Cellular/Molecular

Desert Hedgehog Links Transcription Factor Sox10 to Perineurial Development

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Schwann cells are the main glial cell type in the PNS. They develop along nerves during embryogenesis and rely on the HMG domain containing Sox10 transcription factor for specification, lineage progression, and terminal differentiation. *Sox10* deletion in immature Schwann cells caused peripheral nerve defects in mice that were not restricted to this glial cell type, although expression in the nerve and gene loss were. Formation of the perineurium as the protecting sheath was, for instance, heavily compromised. This resembled the defect observed after loss of *Desert hedgehog* (*Dhh*) in mice. Here we show that Sox10 activates *Dhh* expression in Schwann cells via an enhancer that is located in intron 1 of the *Dhh* gene. Sox10 binds this enhancer in monomeric form via several sites. Mutation of these sites abolishes both Schwann-cell-specific activity and Sox10 responsiveness *in vitro* and in transgenic mouse embryos. This argues that Sox10 activates *Dhh* expression by direct binding to the enhancer and by increasing Dhh levels promotes formation of the perineurial sheath. This represents the first mechanism for a non-cell-autonomous function of Sox10 during peripheral nerve development.

Introduction

Sox10 regulates several aspects of vertebrate nervous system development (Stolt and Wegner, 2010). In the CNS, it is selectively expressed in oligodendrocyte lineage cells in which it affects several stages of development and is indispensable for terminal differentiation and myelination (Stolt et al., 2002; Finzsch et al., 2008). In the forming PNS, Sox10 influences the generation of several cell types, including sympathetic and sensory neurons (Kim et al., 2003; Carney et al., 2006; Reiprich et al., 2008). Even more important is its requirement for the specification of all glia, including Schwann cell precursors along nerves (Britsch et al., 2001; Paratore et al., 2001).

In Schwann cells, Sox10 is expressed throughout development (Kuhlbrodt et al., 1998a). After its role in specification (Britsch et al., 2001), it continues to be functional as Schwann cell precursors develop first into immature Schwann cells and then via promyelinating to myelinating Schwann cells. This lasting requirement has been confirmed by the analyses of mice with hypomorphic *Sox10* alleles and mice in which a loxP-flanked *Sox10* allele was deleted during specific stages of Schwann cell development by Cre recombinase (Schreiner et al., 2007; Finzsch et al., 2010; Bremer et al., 2011; Fröb et al., 2012). Although the essential role of Sox10 for Schwann cell development is evident, much remains to be learned about its mode of action. One of the key issues is the identification of stage-specific Sox10 target genes that are themselves important for peripheral nerve development.

Sox10 activates the expression of peripheral myelin genes during terminal differentiation, including *Mag*, *Mbp*, *Mpz*, *Pmp22*, and Connexin 32 (Peirano et al., 2000; Bondurand et al., 2001; Jones et al., 2007, 2011). Sox10 is also directly involved in the activation of *Krox20*, which is a key activator of peripheral myelin genes (Ghislain and Charnay, 2006; Reiprich et al., 2010). Available evidence suggests that Sox10 first activates and then cooperates with Krox20 to activate the myelination program (Svaren and Meijer, 2008).

Two other Sox10 targets are the genes for the POU domain transcription factor Oct6 and the receptor tyrosine kinase subunit ErbB3 (Jagalur et al., 2011; Prasad et al., 2011). Oct6 is required for Schwann cells to progress to the pro-myelinating stage and to prepare for terminal differentiation (Bermingham et al., 1996; Jaegle et al., 1996), whereas ErbB3 acts as part of the ErbB2/ErbB3 heterodimer that allows Schwann cells to respond in various phases of their development to neuregulin 1 (Newbern and Birchmeier, 2010). Although these known targets help to explain several Sox10 functions, they are by no means sufficient to fully understand the mechanisms by which Sox10 influences peripheral nerve development.

We have discovered recently that Sox10 deletion in immature Schwann cells also led to a dramatic decrease in *Dhh* levels (Finzsch et al., 2010). Here we show that *Dhh* is also a direct target gene of Sox10 in Schwann cells and that, through its influence on *Dhh*, Sox10 exerts a non-cell-autonomous effect on perineurial cells and the formation of the perineurium.

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Materials and Methods

Plasmids. The Dhh genomic region analyzed in this study was located between positions 98720506 and 98739191 of mouse chromosome 15 (mouse genome version mm9). From this region, fragments were obtained by PCR or using restriction nucleases, as indicated in Figures 1*A*, 2*A*, and 3*A*, and inserted into reporter plasmids. For autofluorescence-based reporter gene studies, *pTATA–tdTomato* was used. It carried the red fluorescing GFP derivative *tdTomato* under control of the β-globin minimal promoter. For luminescence-based reporter gene assays, *Dhh–luc* was used. This plasmid is based on *pGL2basic* (Promega) and contained the firefly luciferase gene downstream of the *Dhh* minimal promoter (positions −277 to +31 relative to the transcriptional start site). In the context of the *E4–Dhh–luc* construct (see Fig. 3*A*), potential Sox10 binding sites were mutated using the QuickChange XL Site-Directed Mutagenesis kit (Stratagene). The eukaryotic *pCMV5*-based expression plasmids for full-length Sox10 (*pCMV5–Sox10*) and the short MIC version of Sox10 (*pCMV5–Sox10 MIC*) have been described as well as the *pCAGGS–Sox10–IRES–nls–GFP* plasmid in which *Sox10* and *GFP* reading frames are under control of the chicken β-actin promoter (Kuhlbrodt et al., 1998b; Cossais et al., 2010). For knockdown experiments, *pSuper–Neo–GFP* plasmids (Oligoengine) were used that either expressed a *Sox10*-specific shRNA (targeted region, 5′-CTGCTGTTCC TTCTTGACCT TGCCC-3′) or a control shRNA. *E4–lacZ* transgene constructs contained the *E4* fragment in wild-type or mutant version (see Fig. 7*C*) from the *Dhh* locus in front of the *Dhh* minimal promoter followed by a *lacZ* reporter gene and poly(A) cassette.

Cell culture, transient transfection, extract preparation, electrophoretic mobility shift assays, and reporter gene assays. HEK293 cells were maintained in DMEM containing 10% fetal calf serum (FCS) and transfected by the polyethylenimine technique using 10 μg of pCMV5-based expression plasmid per 100 mm plate. At 48 h after transfection, cells were harvested for extract preparation (Schlierf et al., 2002). After verification of ectopic expression by Western blotting, electrophoretic mobility shift analyses (EMSAs) were performed (Küspert et al., 2011) using ^{32}P-labeled 25 bp double-stranded oligonucleotides containing putative Sox10 binding sites.

