3240 to +443) and the *Mbp* promoter (positions -656 to +31). Luciferase reporters were transfected either alone or in combination with wild-type Sox10, the  $\Delta$ K2 or the Q377X mutant. Data from three independent experiments each performed in duplicate are presented as fold inductions  $\pm$  s.d., with the activity for each luciferase reporter in the absence of co-transfected Sox10 set to 1.

### In situ hybridization and immunohistochemistry

In situ hybridizations (except probe hybridization and final colorimetric detection) were performed automatically on a Biolane HTI (Hölle and Hüttner AG) using DIG-labeled antisense riboprobes for *Mbp*, *Plp*, *Mpz*, *c-Kit*, *Dct* and *Sox10* (Britsch et al., 2001; Stolt et al., 2002).

Primary antibodies for immunohistochemistry included: anti-Sox10 guinea pig antiserum (1:2000) (Maka et al., 2005), anti-Oct6 rabbit antiserum (1:2000) (Friedrich et al., 2005), anti-Phox2b rabbit antiserum (1:2000, gift of C. Goridis, Ecole Normale Supérieure, Paris, France), anti-Olig2 rabbit antiserum (1:10,000, gift of D. Rowitch, DFCI, Boston, MA), anti-B-FABP rabbit antiserum (1:10,000, gift of C. Birchmeier and T. Müller, MDC, Berlin, Germany), anti-Sox10 (Sox1/2/3) rabbit antiserum (1:500) (Tanaka et al., 2004), anti-Th rabbit antiserum (1:1000, Biomol), anti-NF165 mouse monoclonal (1:200, Developmental Studies Hybridoma Bank) and anti-NeuN mouse monoclonal (1:500, Chemicon). Secondary antibodies conjugated to Cy3 immunofluorescent dyes (Dianova) 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).

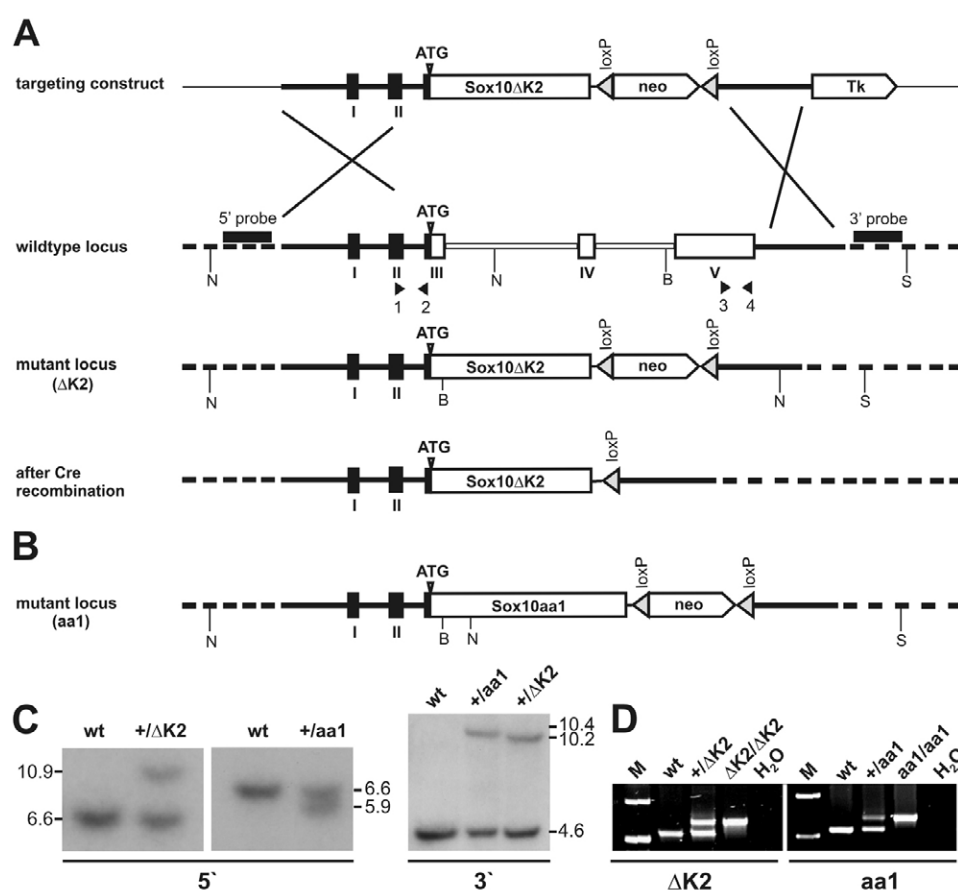
## RESULTS

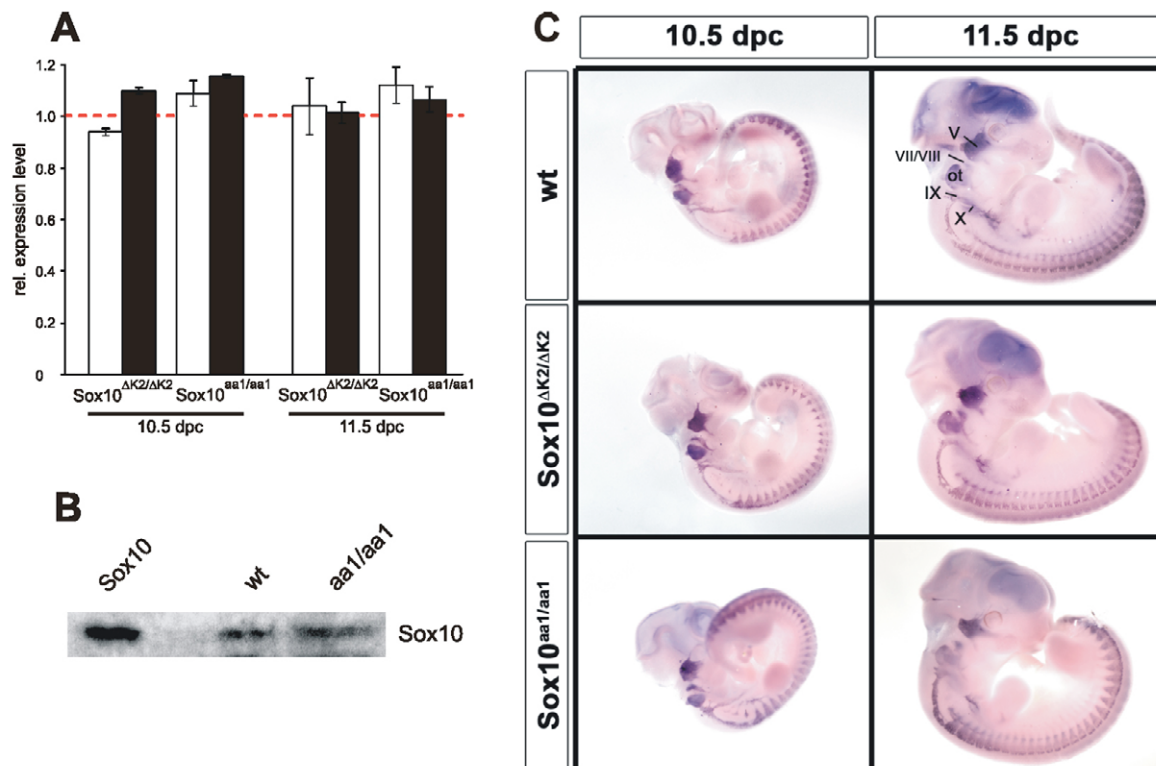
### Generation of mice with mutant Sox10 alleles

