Formation of the node of Ranvier by Schwann cells is under control of transcription factor Sox10

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Abstract

The transcription factor Sox10 is an essential regulator of genes that code for structural components of the myelin sheath and for lipid metabolic enzymes in both types of myelinating glia in the central and peripheral nervous systems. In an attempt to characterize additional Sox10 target genes in Schwann cells, we identified in this study a strong influence of Sox10 on the expression of genes associated with adhesion in the MSC80 Schwann cell line. These included the genes for Gliomedin, Neuronal cell adhesion molecule and Neurofascin that together constitute essential Schwann cell contributions to paranode and node of Ranvier. Using bioinformatics and molecular biology techniques we provide evidence that Sox10 directly activates these genes by binding to conserved regulatory regions. For activation, Sox10 cooperates with Krox20, a transcription factor previously identified as the central regulator of Schwann cell myelination. Both the activating function of Sox10 as well as its cooperation with Krox20 were confirmed in vivo. We conclude that the employment of Sox10 and Krox20 as regulators of structural myelin sheath components and genes associated with the node of Ranvier is one way of ensuring a biologically meaningful coordinated formation of both structures during peripheral myelination.

KEYWORDS
glia, HMG, Krox, node of Ranvier, Schwann cell, Sox

1 | INTRODUCTION

In both peripheral nervous system (PNS) and central nervous system (CNS) of vertebrates, speed and efficiency of information processing depend on myelination and the concomitant formation of nodes of Ranvier, which permit a rapid saltatory mode of conduction. The glial cells that generate myelin and participate in node formation as part of their terminal differentiation program are Schwann cells in the PNS and oligodendrocytes in the CNS.

Intricate gene regulatory networks are in place to guide terminal differentiation of myelinating glia and several transcription factors have been identified over the last years that act as key regulators of differentiation and myelination in Schwann cells and oligodendrocytes (Sock & Wegner, 2019). One of the key regulators in both Schwann cells and oligodendrocytes is the HMG-domain containing Sox10 protein (Kuhlbrodt, Herbarth, Sock, Hermans-Borgmeyer, & Wegner, 1998). This transcription factor functions not only as a lineage determinant in both glial cell types, but also as a major regulator of differentiation and myelination (Britsch et al., 2001; Finzsch et al., 2010; Fröb et al., 2012; Stolt et al., 2002). In feedforward regulatory loops, Sox10 first induces the homeodomain transcription factor Oct6...
regulated with myelin genes and lipid production during Schwann cell are also direct targets of Sox10 and Krox20 and are thus coordinately ergistically activate expression of many genes that code for myelin proteins (such as Mbp and Mpz) as well as enzymes of lipid biosynthesis and thereby directly trigger the production of key components required for myelin formation (Srinivasan et al., 2012; Stolt & Wegner, 2016). Starting out with a screen for Sox10-regulated genes in paranodal junctions (Faivre-Sarrailh & Devaux, 2013; Rasband & Charnay, 2006; Jagalur et al., 2011). Transcription factor Krox20 with Oct6 the zinc finger protein Krox20 (a.k.a. Egr2) (Ghislain & Charnay, 2006; Jagalur et al., 2011). There is ample evidence that Sox10 and Krox20 side, are under the same transcriptional control as myelin proteins and lipid production. Key glial components of PNS nodes are the L1-type cell adhesion molecule Nrcam and the extracellular matrix component Glomедин that are present on and secreted by the nodal Schwann cell microvilli as well as the glial-specific Neurofascin isoform NF155 at paranodal junctions (Faivre-Sarrailh & Devaux, 2013; Rasband & Peles, 2015). Starting out with a screen for Sox10-regulated genes in the mouse Schwann cell line MSC80, we here provide evidence that the key Schwann cell-expressed components of the node of Ranvier are also direct targets of Sox10 and Krox20 and are thus coordinate regulated with myelin genes and lipid production during Schwann cell differentiation.

2 | MATERIALS AND METHODS

2.1 | Cell lines and culture conditions

The mouse MSC80 Schwann cell line, N2a neuroblastoma cell line and human HEK293 kidney cell line were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum. N2a and HEK293 cells were obtained from ATCC. MSC80 cells were a gift from K.-A. Nave (Department of Neurogenetics, Max Planck Institute of Experimental Medicine, Göttingen, Germany). Cell line identity was confirmed by PCR.