For reporter gene assays, rat S16 Schwann cells were maintained in DMEM containing 10% FCS and transfected with SuperFect Transfection Reagent (Qiagen) on 24-well tissue culture plates. For autofluorescence-based assays, cells were transfected with 500 ng of *pTATA–tdTomato*-based reporter plasmids and scored for fluorescence 48 h after transfection using a Leica inverted microscope (DMIRB) equipped with a cooled MicroMax CCD camera (Princeton Instruments). For luminescence-based assays, 500 ng of *Dhh–luc*-based reporter plasmids were used in the presence of 100 ng of pCMV5- or pSuper–Neo–GFP-based expression vectors. Cells were generally harvested 48 h after transfection, except for knockdown experiments in which analysis took place 72 h after transfection. Luciferase activity was determined in the presence of luciferin substrate by detection of chemiluminescence.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) assays were performed on S16 cells as described previously (Schlierf et al., 2006). After crosslinking proteins to DNA in the presence of 1% formaldehyde, chromatin was prepared and sheared to an average fragment length of 200–500 bp using a Sonoplus HD2070 homogenizer (Bandelin). Immunoprecipitations were performed overnight at 4°C using guinea pig antiserum against Sox10 (1:400 dilution) (Maka et al., 2005) as well as guinea pig preimmune serum and protein A Sepharose beads. Quantitative PCR was performed on input and precipitated chromatin after crosslink reversal and purification. The following primer pairs were used at an annealing temperature of 63°C: 5′-GCAGCCAAGA TAACTGTGGC-3′ and 5′-GCAGTACAATGGCCATTCTC-3′ for *E4a*; 5′-CAATGCCCAGTGCCAGGGAAG-3′ and 5′-CTCCCAGCGTTTGG GAGTCG-3′ for *E4b*; 5′-GGCAGAGAGCTGGGATTGTC-3′ and 5′-CC AGGGTTGGCCTATACACG-3′ for Con1; and 5′-CAATTGACATAT GCCAGCCC-3′ and 5′-GATCACACATCTAAGGCCTC-3′ for Con2.

Generation and analysis of transgenic animals. Transgenic mouse embryos were generated by microinjecting the *E4–lacZ* transgene in wild-type or mutant form (*E4m3–lacZ* and *E4mAll–lacZ*; see Fig. 7*C*) as an NheI/KpnI fragment into the male pronucleus of fertilized F1 (C57BL/6 × CBA) oocytes according to standard techniques. Foster mothers were killed when embryos were at 14.5 d postcoitum. Transgenic embryos were identified and genotyped by PCR on embryonic tail DNA using 5′-GCCCAGGAAGATAGTTTGGTG-3′ and 5′-GATAGGTTAC GTTG GTGTAGATGG-3′ as primers under standard PCR conditions.

The *E4–lacZ* transgene was injected as plasmid into the neural tube of live chicken embryos at Hamburger–Hamilton stage 10–11 in the presence of *pCAGGS–IRES–nls–GFP*-based expression plasmids and electroporated as described previously (Cossais et al., 2010). Successfully electroporated embryos were identified 48 h later by GFP expression and dissected.

After fixation, mouse and chicken embryos were cryoprotected in sucrose and frozen at −80°C in tissue freezing medium (Leica). After transverse sectioning on a cryotome, β-galactosidase activity was detected on 20 μm sections by incubation in 1% X-gal at 37°C. For immunohistochemistry, 10 μm cryotome sections were incubated with anti-β-galactosidase goat antiserum (1:500 dilution; Biotrend), anti-Sox10 guinea pig antiserum (1:1000 dilution) (Maka et al., 2005), or anti-GFP mouse monoclonal antibody (1:100 dilution; Roche). Secondary antibodies conjugated to Cy2, Alexa Fluor 488, and Cy3 immunofluorescent dyes (Dianova and Molecular Probes) were used for detection. Sections were analyzed and documented with either a Leica TCS SL confocal microscope or a Leica MZFLIII stereomicroscope equipped with an Axiocam (Carl Zeiss).

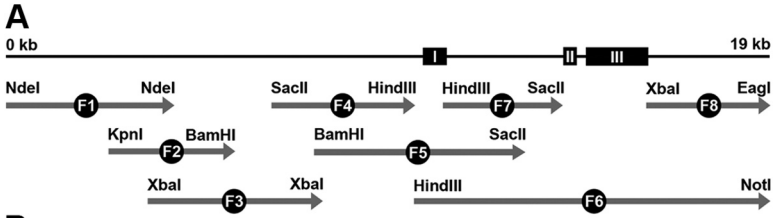
Results

The first intron of the *Dhh* gene has enhancer activity in S16 Schwann cells

Previous studies had shown that Dhh is not only strongly expressed in the male gonad but also in the developing peripheral nerve in which it is predominantly produced from Schwann cells (Bitgood and McMahon, 1995; Parmantier et al., 1999). Loss of this paracrine signaling molecule in mice leads to massive defects of the perineurium, the cellular sheath that surrounds and protects the endoneurium with its axon–Schwann cell units and blood vessels (Parmantier et al., 1999). It also prevents invasion of immune cells into the nerve. The perineurium consists of several layers of flattened fibroblasts that secret collagens and other extracellular matrix molecules as well as poorly characterized signaling factors. Many of the extracellular matrix molecules end up in the epineurium and thereby help to form the outer nerve cover. The signaling molecules from perineurial cells influence many aspects of nerve development. In the absence of Dhh, the perineurium no longer provides an effective barrier between surrounding tissue and nerve. It appears thin, less compacted, and disorganized, with patchy basal lamina and abnormal tight junctions (Parmantier et al., 1999). In Dhh-deficient mice, perineurial cells divide the nerve into mini-fascicles. Alterations in perineurium-derived signals cause defects in myelinating as well as non-myelinating Schwann cells. They lead to an abnormal permeability of the blood–nerve barrier and elevated levels of immune cells in the nerve (Sharghi-Namini et al., 2006). Similar consequences have also been observed in humans in which *Dhh* loss leads to a peripheral neuropathy with mini-fascicle formation and gonadal dysgenesis (Umehara et al., 2000).

Here we analyzed whether *Dhh* is a direct target of Sox10. Transcription of the *Dhh* gene has not been studied, and the regulatory region that mediates its expression in Schwann cells has not yet been identified. However, work by Jaegle et al. (2003) had shown that 19 kb of genomic sequence around the *Dhh* gene are sufficient to achieve expression of a *Cre* transgene in Schwann cells. This region encompasses all three *Dhh* exons as well as upstream and downstream sequences. To identify the part responsible for Schwann-cell-specific expression, we divided the

Dhh locus in eight overlapping fragments (Fig. 1*A*) and combined each with the β-globin minimal promoter (Fig. 1*B*). All eight combinations were then tested for their ability to drive expression of a *tdTomato* reporter gene in the S16 Schwann cell line using autofluorescence of transfected cells as readout (Fig. 1*C*). Combinations of the same β-globin minimal promoter with the *U3* enhancer of the *Sox10* gene or with the enhancer of the *Mpz* gene served as controls and verified that the assay is sensitive enough to detect the activity of Schwann-cell-specific enhancers (LeBlanc et al., 2007; Werner et al., 2007).