To analyze the functional consequences of *Sox10* mutations in vivo, we replaced the wild-type *Sox10* gene sequences by mutant versions in which amino acids 71–73 of Sox10 were changed from CIR to AAA (aa1 mutant) or in which amino acids 233–306 were deleted

( $\Delta$ K2 mutant) (Fig. 1A). The aa1 mutant has previously been found to selectively abolish the ability of Sox10 to form DNA-dependent dimers (Schlierf et al., 2002). Taking into account that the  $\Delta$ K2 mutant affects neither the HMG-domain of Sox10 nor its dimerization domain, its DNA-binding ability is likely to be unaffected. The  $\Delta$ K2 mutant furthermore localized as efficiently as wild-type Sox10 to nuclei in transfected Neuro2a cells (Fig. 1B). There was also no obvious difference in the stability of the  $\Delta$ K2 mutant versus wild-type Sox10 in transfected cycloheximide-treated cells (Fig. 1C). Despite these normal characteristics, Sox10  $\Delta$ K2 exhibited altered transactivation abilities. In luciferase reporter gene assays, activation of the *Mpz* and dopachrome tautomerase (*Dct*) promoters by the  $\Delta$ K2 mutant was 3.6-fold lower than by wild-type Sox10 (Fig. 1D). The residual activity of the  $\Delta$ K2 mutant still elicited a 20- to 23-fold promoter activation, whereas a truncated Sox10 protein without the previously identified C-terminal transactivation domain (Kuhlbrodt et al., 1998b) was completely inactive (Sox10 Q377X in Fig. 1D). In contrast to *Mpz* and *Dct* promoters, the *Mbp* gene promoter, as a third Sox10 target, was activated almost normally by the  $\Delta$ K2 mutant (Fig. 1D). We conclude that the  $\Delta$ K2 mutation selectively affects the transactivation capacity of Sox10 on a subset of its target genes and thus functions biochemically as a weak context-dependent transactivation domain, in contrast to the constitutive transactivation domain in the C-terminal region.

**Fig. 2. Targeted replacement of wild-type Sox10 by mutant Sox10 sequences in mice.** (A) Schematic showing, from top to bottom, the targeting construct for the Sox10  $\Delta$ K2 mutation, the *Sox10* wild-type locus and the mutant locus before and after Cre recombination. The *Sox10* exons (I–V) and the mutant *Sox10*  $\Delta$ K2 open reading frame (ORF) are shown as boxes, the 4.5 kb and 1.5 kb flanking regions as bars. Regions of homology between wild-type locus and targeting vector are depicted as thick black lines, introns III and IV as thin open boxes and surrounding genomic regions not contained in the targeting construct as dashed lines. 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 are the locations of the 5' and 3' probes and the start codon of the *Sox10* gene (ATG). The arrowheads indicate the locations of primers 1,2,3,4 used for quantitative RT-PCR. neo, neomycin resistance cassette; loxP, recognition sites for Cre recombinase; Tk, herpes simplex virus thymidine kinase gene cassette. (B) Schematic of the mutant locus for the Sox10 aa1 mutation. Recombination into the *Sox10* genomic locus was as depicted for the Sox10  $\Delta$ K2 construct. (C) Southern blot analysis of DNA from wild-type (wt) and heterozygous (+/aa1 and +/ $\Delta$ K2) ES cells digested with *Nco*I for use with the 5' probe, and with *Bam*HI/*Sca*I for the 3' probe. The size of bands corresponding to the wild-type (6.6 kb for the 5' probe and 4.6 kb for the 3' probe) and the targeted alleles (10.9 and 5.9 kb, respectively, for the 5' probe; 10.2 and 10.4 kb, respectively, for the 3' probe) are indicated. (D) PCR genotyping of wild-type (wt), heterozygous (+/ $\Delta$ K2 and +/aa1) and homozygous ( $\Delta$ K2/ $\Delta$ K2 and aa1/aa1) mouse embryos at 18.5 dpc. DNA fragments in the size marker (M) are 1.0 kb and 0.5 kb.





**Fig. 3. *Sox10* expression levels and early development of neural crest derivatives in *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* embryos.**

(A) Comparison of *Rpl8*-normalized *Sox10* expression levels in wild-type (set to 1, indicated by dashed line) and age-matched *Sox10<sup>aa1/aa1</sup>* and *Sox10<sup>ΔK2/ΔK2</sup>* mouse embryos at 10.5 and 11.5 dpc following quantitative RT-PCR with primer pairs 1,2 (white bars) and 3,4 (black bars). Experiments were repeated three times with material from two embryos for each genotype and age. (B) Immunoprecipitation of Sox10 from extracts of wild-type (wt) and age-matched *Sox10<sup>aa1/aa1</sup>* (*aa1/aa1*) embryos at 11.5 dpc. Immunoprecipitated material was detected by western blotting with anti-Sox10 rabbit antiserum. Sox10, Sox10-containing extract from transiently transfected Neuro2a cells. (C) Whole-mount in situ hybridization of wild-type (wt), *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* embryos at 10.5 dpc and 11.5 dpc using an antisense riboprobe directed against *Sox10*. V, trigeminal; VII/VIII, facial/acoustic; IX, glossopharyngeal; X, vagus; ot, otic vesicle.

To introduce the *aa1* and  $\Delta K2$  mutations into the *Sox10* genomic locus, all coding regions present in exons 3–5, as well as introns 3 and 4, were removed by homologous recombination and replaced by an uninterrupted *Sox10* reading frame carrying either the *aa1* or the  $\Delta K2$  mutation followed by a neo selection cassette (Fig. 2A,B). Before analysis, the neo selection cassette was removed by Cre-mediated recombination.