2.2 | Genome editing

For CRISPR/Cas9-mediated Sox10 inactivation in MSC80 cells, the guide RNA 5'-GGCCCGTTGCTGGAGACCCGA-3' was cloned into pX330 (Cong et al., 2013). MSC80 cells were co-transfected with EGFP-N1 (Clontech) and the guide RNA and SpCas9 expressing pX330 plasmid. Transfected GFP-positive cells were enriched by FACs and seeded at single cell density in 96-well plates. Homogeneously GFP-expressing clones were analyzed for Sox10 expression by immunocytochemistry using a guinea-pig anti-Sox10 antiserum (1:3000 dilution) (Maka, Stolt, & Wegner, 2005) as primary and Cy3-coupled anti-guinea pig antibodies (Dianova) as secondary detection reagent. Several clonal MSC80 lines without Sox10 protein were obtained.

2.3 | RNA-sequencing and bioinformatical analysis

Total RNA was prepared from independent MSC80 cell clones 1–3 that had undergone CRISPR/Cas9-dependent genome editing to inactivate Sox10 and three different batches of wildtype MSC80 cells using the RNeasy Micro Kit (Qiangen). RNA samples were treated with DNase I to remove contaminating DNA. Quality and purity of samples were evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Germany). Hundred nanograms of total RNA were used for library preparation (Illumina Stranded mRNA Kit). Approximately 39 million reads were generated per library using an Illumina Hiseq 2500 platform sequencer (Next Generation Sequencing Core Facility, FAU Erlangen-Nürnberg) and mapped onto mouse genome mm10 using STAR (version 2.5.1b). Unique mappers were detected using HTSeq count based on ENSEMBL Gene identifier Version 75. Statistical analysis was carried out using DESeq2 R Version 1.8.1. Gene expression values are deposited in GEO under accession number GSE152687.

To identify potential Sox10 target genes that are differentially expressed during Schwann cell differentiation (GSE101153), genes that are at least fourfold downregulated in Sox10-deficient MSC80 cells were compared with genes that are either up-regulated (log2-fold change ≥1.75) or downregulated (log2-fold change ≤–1.75) during Schwann cell differentiation using the Venn webtool on the BEG homepage (http://bioinformatics.psb.ugent.be/webtools/Venn/). Gene ontology (GO) analysis was performed using the GO enrichment, analysis and visualization tool (http://cbl-gorilla.cs.technion.ac.il/) in combination with semantic clustering by REVIGO (http://revigo.irb.hr/) (Supek, Bosnjak, Skunca, & Smuc, 2011). The gene set enrichment analysis tool (GSEA) from the Broad Institute was used to determine whether defined sets of genes show statistically significant, concordant differences between RNA samples from MSC80 wildtype and Sox10-deficient cells (http://software.broadinstitute.org/gsea/index.jsp). The Integrative Genomics Viewer (IGV) was used to visualize select ChIP-Seq peaks from (GSE64703) (Lopez-Anido et al., 2015).

2.4 | Plasmids

Expression plasmids for Sox10 and Krox20 were based on pCMV5 and have been described before (Kuhlbrodt et al., 1998; Wahlbuhl, Reiprich, Vogl, Bosl, & Wegner, 2011). For generation of luciferase reporter plasmids with the following regions were obtained by PCR from rat genomic DNA (see also Figure 3a): ~50 Glgn (m6, chromosome 8, positions 58,820,274-58,821,276), prom Glgn (m6, chromosome 8, positions 58,870,531-58,870,954), +41 Glgn (m6, chromosome 8, positions 58,912,356-58,912,656), ~18 Nfasc (m6, chromosome 13, positions 49,540,812-49,541,300), +68 Nfasc (m6, chromosome 13, positions 49,540,812-49,541,300).
13, positions 49,454,004-49,454,658), –28 Nrcam (rn6, chromosome 6, positions 64,269,051-64,269,623), +27 Nrcam (rn6, chromosome 6, positions 64,325,033-64,325,425), +147 Nrcam (rn6, chromosome 6, positions 64,444,874-64,445,464). They were inserted into the pGL2 reporter plasmid in front of a β-globin minimal promoter and firefly luciferase coding sequence. To replace Sox binding sites by recognition sites for specific restriction enzymes (see Figure 5e), site-directed mutagenesis was performed by PCR using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

2.5 Luciferase assays

For luciferase reporter assays, 50–100 ng pCMV5-based expression plasmids were transfected in combination with 0.5 μg luciferase reporter plasmid (for detailed information, refer to the section on plasmids) in N2a neuroblastoma cells using SuperFect Transfection Reagent (Qiagen) on standard 24-well culture plates. Total pCMV5 amounts were kept constant in all transfections of the experiment. In co-transfections of two transcription factors, expression plasmids were used in equal amounts. All transfections were carried out in triplicates and had comparable transfection efficiencies in the range of 20–30% as determined by co-transfected GFP. At 48 hr post transfection whole cell extracts were prepared and luciferase activities were determined in the presence of luciferin substrate by addition of ATP and detection of chemiluminescence.