C

fragment	U3	Mpz	F1	F2	F3	F4	F5	F6	F7	F8
fluorescence	+	+	–	–	–	–	–	+	+	–

Figure 1. Scanning the *Dhh* genomic region for an enhancer. ***A***, Topology of the *Dhh* genomic region and localization of the fragments *F1–F8* in this 19 kb. Restriction enzymes used for generation of the fragments are indicated. ***B***, Scheme of the reporter construct. Fragments *F1–F8* were inserted in front of the β-globin minimal promoter, followed by *tdTomato* and SV40 poly(A) signal. ***C***, At 48 h after transient transfection of the reporter plasmids, S16 cells were scored for expression of the reporter by detection of tdTomato-specific autofluorescence. The experiment was repeated three times in duplicates. Reporter plasmids that carried the *U3* enhancer or the *Mpz* enhancer in combination with the β-globin minimal promoter served as positive control. +, Autofluorescence detected; −, no autofluorescence.

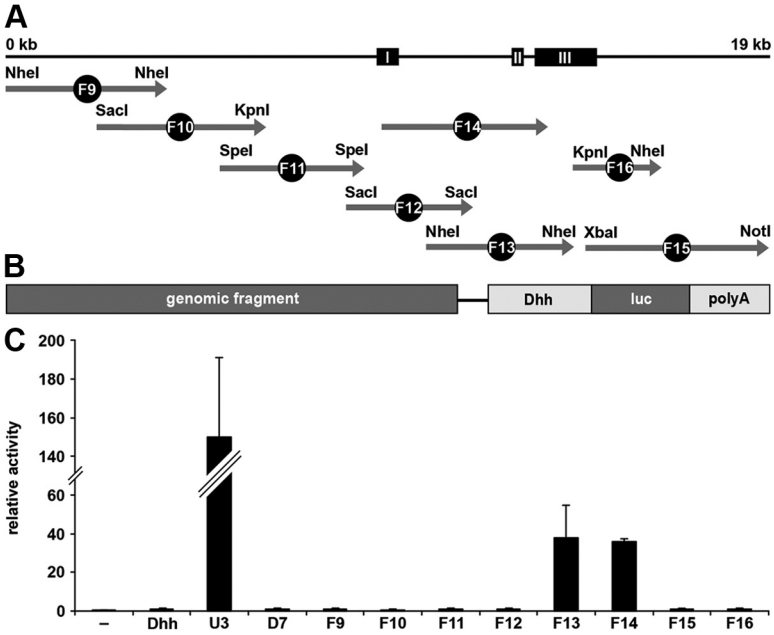


Figure 2. Mapping enhancer activity to intron 1 of the *Dhh* gene. ***A***, Schematic representation of fragments *F9–F16* and their location relative to the *Dhh* gene. Restriction sites used for cloning are indicated at the ends of each fragment. *F14* was generated by PCR. ***B***, Reporter constructs consisted of fragments *F9–F16* inserted in front of *Dhh* minimal promoter (positions −277 to +31, Dhh), luciferase gene, and SV40 poly(A) signal. ***C***, Transient transfections of these reporter constructs were performed in S16 cells. Luciferase activities in extracts from transfected cells were determined 48 h after transfection in three experiments each performed in duplicates. The luciferase activity obtained for a reporter plasmid containing only the minimal *Dhh* promoter (Dhh) was arbitrarily set to 1. Activities in the presence of additional *Dhh* regions were calculated relative to minimal promoter activity and are presented as mean ± SD. A reporter in which the *Dhh* minimal promoter was combined with the *U3* enhancer served as positive control, one in which it was combined with the *D7* enhancer as negative control. −, Reporter plasmid without the minimal promoter.

In contrast to the two known Schwann cell enhancers, none of the four fragments from the upstream region of the *Dhh* gene (*F1–F4*; Fig. 1*A*) exhibited autofluorescence (Fig. 1*C*). Equally inactive as the upstream fragments was *F5*. This fragment spanned the immediate upstream region, exon 1, and part of intron 1 and contained the *Dhh* promoter. It follows that the promoter is not sufficient to secure more than basal expression in S16 cells. In contrast, autofluorescence was observed with fragment *F6* (Fig. 1*A*,*C*). This contained all three exons of the *Dhh* gene, the intervening introns, and the immediate downstream region. A comparable autofluorescence was also obtained with a fragment that corresponded to intron 1 (*F7*; Fig. 1*A*,*C*), whereas a fragment representing the complete downstream region remained inactive (*F8*; Fig. 1*A*,*C*). These results point to enhancer activity in intron 1.

Autofluorescence was fairly weak and is inconvenient for quantification. We were furthermore concerned that enhancer activity may be suboptimal in combination with the heterologous β-globin promoter. Therefore, we switched to a luciferase reporter assay and exchanged the β-globin promoter for a 308-bp-long *Dhh* minimal promoter (−277 to +31) (Fig. 2*B*). This minimal promoter increased luciferase activity barely above levels observed with the promoterless construct. High activity was observed in S16 cells not before the *Dhh* minimal promoter was combined with the *U3* enhancer. This construct served again as positive control (Fig. 2*C*). In contrast, combination of the *Dhh* minimal promoter with the *Sox10 D7* enhancer remained inactive, in agreement with its lack of activity in Schwann cells (Werner et al., 2007).

As observed for combinations with the β-globin promoter, none of the fragments from the *Dhh* upstream region (*F9–F12*; Fig. 2*A*) nor any from the downstream region (*F15* and *F16*; Fig. 2*A*) showed activity in S16 cells when combined with the *Dhh* promoter (Fig. 2*C*). Activity was instead confined to *F13* and *F14*. These fragments strongly overlapped and contained most of the sequences from intron 1, confirming that this intron likely contains the enhancer element.

The *Dhh* enhancer is confined to the second half of intron 1

To determine more exactly the location of the enhancer, we generated subfragments of *F14* (Fig. 3*A*) and tested them again in S16 cells for their ability to induce expression of a luciferase reporter (Fig. 3*B*). Division of *F14* into a larger 2.8 kb fragment spanning the first two-thirds and a smaller 1.4 kb one containing the remaining one-third led to the two, equally inactive *F17* and *F18* fragments.