A similar strategy was previously employed to replace *Sox10* coding sequences by *lacZ* sequences (*Sox10<sup>lacZ</sup>*), *rtTA* sequences (*Sox10<sup>rtTA</sup>*) or *Sox8* coding sequences (*Sox10<sup>Sox8ski</sup>*) (Britsch et al., 2001; Kellerer et al., 2006; Ludwig et al., 2004b). These studies showed that no essential regulatory sequences were removed during recombination and that the inserted sequences were expressed in a *Sox10*-specific pattern throughout embryonic development. Detailed expression studies on the *Sox10<sup>Sox8ski</sup>* allele had also shown that expression levels after recombination do not differ significantly from expression levels of the wild-type allele (Kellerer et al., 2006). To ensure that expression levels of the *Sox10<sup>aa1</sup>* and *Sox10<sup>ΔK2</sup>* alleles were also comparable to that of the wild type, RNA was isolated from homozygous mutant and wild-type embryos at either 10.5 dpc or 11.5 dpc. *Sox10* expression levels were analyzed by quantitative RT-PCR with two different primer pairs (primers 1,2 and 3,4 in Fig. 2A) and found to be indistinguishable between wild-type and mutant genotypes (Fig. 3A). Immunoprecipitation from embryo extracts additionally revealed that wild-type Sox10 and *aa1* mutant protein

were present in similar amounts (Fig. 3B). Although the similar molecular mass of the  $\Delta K2$  mutant and the immunoglobulin heavy chains prevented an analysis of this mutant on the protein level, our results indicate that neither transcript nor protein amounts were significantly altered in the two *Sox10* mutants.

Additionally, it deserves to be mentioned that replacement of the wild-type *Sox10* allele, which has three coding exons, by one in which a continuous reading frame is present, leads to no obvious phenotypic abnormalities (S.S. and M.W., unpublished). The phenotypic changes observed in mice carrying the *Sox10<sup>aa1</sup>* or the *Sox10<sup>ΔK2</sup>* allele are therefore caused by the mutations in the Sox10 protein and not by inadvertently introduced changes in expression levels.

Mice with a single *Sox10<sup>aa1</sup>* or *Sox10<sup>ΔK2</sup>* allele were viable and fertile. In contrast to what has been observed in *Sox10<sup>+/lacZ</sup>* mice, so far we have not observed colonic aganglionosis in the heterozygotes. However, from the second generation onwards, some *Sox10<sup>+/aa1</sup>* mice developed a white belly spot on a C3H background, similar to that observed in *Sox10<sup>+/lacZ</sup>* mice, indicating that the Sox10 *aa1* mutation affects melanocyte development (data not shown).

Homozygous *Sox10<sup>aa1/aa1</sup>* and *Sox10<sup>ΔK2/ΔK2</sup>* mice were also born alive and at expected frequencies and did not exhibit the characteristic body posture of Sox10-deficient mice at birth (data not shown). Whole-mount in situ hybridization studies at 10.5 and 11.5 dpc confirmed that both alleles were expressed in pattern and



intensity indistinguishable from the wild type (Fig. 3C). This again confirms that transcript levels were not significantly altered, but also argues that early development of neural crest derivatives, especially cranial ganglia, dorsal root and sympathetic ganglia, was normal. *Sox10<sup>aa1/aa1</sup>* and *Sox10<sup>ΔK2/ΔK2</sup>* mice thus differ from *Sox10<sup>lacZ/lacZ</sup>* mice, which at this age already show severely hypoplastic cranial ganglia as well as the first signs of degeneration in dorsal root ganglia (DRG) (Britsch et al., 2001; Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998).

Although born at expected frequencies, all *Sox10<sup>ΔK2/ΔK2</sup>* mice and many *Sox10<sup>aa1/aa1</sup>* died within the first hours after birth. Some *Sox10<sup>aa1/aa1</sup>* mice, however, survived for up to 3 days. These *Sox10<sup>aa1/aa1</sup>* survivors failed to thrive and soon exhibited the typical signs of a megacolon. We conclude that both alleles are hypomorphic.

### Analysis of the ENS in *Sox10<sup>aa1</sup>* and *Sox10<sup>ΔK2</sup>* mice

Because of the obvious gastrointestinal innervation defects in *Sox10<sup>aa1/aa1</sup>* mice, we first studied the ENS in both *Sox10* mouse mutants. At 18.5 dpc in the wild type, the gastrointestinal tract was completely colonized by enteric neural crest cells and cytodifferentiation had progressed significantly. As a consequence, enteric neurons (marked by PGP9.5 or NOS, also known as Uchl1 and Nos1, respectively – Mouse Genome Informatics) as well as enteric glia (marked by B-FABP, also known as Fabp7) were present in high numbers in the stomach and in all parts of the small and the large intestine, such as duodenum and caecum. Because all markers yielded similar results, only those for NOS-positive neurons obtained by NADPH diaphorase staining are shown (Fig. 4).

Whereas *Sox10*-deficient embryos lack an ENS throughout the complete gastrointestinal tract (Herbarth et al., 1998; Kapur, 1999; Southard-Smith et al., 1998), the stomach wall of *Sox10<sup>ΔK2/ΔK2</sup>* embryos contained a fully developed ENS at 18.5 dpc (Fig. 4A). Their large intestine was, however, completely devoid of enteric neural crest, enteric neurons or glia (Fig. 4D). Colonization of the gut by ENS cells had stopped in this mouse mutant in the proximal region of the small intestine (Fig. 4C). The region immediately adjacent to the stomach was little affected (Fig. 4B), and although the exact limit of colonization varied between individual embryos of this genotype, it never went beyond the duodenum. By comparison, *Sox10<sup>aa1/aa1</sup>* embryos exhibited a more severe phenotype. Already the stomach wall contained fewer neurons and glia than either the age-matched wild-type or *Sox10<sup>ΔK2/ΔK2</sup>* embryos (Fig. 4A). Hardly any cells with characteristics of enteric neural crest, neurons or glia could be detected in small and large intestine (Fig. 4B-D). Both the *aa1* and *ΔK2* mutations thus affect ENS development, but with different severity.

### Analysis of melanocyte development in *Sox10<sup>aa1</sup>* and *Sox10<sup>ΔK2</sup>* mice

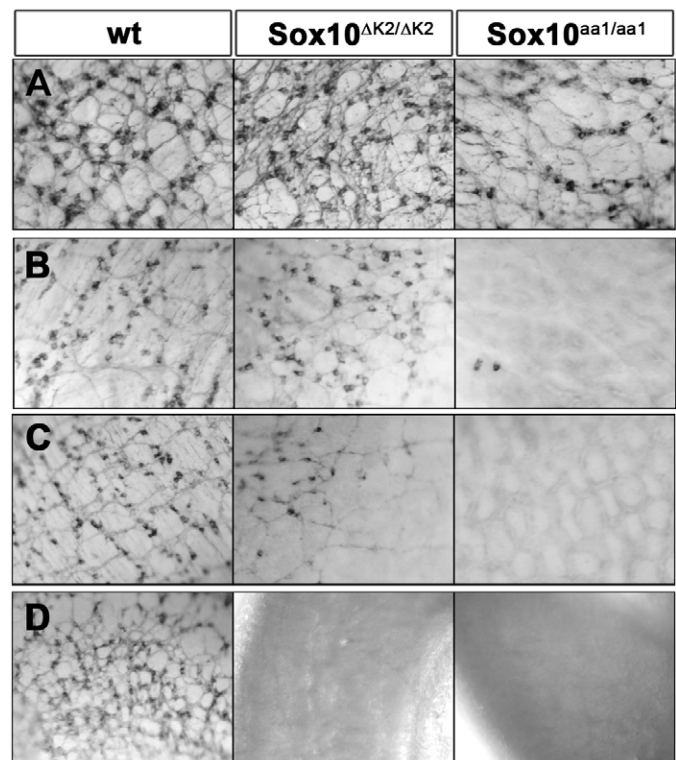
The observation of a white belly spot in some *Sox10<sup>+/aa1</sup>* mice indicated that melanocyte development is disturbed in the *Sox10 aa1* mouse mutant. To confirm this and extend the analysis to the *Sox10 ΔK2* mutant, we investigated melanocyte marker gene expression by whole-mount in situ hybridization in homozygous mutant embryos at 12.5 dpc and compared it with wild-type littermates. Whereas streams of migrating melanoblasts were detected around the eye and in the hindlimb region of wild-type embryos, melanoblast numbers were strongly reduced in both mouse mutants, as assessed using either *Dct* or *kit* oncogene (*c-Kit*) as a marker (Fig. 5A). Quantification in the hindlimb region of *Sox10<sup>aa1/aa1</sup>* mice revealed that *Dct*-positive cells were nearly absent and *c-Kit*-positive