2.6 Western blot

For Western blots, whole cell extracts of MSC80 wildtype and Sox10-deficient cells were prepared by lysing cells in 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA. After addition of NP-40 to 1% final concentration and NaCl to 400 mM, 15 min rotation at 4°C and 5 min centrifugation, glycerol was added to a final concentration of 10%. Whole cell extracts were separated on a 10% polyacrylamide-SDS gel and blotted onto nitrocellulose membranes for antibody-mediated detection of specific proteins. The following antibodies and detection reagents were used: goat anti-Sox10 antiserum (home-made, 1:10000 dilution), rabbit anti-Gapdh antiserum (Santa Cruz, sc-25778,1:3000 dilution), horseradish peroxidase-labeled goat IgG antibody (KPL, 1:3000 dilution) and horseradish peroxidase-labeled protein A (Zymed Laboratories, 1:3000 dilution). Detection was by chemiluminescence using ECL reagent.

2.7 Electrophoretic mobility shift analysis

For preparation of extracts, HEK293 cells were transfected with poly-ethylenimine using 10 μg pCMV5-based expression plasmid per 100 mm plate, and harvested 48 hr post-transfection. The whole cell extracts of HEK293 cells were incubated with poly-dGdC as unspecific competitor and 32P-labeled 29 bp-long double-stranded oligonucleotides containing putative Sox10 binding sites from Gldn, Nfasc and Nrcam ECRs in wildtype and mutant version (see Figure 5a,e) (Kuhlbrodt et al., 1998). As controls for monomeric and dimeric binding of Sox10, oligonucleotides containing sequences of sites B and C/C’ from the Mpz gene were used (Peirano, Goerich, Riethmacher, & Wegner, 2000).

2.8 Mice

Mice were generated on a mixed C3H x C57Bl/6J background that carried two floxed Sox10 alleles (Finzsch et al., 2010) in combination with a Dhh::Cre transgene (Jaegle et al., 2003) or one Sox10loxP (Britsch et al., 2001) and one KroxAloxP (Voiculescu, Charnay, & Schneider-Maunoury, 2000) allele each. Genotyping was performed as described (Finzsch et al., 2010; Hövelmeyer et al., 2005; Sock, Schmidt, Hermans-Borgmeyer, Bösl, & Wegner, 2001). In accordance with animal welfare laws, mice were kept under standard housing conditions with continuous access to food and water and 12:12 hr light-dark cycles as approved by the responsible local committees and government bodies. Sciatic nerve tissue was obtained at postnatal days (P) 7 and 14 from both male and female pups and processed to obtain total RNA or single teased fibers as described (Finzsch et al., 2010; Fröb et al., 2019).

2.9 Immunohistochemistry

Teased fibers from sciatic nerves of P14-old pups were dried on object slides and incubated with rabbit anti-Caspr antiserum (Abcam, #AB34151, 1:10000 dilution) and chicken anti-Nefh antiserum (EnCor Biotechnology Inc., #CPCA-NF-H, 1:10000 dilution) followed by matched secondary antibodies coupled to Cy3 (Dianova, 1:200 dilution) and Alexa488 (Molecular Probes, 1:500 dilution) fluorescent dyes. Visualization and documentation were carried out with a Zeiss Apotome before analysis by ImageJ.

2.10 Quantitative RT-PCR

RNA of sciatic nerve tissue of mouse pups at P7 was prepared, reverse transcribed and used for quantitative PCR on a Biorad CFX96 Real Time PCR System. The following primer pairs were used: 5’-CTGTCGCTGACAATGT-3 and 5’-AGCAGGTGTCACAATACGCA-3 for Gldn, 5’-TTCCGACATGGTGGTCCCTG-3 and 5’-ACGTCGTGCTGACAATGT-3 for Nfasc and 5’-TTCTTTGCTCCCAGACCTTC-3 and 5’-AGCAGGTGTCACAATACGCA-3 for Nrcam. All other primers have been described before (Bremer et al., 2011; Fröb et al., 2012; Weider et al., 2018). Transcript levels were normalized to Rpl8 and Rplp0.
2.11 Statistical analysis

To determine whether differences in length, transcript levels or luciferase activities were statistically significant, a two-tailed Student's t test or one way analysis of variance (ANOVA) with Bonferroni correction was performed (*p ≤ .05; **p ≤ .01, ***p ≤ .001). Results from independent animals or separate transfections were treated as biological replicates (n ≥ 3).