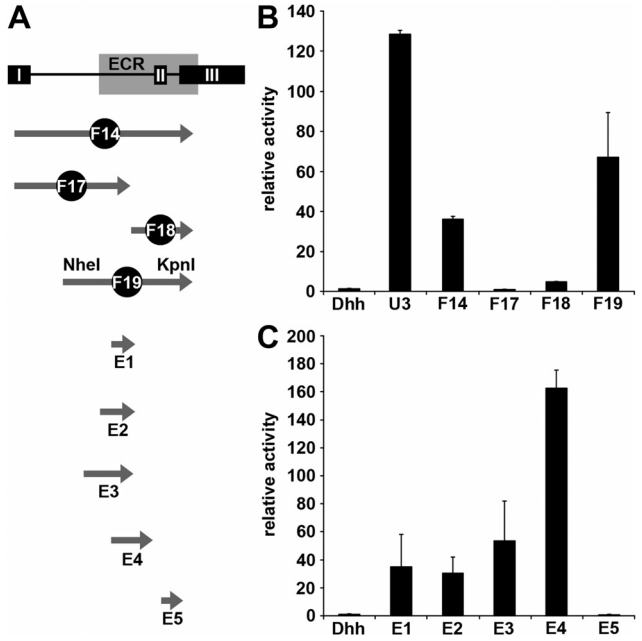


Figure 3. Fine mapping of the *Dhh* enhancer to an evolutionary conserved region in the hind part of intron 1. ***A***, Location of fragments *F14*, *F17–F19*, and *E1–E5* relative to exons I–III of the *Dhh* gene and the region with highest evolutionary conservation (ECR) in the gene. Restriction sites used for cloning are indicated. All other fragments were amplified by PCR. ***B***, ***C***, Luciferase reporter constructs containing one of the *F* (***B***) or *E* (***C***) fragments inserted in front of the *Dhh* minimal promoter were transiently transfected in S16 cells, and luciferase activities were determined 48 h after transfection in three experiments each performed in duplicate. The luciferase activity obtained for a reporter plasmid containing only the minimal *Dhh* promoter (Dhh) was arbitrarily set to 1. Activities in the presence of additional *Dhh* regions were calculated relative to minimal promoter activity and are presented as mean ± SD.

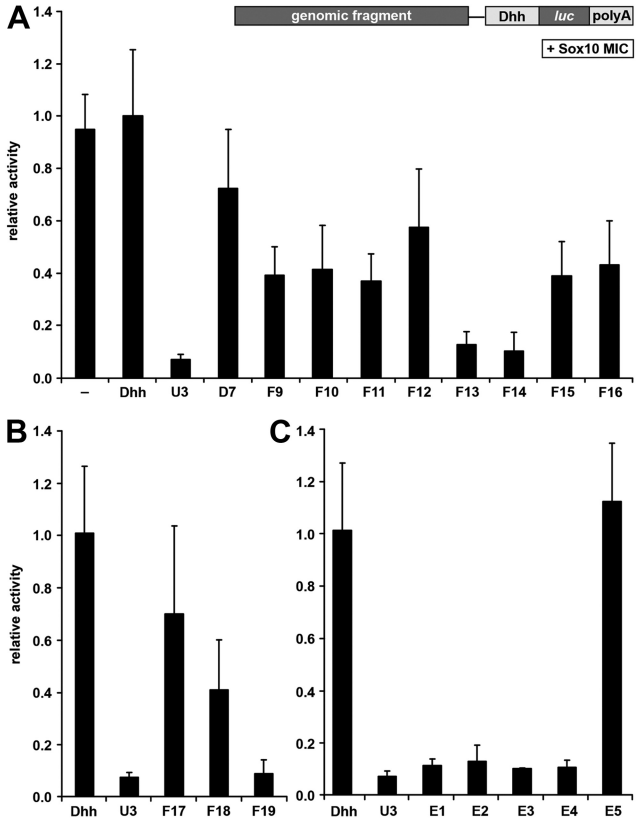


Figure 4. Sox10 responsiveness of the *Dhh* enhancer. Luciferase reporter constructs containing a fragment from the *Dhh* locus (see Figs. 2*A*, 3*A*) inserted in front of *Dhh* minimal promoter and luciferase gene were transiently transfected in S16 cells in the presence of a Sox10 MIC expression plasmid or an empty expression plasmid. Luciferase activities were determined 72 h after transfection in three experiments each performed in duplicate. The luciferase activity obtained for a reporter plasmid in the presence of empty expression plasmid was arbitrarily set to 1. Activities in the presence of the Sox10 MIC expression plasmid were calculated for each luciferase reporter in relation to it and are presented as mean ± SD. ***A***, Fragments *F9–F16*; –, reporter plasmid without the minimal promoter. ***B***, Fragments *F17–F19*. ***C***, Fragments *E1–E5*.

In contrast, enhancer activity was preserved in a 2.8 kb fragment that corresponded to the hind two-thirds of *F14* (Fig. 3*B*).

This *F19* fragment furthermore strongly overlapped with a region that exhibits conservation among mammals and reaches from the middle of intron 1 into exon 3 (Fig. 3*A*, ECR). Conservation is, however, confined to mammals and not seen in other vertebrates. Restricting our search to the evolutionary conserved part of *F19*, we generated five more fragments (Fig. 3*A*, *E1–E5*) and combined them with the *Dhh* minimal promoter in reporter plasmids to assess their enhancer activity in S16 cells. Of these five fragments, all except *E5* exhibited enhancer activity (Fig. 3*C*). Activity varied considerably, with highest levels being found in *E4*. Therefore, we regard the 927-bp-long *E4* region in intron 1 as the core of the *Dhh* enhancer.

The *Dhh* enhancer is responsive to Sox10

Because *Dhh* expression may be under control of Sox10 in Schwann cells (Finzsch et al., 2010), we wanted to find out whether activity of the newly identified *Dhh* enhancer depends on Sox10. To interfere with the function of endogenous Sox10 in S16 cells (Reiprich et al., 2010) and see how this affects activity of the *Dhh* enhancer, we chose a dominant-negative approach. It has been shown that transfection of a Sox10 version truncated immediately behind the DNA-binding HMG domain such as Sox10 MIC (Kuhlbrodt et al., 1998b) suppresses the function of cotransfected wild-type Sox10 (Inoue et al., 2004; Prasad et al., 2011). As a consequence, reporter gene expression in S16 cells should be lowered by cotransfected Sox10 MIC for those constructs that contain Sox10-responsive sequences of the *Dhh* locus. A luciferase reporter containing *U3* enhancer and *Dhh* minimal promoter helped to test the concept (Fig. 4*A*). Its activity in S16 cells was on average 16-fold lower in the presence of Sox10 MIC. Activity of the *Dhh* minimal promoter alone, in contrast, was refractory to Sox10 MIC.

When the genomic *Dhh* fragments that were used to map the enhancer were tested for their responsiveness toward Sox10 MIC in transfected S16 cells by luciferase assays, many showed a slight reduction in activity. However, only those fragments that contained the enhancer were repressed more than threefold. These included fragments *F13* and *F14* from the original screen (Fig. 4*A*), as well as fragments *F19* and *E1–E4* from the fine mapping (Fig. 4*B*,*C*). These findings therefore support the assumption that Sox10 regulates *Dhh* expression in Schwann cells at least partly through this newly identified *Dhh* enhancer.