cells were reduced by two-thirds (Fig. 5C). Taking into account that *c-Kit*, in contrast to *Dct*, also labels cell types other than melanoblasts and that the number of residual *c-Kit*-positive cells was comparable to that in *Sox10*-deficient mice, which lack melanoblasts altogether (Britsch et al., 2001; Southard-Smith et al., 1998), the melanocyte lineage is likely to be absent in *Sox10<sup>aa1/aa1</sup>* mice. This was also corroborated by analysis of the skin of the few *Sox10<sup>aa1/aa1</sup>* survivors at postnatal day 3 (data not shown).

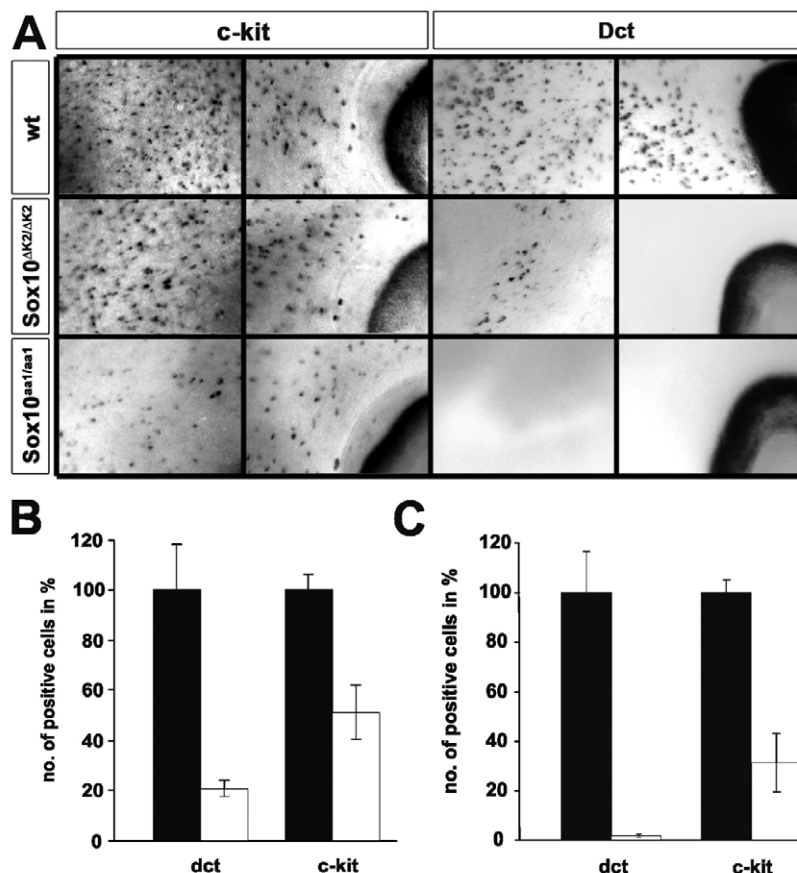
*Sox10<sup>ΔK2/ΔK2</sup>* embryos, by contrast, had melanoblasts at 12.5 dpc (Fig. 5A), although their number corresponded to only 20% of wild-type levels as determined by in situ hybridization for *Dct* (Fig. 5B). *c-Kit* in situ hybridization also revealed a strong reduction, by approximately 50%. This and the absence of a belly spot in adult *Sox10<sup>+/ΔK2</sup>* mice indicate that although both the dimerization domain and the K2 domain participate in melanocyte development, an intact dimerization function appears to be the more important.

### Analysis of the peripheral nervous system in *Sox10<sup>aa1</sup>* and *Sox10<sup>ΔK2</sup>* mice

*Sox10*-deficient mice also lack glial cells throughout the peripheral nervous system (PNS), and this secondarily leads to nerve defasciculation and degeneration of DRG neurons (Britsch et al., 2001). To study the importance of dimerization and the K2 domain for these processes, we analyzed PNS development in *Sox10<sup>aa1/aa1</sup>* and *Sox10<sup>ΔK2/ΔK2</sup>* mice.



**Fig. 4. Analysis of the ENS in *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* embryos.** Stomach (A), duodenum (B,C) and caecum (D) of wild-type (wt), *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* mouse embryos were analyzed at 18.5 dpc by NADPH diaphorase staining. For the duodenum, a region immediately adjacent to the stomach (B) is shown as well as a more distal region (C).



**Fig. 5. Melanocyte development in *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* embryos.** (A) Whole-mount in situ hybridizations were performed on wild-type, *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* mouse embryos at 12.5 dpc using antisense riboprobes against *Dct* and *c-Kit*. (B,C) *Dct*-positive and *c-Kit*-positive cells were quantified in the hindlimb region of wild-type embryos (black bars) and their *Sox10<sup>ΔK2/ΔK2</sup>* (B) or *Sox10<sup>aa1/aa1</sup>* (C) littermates (white bars). Three embryos were counted for each genotype. The number of cells counted in the wild type was set to 100%. Cell numbers for the *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* embryos are presented relative to the wild type as mean±s.d. Differences were statistically significant between wild type and each mutant as well as between the two mutants as determined by Student's *t*-test ( $P \leq 0.001$ ).

Comparison of the DRG in *Sox10<sup>aa1/aa1</sup>* and *Sox10<sup>ΔK2/ΔK2</sup>* embryos with wild-type embryos at 11.5 and 12.5 dpc did not reveal any obvious alterations in the shape or size of DRG. NeuN-positive neurons (NeuN is also known as Neuna60 – Mouse Genome Informatics) and B-FABP-positive glia were present in similar numbers in the two mutants and wild type (Fig. 6). There was also no obvious difference in the number of cells expressing wild-type and mutant Sox10 proteins.