FIGURE 1  Legend on next page.
3 | RESULTS

3.1 | Significant changes of expression of adhesion-related molecules following Sox10 loss in MSC80 cells

Considering that Sox10 is expressed in Schwann cells at all times, there are likely more Sox10-regulated genes than its well-described targets that primarily code for proteins with roles in the regulation of terminal differentiation and myelin sheath formation. To obtain a broader view of Sox10-dependent gene expression in Schwann cells, we used the mouse MSC80 cell line (Boutry et al., 1992). As expected for a Schwann cell line, MSC80 cells expressed Sox10 on both RNA and protein level (Figure 1a,b). Using CRISPR/Cas9-dependent genome editing, we generated several clonal lines with mutations in the Sox10 gene that reduced transcript levels and prevented protein production (Figure 1a,b). From three Sox10-negative clonal lines, and three separate batches of the original MSC80 cells, expression profiles were generated by RNA-Seq. As evident from PCA plots, samples from Sox10-deficient clones clustered separately from samples of Sox10-expressing MSC80 cells (Figure 1c). In the absence of Sox10, 21.4% of all genes exhibited substantially altered expression levels (log2-fold change ≥2; p ≤.05; mean base count ≥20). Of these differentially expressed genes, 13.9% showed increased expression in the absence of Sox10, whereas 7.5% were downregulated (Figure 1d,e). The range of observed expression changes was comparable for up- and downregulated genes (Figure 1e).

Not many of the known myelin-related target genes of Sox10 in Schwann cells were present among the differentially expressed genes, probably because MSC80 cells resemble Schwann cells at an early developmental stage and do not differentiate efficiently in vitro (Boutry et al., 1992). Accordingly, myelin genes such as Mbp and Mpz are expressed at low to undetectable levels. Nevertheless, GSEA of all differentially expressed genes revealed that genes associated with Schwann cell identity, gliogenesis and myelination were less prominently expressed in the absence of Sox10 than in its presence (Figure 1f–h). GO analysis of the upregulated genes revealed that they were associated with several processes including regulation of proliferation, migration/motility, cell death and (intracellular) signal transduction (Figure 1i). Adhesion was another term strongly enriched among the upregulated genes. When downregulated genes underwent GO analysis, adhesion and regulation of development/differentiation were very prominent among the enriched terms (Figure 1j). Considering the enrichment of adhesion-related genes among up- and downregulated genes, Sox10 appears to have a strong impact on the expression of adhesion molecules and the adhesion properties of Schwann cells.

3.2 | Sox10 as a potential regulator of Schwann cell genes associated with node formation

So far, Sox10 has been more frequently found to be an activating rather than a repressing transcription factor (Weider, Reiprich, & Wegner, 2013). Therefore, we focused on the 379 downregulated genes as candidates for direct Sox10 target genes in Schwann cells. Of the downregulated genes, 56 substantially decreased their expression when primary Schwann cells were differentiated in culture (Camarena et al., 2017) (Figure 2a,b). These genes may therefore represent potential Sox10 target genes in early stages of Schwann cell development. GO analysis pointed to an enrichment of genes involved in glial development, cell adhesion, cell communication and synaptic processes among these genes (Figure 2c). An additional group of 27 genes substantially downregulated in Sox10-negative MSC80 cells exhibited increased expression in differentiating Schwann cells and may therefore constitute genes that are direct Sox10 target genes in differentiating Schwann cells although they do not code for any of the known Sox10-dependent major myelin sheath components or proteins involved in lipid metabolism (Figure 2d,e). GO analysis found a strong association of these genes with adhesion, but also with voltage-gated ion channel clustering and action potentials and thus with three terms of relevance to the node of Ranvier (Figure 2e,f).

Among the 27 genes that exhibited decreased expression in Sox10-negative MSC80 cells and increased expression in differentiating Schwann cells we detected Gldn, Nfasc and Nrcam (Figure 2d,e). The corresponding gene products (gliomedin, neurofascin and neuronal cell adhesion molecule) indeed localize to node or paranode and...
contribute to formation of these structures (Faivre-Sarrailh & Devaux, 2013; Rasband & Peles, 2015).

To verify that Schwann cell expression of \textit{Gldn}, \textit{Nfasc} and \textit{Nrcam} depends on Sox10 in vivo, we generated Sox10\(^{Δ/Δ}\) (i.e., Dhh::Cre, Sox10\(^{fl/fl}\)) mice, in which Sox10 was selectively deleted in Schwann cells throughout the PNS starting at the precursor cell stage (Finzsch et al., 2010). From these mice, we isolated sciatic nerves at P7, prepared total RNA and analyzed expression levels. RT-PCR confirmed the strong reduction of \textit{Sox10} expression as a result of its Cre-mediated deletion and an equally strong