Intron 1 of the *Dhh* gene contains potential binding sites for Sox10

Activation of the *Dhh* enhancer may involve direct binding of Sox10. Intron 1 of the *Dhh* gene contains eight potential Sox binding sites, site 1 through site 8 (Fig. 5*A*). These were defined as sites that either conform to the Sox consensus 5′-(A/T)(A/T)CAA(A/T)G-3′ or deviate from this consensus at no more than one position outside the central CAA core. Only site 8 carried two mismatches at the first and last positions. Each of the sites was

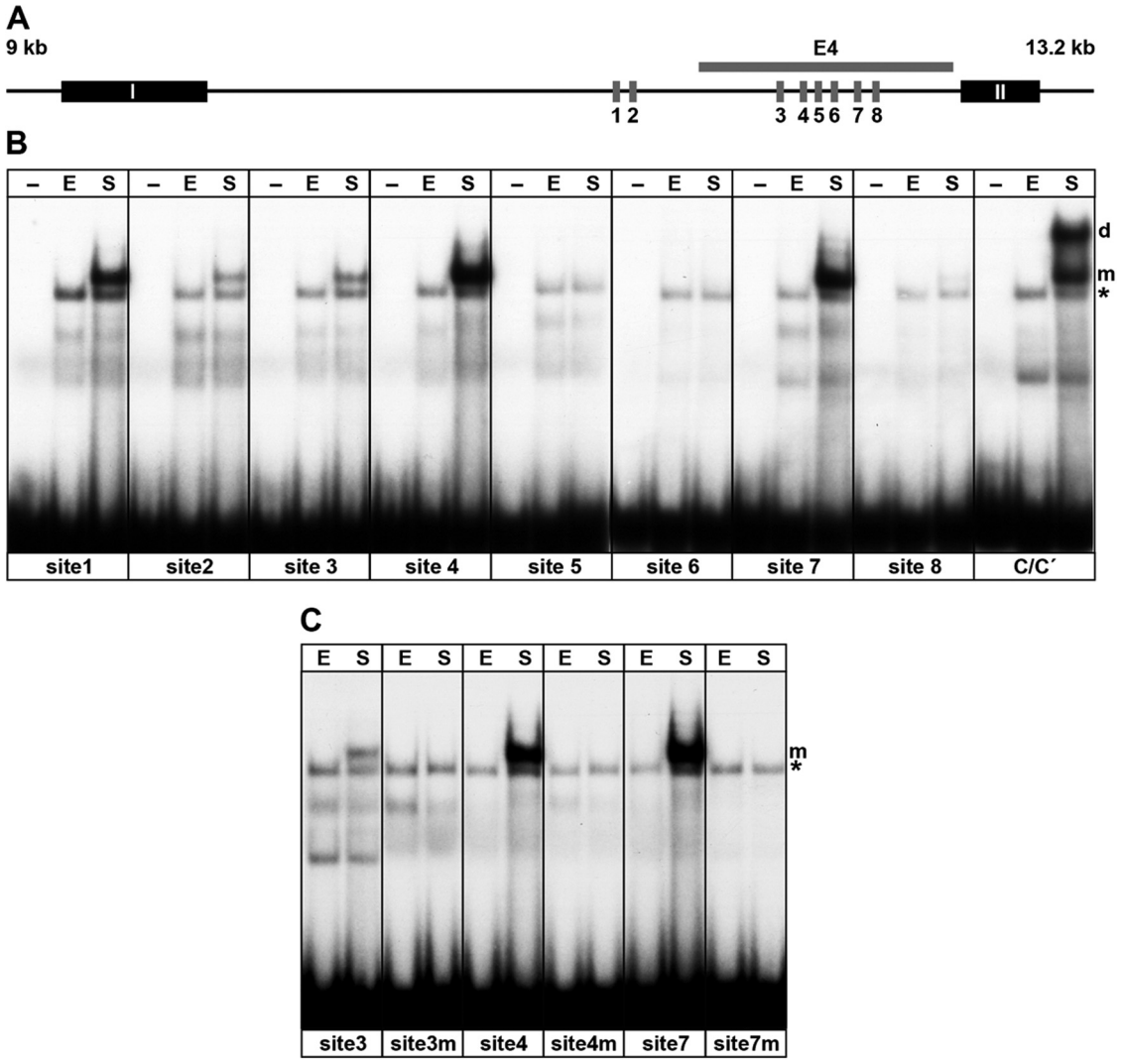


Figure 5. Mapping Sox10 binding sites in intron 1 of the *Dhh* gene. ***A***, Location of the eight potential Sox10 binding sites in intron 1 and relative to the *E4* fragment. ***B***, EMSA with radiolabeled double-stranded oligonucleotides encompassing one of the potential Sox10 binding sites as indicated below the gels. Oligonucleotides were incubated in the absence (−) or presence (*E*, *S*) of protein extracts before gel electrophoresis as indicated above the lanes. Extracts were from mock-transfected HEK293 cells (*E*) or HEK293 cells expressing full-length Sox10 (*S*). Oligonucleotides with site C/C′ from the *Mpz* promoter (Peirano et al., 2000) served as positive control for Sox10 binding and as marker for the mobility of complexes containing either Sox10 monomers (m) or dimers (d). ***C***, EMSA was performed with wild-type and mutant versions (m) for binding sites 3, 4, and 7. Oligonucleotides were incubated in the presence of protein extracts from mock-transfected HEK293 cells (*E*) or HEK293 cells expressing full-length Sox10 (*S*). The position of the Sox10-specific complex is highlighted (m). The asterisk marks a nonspecific complex.

tested in the context of a larger oligonucleotide in EMSA with control and Sox10-containing extract. Site C/C′ from the *Mpz* promoter (Peirano et al., 2000) helped to identify DNA complexes with Sox10 monomers and dimers by their respective mobility (Fig. 5*B*). Of the eight potential binding sites present in intron 1, site 1, site 4, and site 7 bound Sox10 strongly. Weaker binding was additionally detected to site 2 and site 3. All of these sites bound Sox10 as monomers. Dimer binding was not detected.

Because fragment *E4* was the region from intron 1 with strongest enhancer activity, we concentrated our analysis on those sites that were present in this core region and bound by Sox10. These were site 3, site 4, and site 7. Mutations were introduced into the CAA core of each of these sites, and the consequence of these mutations on binding was then tested in EMSA. As evident from Figure 5*C*, all mutations effectively abrogated Sox10 binding to the respective sites.