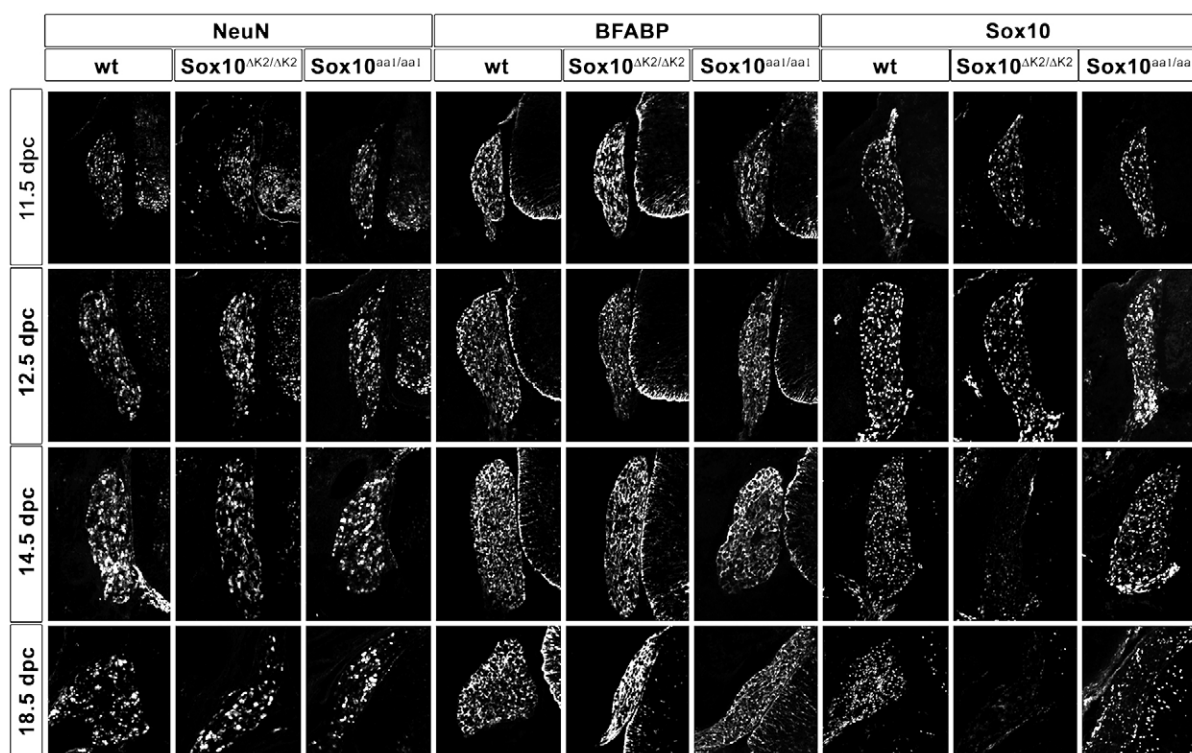
Ganglia still appeared normal in their morphology in both mutants at 14.5 dpc and expressed NeuN as well as B-FABP. However, the mutant Sox10 protein had almost completely disappeared in the DRG of *Sox10<sup>ΔK2/ΔK2</sup>* embryos (Fig. 6), which is likely to be due to reduced expression levels as the abundance of *Sox10* transcripts was also severely reduced (data not shown). By contrast, other Sox10-expressing tissues continued to express the mutant Sox10 protein in the *Sox10<sup>ΔK2/ΔK2</sup>* embryos. The loss of Sox10 expression led to a substantial increase in apoptosis in DRG of *Sox10<sup>ΔK2/ΔK2</sup>* mice at 14.5 dpc that not only affected the normally Sox10-expressing satellite glia but also the sensory neurons (data not shown). As a consequence, DRG were severely reduced in size at 18.5 dpc in *Sox10<sup>ΔK2/ΔK2</sup>* embryos. *Sox10<sup>aa1/aa1</sup>* embryos continued to express the mutant Sox10 protein in their DRG. In size and marker gene expression, DRG in *Sox10<sup>aa1/aa1</sup>* embryos were not affected even at 18.5 dpc. Only the slight changes in DRG shape indicated that minor defects also existed in this genotype. In summary, these results indicate that generation of DRG is dependent neither on an intact dimerization function, nor on the presence of the K2 domain. Further development and maintenance of DRG on the other hand appears to specifically require the activity of the K2 domain of Sox10. At those times when the defect became

apparent, Sox10 is predominantly expressed in satellite glia of DRG, arguing that the K2 domain might be particularly important in this cell type.

We also analyzed development of Schwann cells in *Sox10<sup>aa1/aa1</sup>* and *Sox10<sup>ΔK2/ΔK2</sup>* mice as the second main type of glial cell in the PNS. Schwann cells along peripheral nerves, visualized by immunohistochemistry for NF165 (also known as Nefm – Mouse Genome Informatics), contained Sox10 protein at all times of embryonic development in both mutants and appeared similar to the wild type (Fig. 7 and data not shown). At 18.5 dpc, many of the Schwann cells along wild-type spinal nerves had entered the promyelinating stage in which they express the transcription factor Oct6 (also known as Pou3f1 – Mouse Genome Informatics) (Bermingham et al., 1996; Jaegle et al., 1996). Oct6 expression was present in spinal nerves of *Sox10<sup>ΔK2/ΔK2</sup>* embryos at 18.5 dpc. Peripheral nerves from age-matched *Sox10<sup>aa1/aa1</sup>* embryos, by contrast, lacked significant Oct6 expression (Fig. 7). Instead, Schwann cells along the nerve of *Sox10<sup>aa1/aa1</sup>* embryos continued to express the SoxB1 transcription factor Sox2 which, in the majority of wild-type Schwann cells, is already downregulated at this age and is a marker for immature Schwann cells (Le et al., 2005). Schwann cells in *Sox10<sup>aa1/aa1</sup>* mice thus remained in the immature stage and did not enter the promyelinating stage. As a consequence, expression of the peripheral myelin genes *Mbp* and *Mpz* was not detected along the nerves of *Sox10<sup>aa1/aa1</sup>* mice at 18.5 dpc, in contrast to the wild type (Fig. 7).