\begin{figure}[h]
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\caption{\textit{Gldn}, \textit{Nfasc} and \textit{Nrcam} as Sox10 target genes in MSC80 cells and in vivo. (a, d) Venn diagram showing overlap of genes downregulated in Sox10-negative MSC80 cells (light blue) and exhibiting decreased (a) or increased (d) expression in cultured primary rat Schwann cells (light red) upon differentiation according to (Camarena et al., 2017). (b, e) List of the 56 genes (b) with reduced expression in Sox10-negative MSC80 and differentiating Schwann cells, and the 27 genes (e) with reduced expression in Sox10-negative MSC80 cells and increased expression in differentiating Schwann cells. (c, f) GO analysis for biological processes enriched in gene sets shown in (b, e). Processes are sorted by statistical significance. reg, regulation of; neg, negative. (g) Comparison of transcript levels for \textit{Sox10}, \textit{Krox20}, \textit{Mbp}, \textit{Gldn}, \textit{Nfasc} and \textit{Nrcam} in sciatic nerves prepared from Sox10\(^{Δ/Δ}\) mice and controls at P7 by quantitative RT-PCR. Values for each gene in wildtype controls were set to 1 and levels in mutants were set in relation to this (\(n=6\)). Statistical significance was determined by Student's \(t\) test (\(*p ≤ .05; **p ≤ .01; ***p ≤ .001\))}
\end{figure}
decrease in expression of the central regulator of myelination Krox20 and the myelin gene Mbp as known Sox10 target genes (Figure 2g). Strong reductions were also observed for Gldn, Nfasc and Nrcam transcripts thus confirming the Sox10 dependence of their Schwann cell expression in vivo in agreement with our bioinformatic prediction.

3.3 Sox10-responsive regions of genes associated with node formation in Schwann cells

If Gldn, Nfasc and Nrcam are direct Sox10 target genes, there should be Sox10 binding sites within or near these genes. Previous ChIP-Seq studies on sciatic nerves with antibodies against Sox10 (Lopez-Anido et al., 2015) had detected such binding (Figure 3a). For Gldn, Sox10 binding had been mapped to the promoter as well as two regions localized 50 kb upstream and 41 kb downstream of the transcriptional start site (TSS). All three Gldn-associated regions furthermore exhibited substantial sequence conservation among rat, mouse and human genomes. Promoter and 50 kb upstream region were even conserved in chicken. Sox10 binding was also detected 18 kb upstream and 68 kb downstream of the Nfasc TSS as well as 28 kb upstream, 27 downstream and 147 kb downstream of the Nrcam TSS (Figure 3a). All Sox10 binding regions in the Nfasc and Nrcam loci showed strong sequence conservation among rat, mouse and human genomes with the regions
downstream of the TSS being localized in the large first intron of the respective gene. In the following studies, each of these evolutionary conserved regions (ECR) is named according to its localization relative to the TSS (in kb, with upstream sequences in negative and downstream sequences in positive numbers) and the corresponding gene with exception of the Gldn promoter region (referred to as Gldn prom).

To analyze whether identified ECRs can respond in their activity to the presence of Sox10, we combined each region with a minimal promoter and a luciferase reading frame in reporter plasmids. All reporter plasmids were transiently transfected into N2a neuroblastoma cells in the presence or absence of Sox10 expression plasmids. These cells were chosen because N2a cells are reproducibly transfected with good efficiency and share a neural crest origin with Schwann cells, but do not exhibit endogenous expression of the key Schwann-cell specific transcription factors such as Sox10. Among Gldn ECRs, only the −50 Gldn mediated a robust Sox10-dependent reporter gene activation (Figure 3b). In case of the Nfasc ECRs, both −18 Nfasc and +68 Nfasc were significantly activated by Sox10 (Figure 3c). Substantial Sox10-dependent activation was also detected for all three Nrcam ECRs, with −28 Nrcam being most effective (Figure 3d). However, compared to regulatory regions from previously analyzed myelin genes, Sox10-dependent activation rates are modest (Bondurand et al., 2001; Fröb et al., 2019; Peirano et al., 2000).

### 3.4 Schwann cell genes associated with node formation as joint common targets of Sox10 and Krox20

Many genes that are expressed in Schwann cells as part of the differentiation program are jointly regulated by Sox10 and Krox20 (Srinivasan et al., 2012). Therefore, we asked how the Sox10-responsive ECRs from the Gldn, Nfasc and Nrcam genes would respond to Krox20 alone or in combination with Sox10 (Figure 4a–f). Intriguingly, all ECRs were activated by Krox20, most of them more strongly by Krox20 than by Sox10. Reporter plasmids with the −28 Nrcam and the +27 Nrcam ECRs exhibited comparable induction rates for Sox10 and Krox20 (Figure 4d,e). All ECRs furthermore exhibited higher activities in the presence of both Sox10 and Krox20 than in