Mutations were introduced in the context of *E4* into each of these sites as single mutations or in combinations, and the consequences on enhancer activity were assessed in luciferase reporter gene assays in transfected S16 cells (Fig. 6*A*). Compared with wild type, all *E4* mutants exhibited dramatically reduced enhancer activity. Wild-type *E4* enhanced the activity of the *Dhh* minimal promoter 150-fold, but activation rates were down to sixfold for the site 3 mutant (Fig. 6*A*, *E4m3*). The site 4 mutation (*E4m4*) reduced activation rates to ninefold, and the site 7 mutation (*E4m7*) allowed a residual 14-fold activation. *E4* mutants with simultaneous mutation of two sites exhibited even lower activation rates. These results argue that *E4* enhancer activity in S16 cells depends on the three identified Sox10 binding sites.

We also analyzed to what extent the three mutations altered *E4* responsiveness toward Sox10 by cotransfecting all *E4* mutants with the dominant-negative Sox10 (Fig. 6*B*). Sox10 MIC not only effectively reduces the activity of wild-type *E4* but also the remaining activity of *E4m4*, *E4m7*, and the *E4m4m7* double mutant. In contrast, the residual activity of the *E4m3* mutant or all *E4* double and triple mutants with mutant site 3 were refractory to Sox10 MIC.

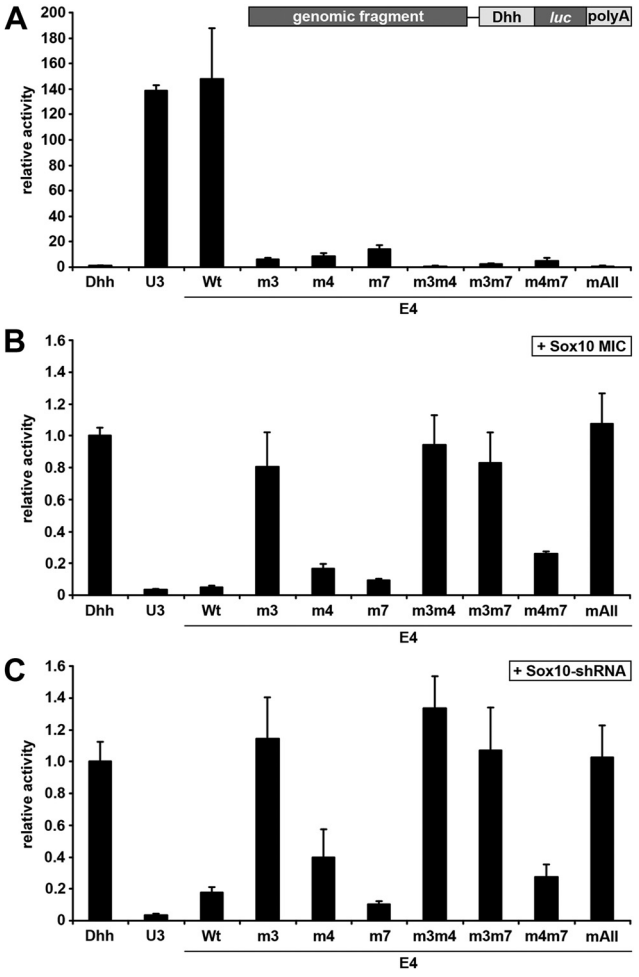


Figure 6. Functional relevance of Sox10 binding sites in the *Dhh* enhancer. ***A***, Luciferase reporter constructs containing the *E4* fragment in wild-type or mutant versions were transiently transfected in S16 cells, and luciferase activities were determined 48 h after transfection. Mutant *E4* versions carried inactivating mutations in Sox10 binding site 3 (m3), site 4 (m4), site 7 (m7), or combinations thereof (m3m4, m3m7, m4m7, mAll). The luciferase activity obtained for a reporter plasmid containing only the minimal *Dhh* promoter (Dhh) was arbitrarily set to 1. Activities in the presence of *E4* were calculated relative to minimal promoter activity and are presented as mean ± SD. ***B***, The same luciferase reporter constructs were also transfected in S16 cells in the presence of a Sox10 MIC expression plasmid or an empty expression plasmid. The luciferase activity obtained for a reporter plasmid in the presence of empty expression plasmid was arbitrarily set to 1. Activities in the presence of the Sox10 MIC expression plasmid were normalized for each luciferase reporter to this value and are presented as mean ± SD. ***C***, Additionally, transfections of luciferase reporters were performed in the presence of Sox10-specific shRNA and scrambled shRNA. Luciferase activities were determined, and the ratio of activities in the presence of Sox10-specific shRNA versus scrambled shRNA was calculated. Values were then normalized to the relative activity of the reporter plasmid containing only the minimal promoter and are presented as mean ± SD. All experiments were performed three times in duplicates.

Very similar results were also obtained when S16 cells were transfected with the *E4* reporter plasmids in the presence of Sox10-specific shRNAs (Fig. 6*C*). The activity of the Dhh minimal promoter was only marginally affected by the presence of Sox10–shRNA or scrambled shRNA. For reporter plasmids that additionally contained the *U3* or the *E4* enhancer, activity was reduced to a residual 5–20% in the presence of Sox10–shRNA. Similar to the wild-type, several *E4* mutants were responsive to the Sox10–shRNA, arguing that they still contain residual functional Sox10 binding sites. These included *E4* versions with site 4, site 7, and combined site 4/7 mutations. In contrast, site 3 mutations abolished this responsiveness when present alone or in combination with other mutations (Fig. 6*C*). Both cotransfections with Sox10-specific shRNA and Sox10 MIC therefore lead us to conclude that site 3 is most important among the three Sox10 binding sites present in *E4*.

Considering that we detected Sox10 binding to three sites and found mutation of these sites to reduce activity and Sox10 responsiveness, it is reasonable to assume that Sox10 exerts its stimulatory effect on *E4* by direct binding. To verify this assumption, we performed ChIP from S16 cells with Sox10-specific antibodies (Fig. 7*A*,*B*). These antibodies readily precipitated chromatin fragments from the *E4* enhancer, leading to their enrichment in the precipitate relative to two control fragments from the upstream region of the *Dhh* gene (Fig. 7*B*). No such enrichment was observed with control antibodies. Interestingly, the enrichment was much higher for the *E4* region that contained site 3 and site 4 than for the region with site 7 (Fig. 7*B*, compare *E4a*, *E4b*). ChIP thus confirms the presence of Sox10 on the *E4* enhancer in S16 Schwann cells.