Although Schwann cells from *Sox10<sup>ΔK2/ΔK2</sup>* mice expressed Oct6 and were thus likely to have entered the promyelinating stage, they continued to express Sox2 and therefore also behaved





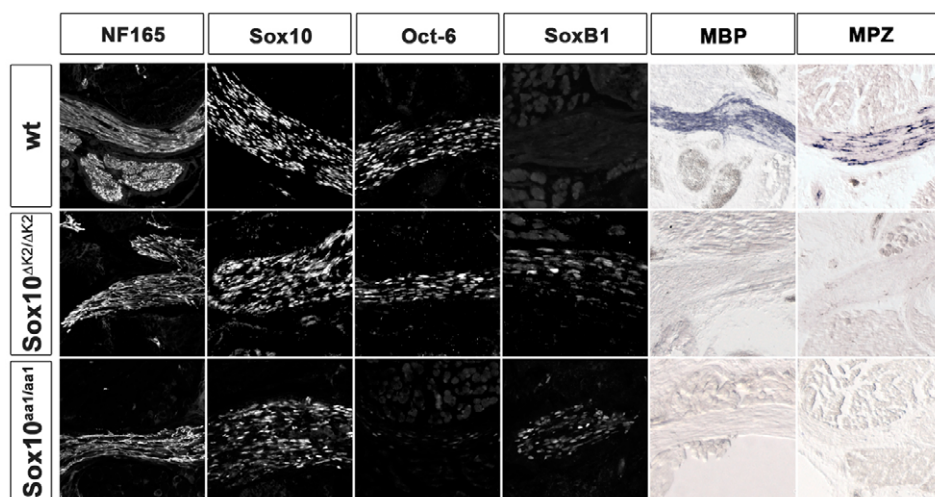
**Fig. 6. Analysis of DRG in *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> embryos.** Immunohistochemistry was carried out on transverse sections through DRG of wild-type, *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> mouse embryos at 11.5, 12.5, 14.5 and 18.5 dpc using antibodies against the neuronal marker NeuN, the glial marker B-FABP and against Sox10. The spinal cord is to the right of the dorsal root ganglion.

aberrantly (Fig. 7). Similar to Schwann cells from *Sox10*<sup>aa1/aa1</sup> mice, Schwann cells from *Sox10*<sup>ΔK2/ΔK2</sup> mice did not express myelin genes indicating that they also did not reach the myelinating stage.

In contrast to DRG and peripheral nerves, sympathetic ganglia contain comparatively few glial cells. Neither at 12.5 dpc nor at 18.5 dpc did we detect significant changes in the development of rostral sympathetic chain ganglia of *Sox10*<sup>aa1/aa1</sup> and *Sox10*<sup>ΔK2/ΔK2</sup> embryos. Immunohistochemistry with antibodies directed against Sox10, Phox2b and tyrosine hydroxylase (Th) yielded numbers of positive cells and signal intensities in the mutants that were comparable to the wild type (Fig. 8 and data not shown).

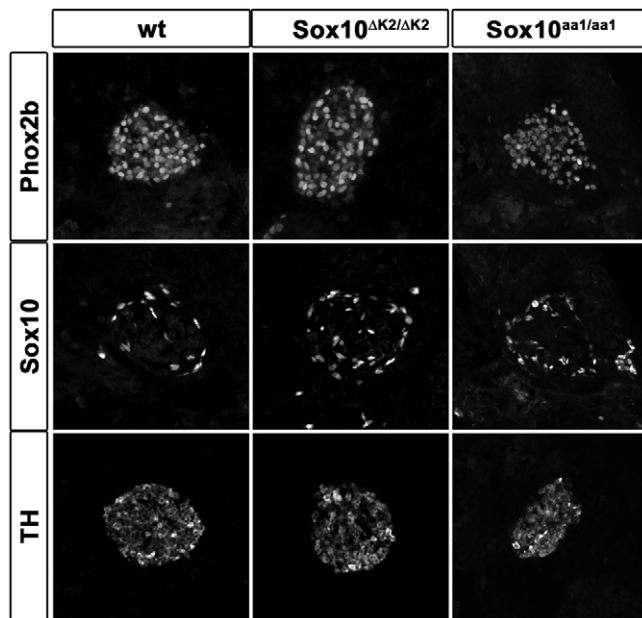
### Analysis of oligodendrocyte development in *Sox10*<sup>aa1</sup> and *Sox10*<sup>ΔK2</sup> mice

Finally, we analyzed oligodendrocyte development in the spinal cord of both mouse mutants. Because the mutant Sox10 proteins were expressed throughout oligodendrocyte development at levels comparable to the wild type (data not shown), we used Sox10 expression to follow oligodendrocyte development. Oligodendrocyte precursors were specified in the correct spatiotemporal pattern at 12.5 dpc in the ventral part of the spinal cord of both mouse mutants (Fig. 9A). In the following days, oligodendrocyte precursors spread throughout the mantle zone of the spinal cord and finally started to accumulate in the marginal zone



**Fig. 7. Peripheral nerve development in *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> embryos.**

Immunohistological analysis of spinal nerves from wild-type, *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> mouse embryos at 18.5 dpc was carried out using antibodies against NF165, Sox10, Oct6 and SoxB1 (*Sox10*<sup>ΔK2/ΔK2</sup>). Expression of *Mbp* and *Mpz* was additionally visualized in all three genotypes by in situ hybridization with specific antisense probes.



**Fig. 8. Development of sympathetic ganglia in *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> embryos.** Immunohistochemical staining of sympathetic ganglia was performed on wild-type, *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> mouse embryos at 12.5 dpc using antibodies against Phox2b, Sox10 and Th.

at 18.5 dpc in preparation for terminal differentiation. Both migration pattern and marginal zone accumulation were normal in *Sox10*<sup>aa1/aa1</sup> and *Sox10*<sup>ΔK2/ΔK2</sup> embryos, arguing that there is no defect in oligodendrocyte precursors. Comparing Olig2 expression in control and mutant mice, we also detected no changes in the distribution or number of oligodendroglial cells (Fig. 9B and data not shown).

In situ hybridization with antisense riboprobes for the myelin genes *Mbp* and *Plp* (also known as *Plp1* – Mouse Genome Informatics) at 18.5 dpc revealed that in contrast to *Sox10*-deficient embryos (Stolt et al., 2002), terminal differentiation took place at significant rates in both *Sox10*<sup>aa1/aa1</sup> and *Sox10*<sup>ΔK2/ΔK2</sup> embryos (Fig. 10A). Quantification revealed a slight reduction, to ~60–70% of wild-type levels, in the number of myelinating oligodendrocytes in *Sox10*<sup>ΔK2/ΔK2</sup> embryos (Fig. 10B). By contrast,

terminal oligodendrocyte differentiation and myelination proceeded at wild-type rates in *Sox10*<sup>aa1/aa1</sup> embryos (Fig. 10C). Oligodendrocyte development is thus surprisingly little affected in the two mutants.