![Figure 4](image-url)

**FIGURE 4** Joint activation of Gldn, Nfasc and Nrcam ECRs by Sox10 and Krox20. (a–f) Luciferase assays in N2a cells transiently transfected with reporter genes under control of regulatory regions from the Gldn (a), Nfasc (b, c) and Nrcam (d–f) genes in the absence (−) or presence of Sox10, Krox20 or a combination thereof. Reporter gene expression was determined for each reporter construct as fold induction ± SEM over expression in the absence of co-transfected effectors (n = 3). The statistical significance of an increased reporter gene expression in the combined presence of Sox10 and Krox20 was determined by one way analysis of variance (ANOVA) with Bonferroni correction (*p ≤ .05; **p ≤ .01; ***p ≤ .001)
the presence of only one of these transcription factors. Responses ranged from slightly more than additive (as in the case of −50 Gldn and −18 Nfasc, Figure 4a,b) to almost or more than multiplicative (as in the case of +27 Nrcam and +147 Nrcam, Figure 4e,f). These more than additive to multiplicative activation rates point to synergism between Sox10 and Krox20 and argue that the identified ECRs of the Gldn, Nfasc and Nrcam genes are under joint control of Sox10 and Krox20.

3.5 | Functional Sox10 binding sites in Gldn, Nfasc and Nrcam regulatory regions

To study the molecular mode of Sox10 action in more detail, we searched in a selection of ECRs for the presence of sequences that conformed to the general consensus for Sox binding sites 5'- (A/T) (A/T)CAA(A/T)G-3', allowing one mismatch at most (Guth & Wegner, 2008). In case of the −50 Gldn, we identified 8 potential

![Figure 5](image-url)
binding sites with one mismatch and two sites fully conforming to the consensus (Figure 5a). In case of the +68 Nfasc, one of seven potential binding sites was without mismatch, the others had one. Among the 13 potential binding sites within −28 Nrcam, one fitted the consensus exactly. Given the large number of potential binding sites within each ECR, we chose to concentrate on those without mismatch.

To analyze the ability of these sites to interact with Sox10, we performed electrophoretic mobility shift analysis using whole cell extracts of transfected HEK293 cells as protein source. Of the two potential binding sites within the −50 Gldn, only Site 2 exhibited substantial binding (Figure 5b). In several regulatory regions with Schwann cell activity, we have previously observed cooperative binding of two Sox10 molecules as a dimer to a consensus site and a closely spaced sequence with resemblance to a Sox binding site, as exemplified by the C/C' region of the Mpz promoter (Peirano et al., 2000). The running behavior of the complex between Sox10 and Site 2, however, did not have the same mobility as a Sox10 dimer bound to C/C'. Mobility rather resembled a complex containing the B region of the Mpz promoter that is known to bind Sox10 monomers (Peirano et al., 2000). Mutation of the predicted binding site strongly reduced complex formation (Figure 5b).

In case of the mismatch-free site in +68 Nfasc, a complex was obtained with a mobility similar to C/C' indicating binding of a Sox10 dimer (Figure 5c). Closer inspection of the surrounding sequence revealed a second potential Sox10 binding site with two mismatches in a head-to-tail arrangement and with spacing of three nucleotides (Figure 5e). Mutation of the site that fully conforms to the consensus (referred to as Site1a) abolished Sox10 binding, whereas mutation of the site with two mismatches (referred to as Site1b) reduced dimeric to monomeric binding. These results confirm that the identified sequences are involved in mediating the binding of Sox10 dimers. For the −28 Nrcam, we detected again binding of Sox10 monomers to the site that matched the consensus (Figure 5d). Sox10 binding was lost upon mutation of the potential binding site.

The same mutations were introduced for each of the verified sites into the context of the ECRs and consequences were analyzed in luciferase reporter assays in transiently transfected N2a cells (Figure 6). In case of −50 Gldn, Sox10-dependent reporter gene activation was only slightly reduced, and the decrease did not reach statistical significance (Figure 6a). For +68 Nfasc and −28 Nrcam, the binding site mutations exhibited a more pronounced and statistically significant effect on Sox10-dependent induction rates of reporter gene expression (Figure 6b,c). None of the ECRs lost its Sox10-responsiveness completely, arguing that other Sox10 binding sites are present within the ECRs, likely among the sites with one mismatch to the consensus.

As expected, responsiveness of the ECRs to Krox20 was not substantially altered by mutation of the Sox binding sites. However, cooperative activation by Sox10 and Krox20 was strongly reduced for all ECRs, including −50 Gldn where the effect of the mutation on Sox10-dependent activation was not significant. These results argue that interference with the normal Sox10 binding pattern to the ECRs severely impacts their cooperative activation by Sox10 and Krox20.