Schwann-cell-specific activity of the *Dhh* enhancer depends on Sox10 *in vivo*

Although the ChIP results in S16 cells are suggestive, they do not prove that *E4* is truly active as a Sox10-dependent Schwann cell enhancer *in vivo*. To confirm enhancer activity *in vivo*, we generated transgenic constructs in which *E4* was placed in front of the *Dhh* minimal promoter and a *lacZ* reporter gene (Fig. 7*C*, *E4-lacZ*). Injection of the *E4–lacZ* transgene into the male pronucleus of fertilized oocytes allowed us to generate five transgenic embryos (Fig. 7*F*) that were analyzed at embryonic day 14.5 by X-gal staining and immunohistochemistry (Fig. 7*D*,*E*). At the time of analysis, the embryos contained on average one to nine copies of the transgene per cell as determined by quantitative PCR (Fig. 7*F*). Four of the five embryos exhibited staining along the peripheral nerve. There was some variation in staining intensity and in the number of stained cells. Nevertheless, staining along the nerve was clearly detectable. Immunohistochemistry confirmed that β-galactosidase was present in the cytoplasm of those cells along the nerve that contain Sox10 in their nuclei and are thereby defined as Schwann cells (Fig. 7*E*). We therefore conclude that *E4* is able to target transgene expression to Schwann cells *in vivo*.

In addition to transgenes with wild-type *E4*, we also injected transgenes in which all three Sox10 binding sites (*E4mAll–lacZ*) or site 3 (*E4m3–lacZ*) were mutated (Fig. 7*C*). Of the four transgenic embryos obtained for *E4mAll–lacZ*, none exhibited significant activity in peripheral nerves (Fig. 7*D*,*F*). Similarly, none of the seven embryos obtained for *E4m3–lacZ* at embryonic day 14.5 exhibited expression of the *lacZ* reporter along the nerve (Fig. 7*D*,*F*). The activity observed for the *lacZ* transgene *in vivo* thus crucially depends on *E4* and not the minimal promoter, and within *E4* on the identified Sox10 binding sites, in particular on site 3.

Finally, we electroporated the *E4–lacZ* transgene into the neural tube of early chicken embryos in the presence of GFP as electroporation marker. Considering that the neural tube at this stage consists of highly proliferative, Sox10-negative neuroepithelial cells, the lack of activity of the *lacZ* transgene was not surprising (Fig. 7*G*,*H*). However, lacZ activity became readily visible in the electroporated half of the neural tube after coelectroporation of Sox10 (Fig. 7*I*,*J*), arguing that *Dhh* enhancer activity indeed depends on this transcription factor *in vivo*.