## DISCUSSION

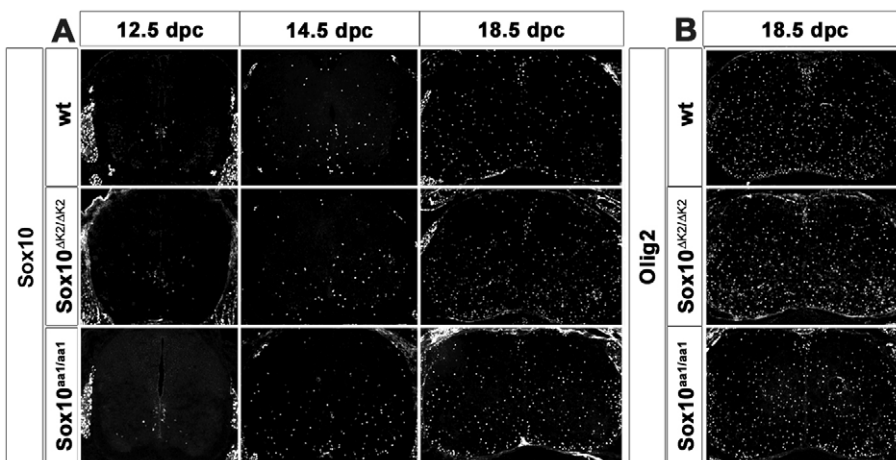
The importance of the DNA-binding and transactivation domains for a transcription factor is almost self-evident and has been confirmed for Sox10 in vitro (for a review, see Wegner, 2005) and by the existence of mutations in human patients that either lead to a loss of DNA-binding owing to amino acid changes in the HMG-domain, or to a loss of the constitutive C-terminal transactivation domain (see Inoue et al., 2004).

Transcription factors also contain regions with more subtle functions. For Sox10, these include a domain that is in direct apposition to the beginning of the HMG-domain and which has been found in vitro to mediate DNA-dependent dimerization (Peirano et al., 2000; Peirano and Wegner, 2000). There is also a region in the middle of the protein that is fully conserved between Sox10 and its relatives Sox8 and Sox9, and which has transactivation potential in Sox8 (Schepers et al., 2000; Wegner, 1999).

Here, we replaced wild-type Sox10 in the mouse by mutant versions that interfered with the function of these domains. For the aa1 variant, three consecutive amino acid residues were substituted. This had previously been shown to interfere with DNA-dependent dimerization (Schlierf et al., 2002). In the ΔK2 mutant, the complete conserved region was removed. Although we replaced the three coding exons of *Sox10* by a single continuous open reading frame during the process, expression rates were not significantly altered. The defects observed in mice homozygous for the *Sox10*<sup>aa1</sup> or the *Sox10*<sup>ΔK2</sup> allele are therefore primarily due to the changes in the Sox10 sequence.

Compared with a mouse with Sox10 deficiency, *Sox10*<sup>aa1/aa1</sup> and *Sox10*<sup>ΔK2/ΔK2</sup> mice have a milder hypomorphic phenotype. Furthermore, there are significant differences between the two mouse mutants and these differences, as well as the phenotypic similarities between them, offer interesting insights into the function of Sox10.

We detected no change in neural crest formation and emigration in either mouse mutant, indicating that early neural crest development is not particularly sensitive to either mutation. If we take into account that Sox8 can replace Sox10 during this phase in the mouse (Kellerer et al., 2006) and in chicken (Cheung and Briscoe, 2003), neither process appears to require the presence of a



**Fig. 9. Development of oligodendrocyte precursors in *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> embryos.** Specification and subsequent distribution of oligodendrocyte precursors throughout the spinal cord of wild-type, *Sox10*<sup>ΔK2/ΔK2</sup> and *Sox10*<sup>aa1/aa1</sup> mouse embryos was analyzed on transverse sections from the forelimb region at 12.5, 14.5 and 18.5 dpc by immunohistochemistry with antibodies directed against Sox10 (A) and Olig2 (B).



particular SoxE protein. Accordingly, the temporal pattern with which SoxE proteins are switched on and function during early neural crest development varies among vertebrate species (O'Donnell et al., 2006).

Melanocyte and ENS development, on the other hand, are significantly affected in both mouse mutants. Because these two processes were also defective in *Sox10<sup>Sox8ki/Sox8ki</sup>* mice, they appear to be exquisitely sensitive to changes in Sox10 and reliant on an intact Sox10 protein. Such an assumption is also supported by the fact that the vast majority of heterozygous *SOX10* mutations identified to date in human patients lead to defects in melanocyte and ENS development and manifest as a combination of Waardenburg syndrome and Hirschsprung disease (Inoue et al., 2004; Pingault et al., 1998).

ENS and melanocyte development were more affected in the *Sox10<sup>aa1/aa1</sup>* than in the *Sox10<sup>ΔK2/ΔK2</sup>* mutant, pointing to clear differences between the two hypomorphic alleles. In fact, the melanocyte defect in the *Sox10<sup>aa1/aa1</sup>* mutant was comparable to the Sox10-deficient situation, arguing that DNA-dependent dimerization plays an essential role during melanocyte development.

Two target genes, *Mitf* and *Dct*, have so far been identified for Sox10 in the melanocyte lineage (Bondurand et al., 2000; Britsch et al., 2001; Lee et al., 2000; Potterf et al., 2000; Potterf et al., 2001; Verastegui et al., 2000). In zebrafish, Sox10 function in melanocytes is mainly mediated through *mitf* activation (Elworthy et al., 2003). Taking into account that the *Mitf* promoter, in contrast to the *Dct* promoter, does not contain dimeric Sox10 binding sites (Bondurand et al., 2000; Ludwig et al., 2004a), a strong effect on melanocyte development was not necessarily expected for the *Sox10<sup>aa1/aa1</sup>* mutant.

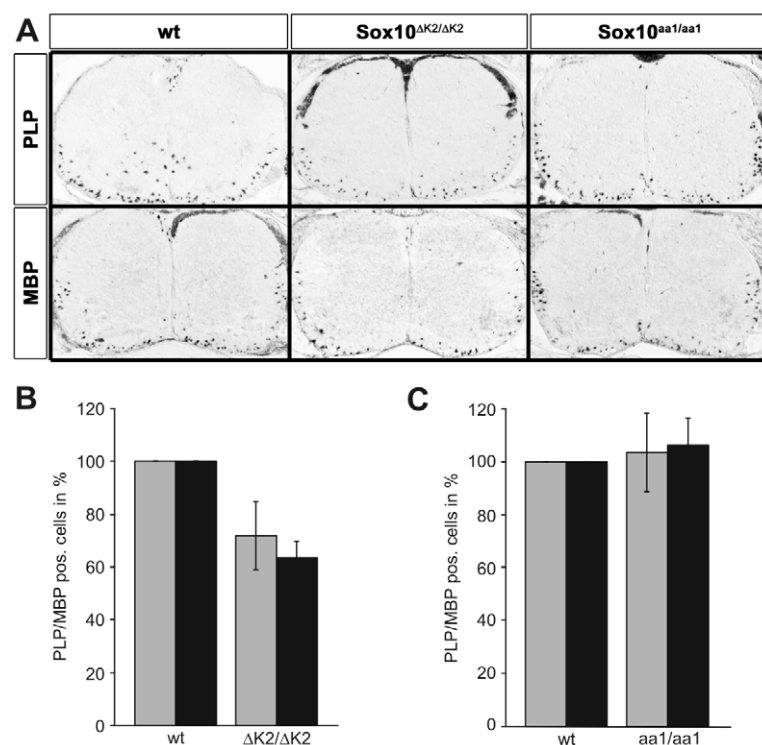
Several possibilities exist to explain the severity of the melanocyte defect in the *Sox10<sup>aa1/aa1</sup>* mutant. In addition to the *Mitf* promoter, Sox10 might require additional regulatory regions to activate *Mitf* expression in vivo and the activity of these regulatory regions might depend on dimeric Sox10 binding. The existence of a distal enhancer

with Sox10-binding capability has indeed been reported for the *Mitf* gene (Watanabe et al., 2002). Alternatively, and in contrast to zebrafish, Sox10 might also work through pathways other than *Mitf* activation in mouse melanocytes. Evidence for such interspecies differences exist (Hou et al., 2006). Instead of *Mitf*, additional melanocytic target genes might then require dimeric Sox10 binding. Finally, it cannot be ruled out that the amino acid substitutions in the aa1 mutant affect other as yet unidentified functions in addition to DNA-dependent dimerization.