**Figure 6** Effect of Sox10 binding site removal on inducibility of Gldn, Nfasc and Nrcam ECRs. (a–c) Luciferase assays in N2a cells transiently transfected with reporter genes under control of the −50 Gldn (a), +68 Nfasc (b) and −28 Nrcam (c) ECRs with intact (wt) or inactivated (mut) Sox10 binding sites, in the presence of Sox10, Krox20 or a combination thereof (both). Reporter gene expression was determined for each reporter construct as fold increase ± SEM (n = 3) over expression in the absence of co-transfected effectors (arbitrarily set to 1). Differences were statistically significant as determined by Student's t test (**p ≤ .05; ***p ≤ .01; ****p ≤ .001).

3.6 Genetic interaction of Sox10 and Krox20 during PNS node formation in vivo

To verify that node formation in peripheral nerves jointly depends on Sox10 and Krox20 as indicated by reporter gene assays in vitro, we generated mice with constitutive heterozygous deletion of Sox10 and Krox20 (Sox10<sup>+/loxZ</sup>, Krox20<sup>+/cre</sup> mice, referred to as dhet). Using RT-PCR we compared Gldn, Nfasc and Nrcam expression levels in sciatic nerves of these mice at P7 with expression levels in sciatic nerves of the single heterozygous mice that corresponded to standard
expression levels for this postnatal age. Relative to single heterozygous mice, transcript levels for *Gldn*, *Nfasc* and *Nrcam* were substantially reduced in dhet mice, thus confirming the joint impact of Sox10 and Krox20 on the expression of these genes (Figure 7a).

Intriguingly, immunohistochemical staining of teased single fibers from sciatic nerves of wildtype, single and double heterozygous mice at P14 using antibodies directed against Nefh (green) and Caspr (red). Position and size of nodes are marked by bars. Scale bar: 5 μm. (c–e) Length determination of internodes (c), nodes (d) and paranodes (e) in sciatic nerves of wildtype (wt, white bars) as well as single and double heterozygous mice at P14 as mean ± SEM. Between 84 and 98 nodes, paranodes and internodes were measured in teased fiber preparations from three different animals per genotype. The legend at the bottom refers to panels (a), (c), (d), (e). Differences were statistically significant as determined by one way analysis of variance (ANOVA) with Bonferroni correction (*p ≤ .05; **p ≤ .01; ***p ≤ .001)

**FIGURE 7** Effect of Sox10 and Krox20 on *Gldn*, *Nfasc* and *Nrcam* expression in vivo and peripheral nerve fiber characteristics. (a) Comparison of transcript levels for Sox10, Krox20, Mbp, Gldn, Nfasc and Nrcam in sciatic nerves prepared from mice with double (dhet, black bars) or single heterozygosity of Sox10 (Sox10het, light gray) and Krox20 (Krox20het, dark gray) at P7 by quantitative RT-PCR. Highest wildtype-corresponding expression levels for each gene were set to 1 (red horizontal line) and levels in other genotypes were set in relation to this (n = 3). (b) Immunohistochemical staining of teased single fibers prepared from sciatic nerves of wildtype, single and double heterozygous mice at P14 using antibodies against Nefh and Caspr. Position and size of nodes are marked by bars. Scale bar: 5 μm. (c–e) Length determination of internodes (c), nodes (d) and paranodes (e) in sciatic nerves of wildtype (wt, white bars) as well as single and double heterozygous mice at P14 as mean ± SEM. Between 84 and 98 nodes, paranodes and internodes were measured in teased fiber preparations from three different animals per genotype. The legend at the bottom refers to panels (a), (c), (d), (e). Differences were statistically significant as determined by one way analysis of variance (ANOVA) with Bonferroni correction (*p ≤ .05; **p ≤ .01; ***p ≤ .001)
activation of Glnd, Nfasc and Nrcam expression by Sox10 and Krox20 and therefore support a potential role of this cooperativity in node formation and saltatory conduction.

4 | DISCUSSION

By performing a genome-editing approach in a mouse Schwann cell line, we show in this manuscript that the transcription factor Sox10 — aside from its importance for the expression of myelin sheath components — has a strong impact on the expression of molecules associated with cell-to-cell and cell-to-ECM adhesion in peripheral glial cells. This fits to other observations. Schwann cells are derived from the neural crest and it has been previously shown that Sox10 and the closely related Sox9 are intricately involved in regulating neural crest induction, survival, delamination and migration (Cheung et al., 2005). Especially the two latter processes require substantial adjustments in the cell’s adhesion properties. It has furthermore been shown for the related Sox9 in chondrocyte development that many of its direct target genes encode either structural components of cartilage or adhesion molecules (Lefebvre, Angelozzi, & Haseeb, 2019).