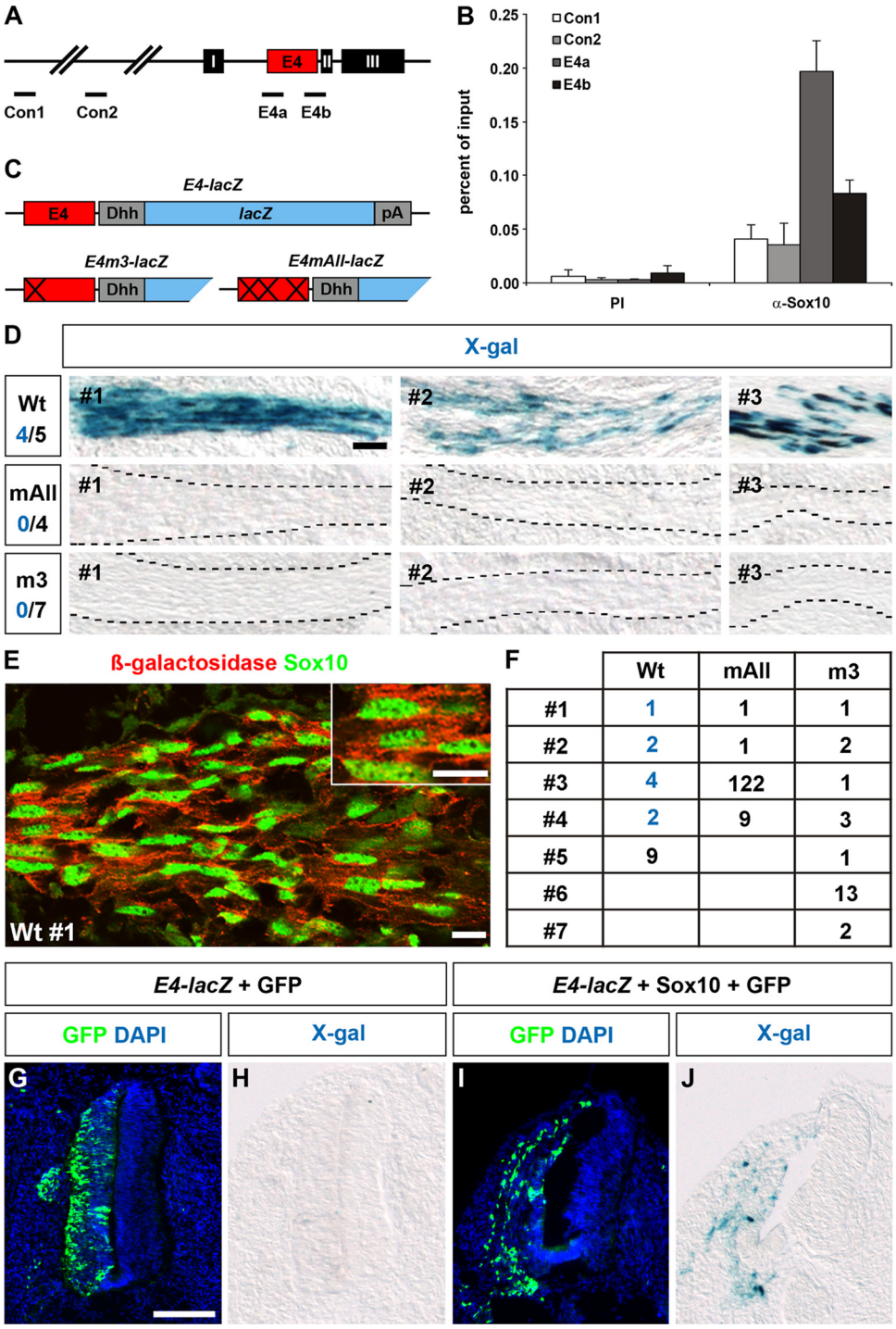


Figure 7. *In vivo* requirement of Sox10 binding for *Dhh* activity. ***A***, Schematic representation of the location of regions from the *Dhh* locus probed by PCR during ChIP studies, including the front (E4a, positions +2363 to +2779) and hind (E4b, positions +2929 to +3293) part of *E4* and two control fragments from the distal upstream region of the *Dhh* gene (Con1, positions −118661 to −119032; Con2, positions −78544 to −78886). ***B***, ChIP was performed on S16 cells using antibodies directed against Sox10 (α-Sox10) and control preimmune serum (PI). Quantitative PCR was applied on the immunoprecipitate. Values for each fragment correspond to the percentage of material precipitated from the input. ***C***, Schematic representation of the transgenic constructs consisting of *E4* in wild-type (E4) or mutant (E4m3 and E4mAII) versions, the minimal *Dhh* promoter (Dhh), the *lacZ* marker gene (lacZ), and an SV40 poly(A) signal (pA). ***D–F***, Analysis of transgenic mouse embryos. ***D***, LacZ activity was detected colorimetrically using X-gal substrate on transverse sections of 14.5-d-old transgenic mouse embryos. Only spinal nerve (marked by dotted lines) is shown. The number of embryos that exhibited X-gal staining in the nerve relative to the total number of transgenic embryos obtained for *E4–lacZ* (Wt), *E4mAII–lacZ* (mAII), and *E4m3–lacZ* (m3) by pronucleus injection are given on the left. Scale bar, 50 μm. ***E***, Coimmunohistochemistry was performed on transverse sections of 14.5-d-old *E4–lacZ* mouse embryos using antibodies directed against β-galactosidase (in red) in combination with antibodies directed against Sox10 (in green). The confocal image is confined to spinal nerve. The inlay shows a higher resolution. Scale bars, 10 μm. ***F***, Summary of mouse embryos obtained for each transgene and analyzed during this study including the determined number of transgene copies present on average in each embryo per cell. ***G–J***, Analysis of electroporated chicken embryos. Chicken embryos were electroporated with the *E4–lacZ* in the presence of *pCAGGS–IRES–nls–GFP* (***G***, ***H***) or *pCAGGS–Sox10–IRES–nls–GFP* (***I***, ***J***). Electroporated neural tube regions were identified 48 h later on transverse sections by unilateral GFP expression (green) using DAPI as a nuclear counterstain (blue) (***G***, ***I***). LacZ activity was detected on immediately adjacent sections using X-gal substrate (***H***, ***J***). Scale bar (in ***G***): ***G–J***, 200 μm.

Discussion

In this study, we have mapped the regulatory region that is likely responsible for *Dhh* expression in Schwann cells by a combination of cell culture and transgenic studies. Schwann-cell-specific expression was not mediated by the promoter but rather by an enhancer within the first intron of the *Dhh* gene. The enhancer was localized in the hind part of intron 1, and the *E4* region constituted its core.

It is unclear at present whether *E4* is identical to the enhancer or whether surrounding sequences additionally contribute to enhancer activity. Two observations argue for a role of *E4* flanking sequences in enhancer activity. Although *E4* effectively directed reporter gene expression to Schwann cells in transgenic embryos, expression levels were quite variable. This is a consequence of different copy numbers and integration sites in the transgenic embryos. However, the fact that activity is fairly sensitive to these parameters may indicate that *E4* lacks some of the elements that stabilize enhancer activity *in situ*. Additionally, *E4* is embedded in a larger region that exhibits sequence conservation among mammalian species, and this conservation may reflect the exact dimensions of the enhancer.

It is also noteworthy that conservation was only observed among mammals. Already in monotherians, the conserved region is much smaller than in metatherians and confined to 190 bp of *E4* surrounding site 3 and site 4. No conservation was detected in other vertebrates in agreement with the fact that *Dhh* is a hedgehog gene specific to mammals (O'Hara et al., 2011).

We have also shown that the *Dhh* enhancer and its *E4* core contain binding sites for Sox10 and that they respond to its presence. As a consequence, deletion of the Sox10 binding sites led to a dramatic decrease of enhancer activity in cultured cells and abolished Schwann-cell-specific activity *in vivo*. Considering further that Sox10 was detected on this enhancer by ChIP and that the presence of Sox10 allows this enhancer to function in neuroepithelial cells after electroporation *in vivo*, there is strong evidence that Sox10 contributes to the Schwann-cell-specific activity of the *Dhh* enhancer by directly binding to these sites.

Among the three sites identified, site 3 is the most important for Sox10-dependent activity, despite a rather modest Sox10 binding *in vitro* when compared with site 4 and site 7. This shows that binding strength *in vitro* is not a completely reliable predictor for functional importance *in vivo*. Several reasons for the discrepancy can be envisaged. It is possible that the accessibility of the sites is different in chromatin *in vivo* compared with naked DNA *in vitro*. Some of the sites may be more effectively masked in nucleosomes than others. Binding of other transcription factors to the enhancer has to be taken into account as well. Competitive binding to overlapping sites may reduce accessibility, whereas transcription factor binding to adjacent sites may allow additional contacts between the two bound proteins and thereby facilitate binding. In this respect, it is intriguing that there is a potential binding site for Krox20 adjacent to site 3. Both sites are separated by 11 bp, and the distance between their centers corresponds exactly to two helical turns. This is closer than most previously identified composite binding elements for Sox10 and Krox20 (Jones et al., 2007). It also differs from previously identified sites in that it combines the Krox20 site with a monomeric rather than a dimeric Sox10 binding site. Additional sites for Klf, Nfat, and YY1 proteins are a bit farther away. Future experiments will have to probe their relevance.

It is also worth mentioning that all of the identified sites bind Sox10 as monomer. Similar to the other SoxE proteins Sox8 and Sox9, Sox10 can bind to DNA as monomer or dimer (Wegner, 2010), and it has been shown for at least two Sox10-responsive enhancers that a dimer site cannot simply be replaced by a monomer site without a significant loss in enhancer activity (Peirano and Wegner, 2000; Jagalur et al., 2011). It has even been postulated that dimeric binding is such a crucial feature that it can be used to predict Sox10-dependent neural-crest enhancers (Antonellis et al., 2008). For Schwann-cell-specific enhancers that are activated by Sox10, there seems to be no strict reliance on dimeric binding. Some Schwann-cell-specific enhancers only contain dimer sites and crucially depend on them (i.e., the *Oct6 SCE*) (Jagalur et al., 2011), whereas others contain only monomeric sites (i.e., the *Dhh* enhancer), yet others carry both monomeric and dimeric sites and need both for their activity (i.e., *Krox20* myelinating Schwann cell enhancer, *U3* enhancer of the *Sox10* gene) (Reiprich et al., 2010; Wahlbuhl et al., 2012). Considering that Sox10 monomers affect the overall topology of the enhancer in a different way than dimers, the most likely explanation is that each enhancer contains the type of site that is best suited at its specific position to guarantee the multiprotein complex formation of the enhanceosome.

The identification of *Dhh* as a direct target gene also provides the first mechanistic explanation for a non-cell-autonomus function of Sox10. By regulating *Dhh* expression in Schwann cells, Sox10 influences the development of perineurial cells and is responsible for formation and proper function of the perineurium. Loss or inactivation of Sox10 should therefore be associated invariably with defects in the perineurial sheath. The compromised blood–nerve and tissue–nerve barriers should furthermore lead to elevated immune cell numbers in the nerve, which in turn may evoke a chronic inflammation. The fact that these changes are indeed observed in mouse models with Sox10-deficient Schwann cells (Finzsch et al., 2010) confirms the relevance of this effector–target gene relationship for normal nerve development. Disturbance of Dhh signaling to the perineurial cells may also occur in patients with *Sox10* mutations and contribute to disease manifestation and pathology in humans.

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