Compared with the defects in ENS and melanocyte development, developmental alterations in peripheral ganglia and nerves had a later onset and were milder. Interestingly, they were more pronounced in both *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* mutants than in the *Sox10<sup>Sox8ki/Sox8ki</sup>* mutant, arguing that a fully functional Sox8 protein with intact DNA-dependent dimerization function and K2 domain is better able to substitute for Sox10 in PNS development than the Sox10 mutants. As the PNS defects in the *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* embryos appeared no earlier than 14.5 dpc, they also uncover novel functions for Sox10 in peripheral glia that were impossible to see in the Sox10-deficient mutant, in which peripheral glia are not specified and thus completely missing (Britsch et al., 2001).

In *Sox10<sup>ΔK2/ΔK2</sup>* embryos, DRG develop normally and first contain a near normal complement of neurons and glial cells. However, from 14.5 dpc onwards, Sox10 expression is extinguished. Despite the fact that cells specified to a glial fate remain detectable for some time after the loss of Sox10 expression, DRG begin to degenerate and lose glial cells as well as neurons.

Taking into account that the stability of the  $\Delta K2$  protein was similar to that of wild-type Sox10 in tissue culture and that  $\Delta K2$  transcripts in DRG were also reduced, we assume that Sox10 expression is selectively downregulated in DRG of *Sox10<sup>ΔK2/ΔK2</sup>* embryos. The K2 domain might in fact be needed to maintain Sox10 expression in DRG glia by a positive autoregulatory feedback mechanism.



**Fig. 10. Terminal differentiation of oligodendrocytes in *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* embryos.** (A) In situ hybridizations with probes specific for *Plp* and *Mbp* were performed on transverse spinal cord sections from the forelimb region of wild-type, *Sox10<sup>ΔK2/ΔK2</sup>* and *Sox10<sup>aa1/aa1</sup>* mouse embryos at 18.5 dpc. (B) *Plp*-positive (black bars) and *Mbp*-positive (gray bars) cells were counted on at least 20 sections from three independent embryos for wild-type and *Sox10<sup>ΔK2/ΔK2</sup>* spinal cords. The number of cells counted in the wild type was set to 100%. Cell numbers for the *Sox10<sup>ΔK2/ΔK2</sup>* are presented relative to the wild type as mean ± s.d. Statistically significant differences were observed using Student's *t*-test for *Plp* ( $P \leq 0.001$ ) and *Mbp* ( $P \leq 0.01$ ). (C) Similar quantifications for *Sox10<sup>aa1/aa1</sup>*. No statistical difference in the number of *Plp*- and *Mbp*-positive cells was detected compared with the wild type.

Because of the dearth of specific markers for glial cells in the DRG, it is impossible at present to exactly define at which stage after specification satellite glia development is disrupted in the *Sox10<sup>ΔK2/ΔK2</sup>* mutant. It nevertheless shows that under normal conditions, Sox10 is not only important during the specification event, but also has additional roles during later phases of differentiation and maturation of satellite glia. Considering the preferential expression of Sox10 in glia rather than DRG neurons, the simplest explanation for the observed phenotype in the *Sox10<sup>ΔK2/ΔK2</sup>* mutants would thus be a primary failure of satellite glia to differentiate and to produce the signaling molecules needed for neuronal survival. According to this model, the neuronal loss would be secondary to the glial defect. Interestingly, DRG are one of the few tissues in which loss of the K2 domain has a stronger effect than loss of the DNA-dependent dimerization function.

Glial development was also disturbed along peripheral nerves. However, no significant loss of Schwann cells was observed in either mouse mutant and the mutant Sox10 proteins remained strongly expressed throughout embryonic development. The glial cells along the nerve also developed normally into Sox2-expressing immature Schwann cells. In the case of the *Sox10<sup>aa1/aa1</sup>* mutant, Schwann cells failed to enter the promyelinating stage and did not turn on Oct6 expression. Schwann cells in the *Sox10<sup>ΔK2/ΔK2</sup>* mutant, by contrast, progressed into an Oct6-positive stage, but then also failed to turn on myelin genes. Our analysis therefore also proves for the first time that a fully functional Sox10 is needed for the final two stages of the development of myelinating Schwann cells. This is consistent with biochemical evidence that Sox10 directly activates expression of Krox20 (also known as Egr2 – Mouse Genome Informatics) as the master regulator of peripheral myelination and of several peripheral myelin genes (Bondurand et al., 2001; Ghislain et al., 2002; Peirano et al., 2000). However, our study provides the first genetic evidence and points to the fact that the peripheral neuropathy observed in some patients with heterozygous *SOX10* mutations might be due to defective terminal differentiation of Schwann cells rather than to early Schwann cell specification defects (Inoue et al., 2004). The fact that the two Sox10 mutations affect development of Schwann cells and satellite glia differently furthermore corroborates the distinct nature of these two glial populations in the PNS.

It is also interesting that Schwann cell development is more sensitive to alterations to the functional domains of Sox10 than to an exchange of Sox10 for the related Sox8 (Kellerer et al., 2006). This observation on Schwann cells furthermore contrasts with that on oligodendrocytes, which are more severely affected in the *Sox10<sup>Sox8ki/Sox8ki</sup>* mutant than in either the *Sox10<sup>ΔK2/ΔK2</sup>* or the *Sox10<sup>aa1/aa1</sup>* mutant. Surprisingly, the onset of terminal oligodendrocyte differentiation even appears to be completely normal in the *Sox10<sup>aa1/aa1</sup>* mutant.

Our study has yielded important information regarding the cell types and developmental steps at which DNA-dependent dimerization or the K2 domain becomes relevant for Sox10 function. The differential effect of the  $\Delta K2$  mutation on several tissues furthermore supports the data from biochemical studies that define this region as a promoter-specific transactivation domain. In future experiments, it will be interesting to determine which Sox10 target genes might explain the specific defects in the *Sox10<sup>aa1/aa1</sup>* mutant, and identify the Sox10 interaction partners that mediate K2 domain function in select tissues.

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