Among the Sox10-dependent adhesion-associated molecules, we identified Schwann cell-expressed proteins with relevance for formation of node and paranode, that is, Glnd, Nfasc and Nrcam. This argues that Sox10 may not only control myelin sheath formation by regulating the expression of structural components of the myelin sheath and enzymes of lipid biosynthetic pathways in Schwann cells (Lopez-Anido et al., 2015; Svaren & Meijer, 2008). It may also directly influence formation of the node. In line with the assumption that Sox10 regulates the Schwann cell-specific expression of nodal and paranodal components in vivo, RT-PCR analyses on sciatic nerves of mice with a Schwann cell-specific Sox10 deletion confirmed a dramatic downregulation of Glnd, Nfasc and Nrcam levels.

Mining of published ChIP-Seq data furthermore identified regions in the vicinity of each gene that were bound by Sox10 in Schwann cells. The majority of these regions were able to activate reporter gene expression in a Sox10-dependent manner in a neural crest-related cell line. Sox10 binding to these regions was furthermore confirmed in vitro and inactivation of Sox10 binding sites substantially reduced the responsiveness of these regions to Sox10 in reporter assays. We therefore conclude that Sox10 directly controls Schwann cell expression of Glnd, Nfasc and Nrcam by binding to regulatory regions in the vicinity of these genes and activating them. In vitro studies of regulatory regions from genes that code for components of the myelin sheaths had shown that some contain sites that bind only one Sox10 molecule, whereas others allow the cooperative binding of two Sox10 molecules (Bondurand et al., 2001; Peirano et al., 2000). Although there is evidence that in a particular regulatory region monomeric and dimeric sites cannot be exchanged (Peirano & Wegner, 2000; Schlierf, Ludwig, Klenovsek, & Wegner, 2002), the reason for the existence of these different types of binding sites is still a matter of speculation (Wegner, 2010). Whereas regulatory regions of Glnd and Nrcam contain monomeric sites, the ECR 68 kb downstream of the transcriptional start of the Nfasc gene harbors a dimeric site. It is currently unknown whether there is a functional relevance to this different binding behavior of Sox10.

In case of genes that code for structural components of the myelin sheath or that influence lipid metabolism in Schwann cells, many regulatory regions were found to bind both Sox10 and Krox20 and be cooperatively activated by both factors (Srinivasan et al., 2012). Similarly, nodal and paranodal components appear to be under joint control of Sox10 and Krox20, with some of the responsible regulatory regions exhibiting synergistic activation. Analysis of Glnd, Nfasc and Nrcam expression levels in sciatic nerves of compound mutant mice with heterozygous mutations in the Sox10 and Krox20 genes provide further evidence for the relevance of this finding in vivo.

By placing the expression of Glnd, Nfasc and Nrcam under control of the same transcription factors as lipids and protein components of the myelin sheath, a biologically meaningful coupling of these expression modules is achieved. However, there is an intriguing difference. In case of myelin gene regulatory regions, Sox10 is usually the strong activator and assisted by a less active Krox20 (Bondurand et al., 2001; Peirano et al., 2000). In case of the Glnd, Nfasc and Nrcam regulatory regions, the roles of Sox10 and Krox20 appear reversed with Krox20 being the stronger activator. While we do not know the functional relevance of this observation, it may point to a fine-tuning mechanism that permits variation in the exact regulation of myelin genes versus nodal and paranodal genes despite usage of the same transcription factors.

A coordinated expression of myelin sheath components on the one hand and node-related proteins on the other hand would also make sense for oligodendrocytes of the CNS. It is currently unknown whether Sox10 also influences expression of nodal and paranodal proteins in oligodendrocytes. Analysis of Sox10 ChIP-Seq peaks in rat spinal cord (Lopez-Anido et al., 2015) revealed Sox10 binding in one Glnd-associated, three Nfasc-associated and two Nrcam-associated regions, arguing that expression of node-related genes may also be under control of Sox10 in the CNS. However, apart from +147 Nrcam, bound regions differ in Schwann cells and oligodendrocytes. Additionally, Sox10 may require another cooperation partner because Krox20 does not substantially contribute to oligodendrocyte differentiation and CNS myelination (Sock & Wegner, 2019). Considering that many of the functions performed by Krox20 in differentiating Schwann cells are performed by Myrf in oligodendrocytes (Hornig et al., 2013), it will be interesting to see in future studies whether Myrf has a say in the regulation of nodal and paranodal gene expression in oligodendrocytes.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in this published article or were deposited in GEO under accession number GSE152687 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1526872).

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