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SOX4 inhibits oligodendrocyte differentiation of embryonic neural stem cells *in vitro* by inducing *Hes5* expression

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A B S T R A C T

SOX4 has been shown to promote neuronal differentiation both in the adult and embryonic neural progenitors. Ectopic SOX4 expression has also been shown to inhibit oligodendrocyte differentiation in mice, however the underlying molecular mechanisms remain poorly understood. Here we demonstrate that SOX4 regulates transcriptional targets associated with neural development in neural stem cells (NSCs), reducing the expression of genes promoting oligodendrocyte differentiation. Interestingly, we observe that SOX4 levels decreased during oligodendrocyte differentiation *in vitro*. Moreover, we show that SOX4 knockdown induces increased oligodendrocyte differentiation, as the percentage of Olig2-positive/2′,3′-Cyclic-nucleotide 3′-phosphodiesterase (CNPase)-positive maturing oligodendrocytes increases, while the number of Olig2-positive oligodendrocyte precursors is unaffected. Conversely, conditional SOX4 overexpression utilizing a doxycycline inducible system decreases the percentage of maturing oligodendrocytes, suggesting that SOX4 inhibits maturation from precursor to mature oligodendrocyte. We identify the transcription factor *Hes5* as a direct SOX4 target gene and we show that conditional overexpression of *Hes5* rescues the increased oligodendrocyte differentiation mediated by SOX4 depletion in NSCs. Taken together, these observations support a novel role for SOX4 in NSC by controlling oligodendrocyte differentiation through induction of *Hes5* expression.

1. Introduction

Neural stem cells (NSCs) are multipotent progenitor cells residing throughout both the developing and the adult brain in specialized niches of the subventricular zone (SVZ) and the subgranular layer (SGL) of the hippocampal dentate gyrus (Alvarez-Buylla et al., 2001). During corticogenesis, the radial glia, which are considered embryonic NSCs, locate at the ventricular zone (VZ) and differentiate into neurons, astrocytes and oligodendrocytes while migrating towards the cortical plate (CP) (Fishell and Kriegstein, 2003; Götz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). Growing evidence demonstrates that NSCs transplantation shows potential in treating pathological conditions of the central nervous system (CNS) such as hypoxic-ischemic encephalopathy, multiple sclerosis, Alzheimer's disease and cerebral stroke (Bacigaluppi et al., 2016; Blurton-Jones et al., 2009; Braccioli et al., 2017; Daadi et al., 2010; Martino and Pluchino, 2006; Park et al., 2002). NSC differentiation is a finely tuned process regulated amongst others by the Notch pathway, which is involved not only in neural progenitor maintenance in the adult and embryonic compartment, but also in fate decisions and lineage commitment (Ables et al., 2011; Louvi and Artavanis-Tsakonas, 2006). For instance, the Notch target gene Hes5, a basic-helix-loop-helix transcriptional repressor, has been described as negatively regulating neural and oligodendrocyte differentiation while promoting NSC maintenance (Kondo and Raff, 2000; Liu et al., 2006; Ohtsuka et al., 1999; Ohtsuka et al., 2001).

SOX4 is a transcription factor belonging to SoxC group of the SRY-related HMG-box (SOX) family, which are important for embryonic development and tissue homeostasis, as well as cancer progression (Vervoort et al., 2013a; Wegner, 2010). The SoxC family consists of SOX4, SOX11 and SOX12, which share a high level of identity within the high-mobility group (HMG) domain and the C-terminal transactivation domain (TAD) (Dy et al., 2008). SOX4 expression overlaps with other SoxC factors in several compartments during embryogenesis, including mesenchymal tissue, peripheral and central nervous system (Vervoort et al., 2013a). During chick development, SOX4 and SOX11 have been shown to induce the expression of neuronal genes such as βIII-tubulin and MAP2, suggesting they may regulate the late stages of neuronal differentiation (Bergsland et al., 2006). Additionally, SOX4 and SOX11 promote neural progenitor proliferation and survival (Bhattaram et al., 2010). Moreover, Mu and colleagues demonstrated that SOX4 and SOX11 promote neurogenesis in the SGL by activating

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doublecortin (DCX) expression (Mu et al., 2012). Although highly homologous, SOX4 and SOX11 have been show to play *non*-redundant roles by regulating separate population of neurons during corticogenesis: SOX11 interacts with Neurogenin1 in early born neurons hence activating the pro-neural gene NeuroD1, while SOX4 is expressed by intermediate progenitor cells and promotes their maintenance by interacting with Neurgenin2 to activate Tbrain2 (Chen et al., 2015). SOX4 has also been reported to regulate gliogenesis, as prolonged expression of a SOX4 transgene under the control of the human GFAP promoter activating transgene expression in radial glia and astrocytes, leads to defects in the morphology of the cerebellum and ataxia in mice (Hoser et al., 2007). Moreover, the expression of a SOX4 transgene under control of the myelin basic protein (MBP) promoter, which is active in differentiating oligodendryocytes, impairs myelination in the whole CNS (Potzner et al., 2007). Accordingly, it has been shown that ectopic expression of SOX4 in Schwann cells under control of myelin protein zero (MPZ) gene and the enhancer of MBP inhibits myelination and worsens neuropathic phenotype in a mouse model of Charcot–Marie–Tooth 4C disease (Bartesaghi et al., 2015). Taken together these observations suggest that SOX4 negatively regulates glial differentiation, however the underlying molecular mechanisms regulated by SOX4 remain unknown. To this end we explored the role of SOX4 during embryonic NSC differentiation. We combined global transcriptional profiling with *in vitro* analyzes, identifying SOX4 as a negative regulator of oligodendrocyte differentiation. Finally, we identify *Hes5* as a target of SOX4, which may mediate SOX4 repression of NSC oligodendrocyte differentiation. Taken together these findings indicate a unique role for SOX4 in regulating oligodendrocyte differentiation of NSCs.

2. Results

2.1. Genome-wide analysis of SOX4 DNA-binding in NSCs

SOX4, together with its homologous SOX11, has been previously described to promote differentiation of both embryonic and neural progenitor differentiation (Bergsland et al., 2006; Mu et al., 2012). However, due to the high redundancy between SOX4 and SOX11, the role of SOX4 alone in the regulation of embryonic NSC biology has not been properly evaluated. In order to determine the specific function of SOX4 in NSC, we first investigated the genome-wide binding of SOX4 through chromatin-immunoprecipitation combined with sequencing (ChIP-seq) using NSCs cultured as neurospheres derived from the prefrontal cortex of E14.5 CD-1 embryos. SOX4-bound regions were effectively detected as exemplified by peaks mapped at the region of the genes *Id2* and *Adi1* (Fig. 1A). Analysis of the distribution of SOX4-binding events revealed an enrichment for SOX4-binding in promoter regions when compared to random genomic sequences (Fig. 1B). We also detected enrichment for SOX4 binding in 5′-untraslated regions (5′UTR) (Fig. 1B) and observed enrichment for SOX4 binding around transcriptional start sites (TSS) (Fig. 1B and E). SOX4-signal was enriched around the center of the peaks, thus validating the peak-calling procedure (Fig. 1C). The SOX4-motif was also enriched at the center of the peaks, hence confirming sequence-specific binding (Fig. 1D). *De novo* motif analysis identified SOX4-motif as the most enriched motif, followed by the binding-motives of the transcription factors Interferon Regulatory Factor 6 (IRF6), E2F Transcription Factor 1 (E2F1) and YY1 (Fig. 1F). To identify the genes that are neighboring SOX4-bound regions, the genes whose TSS was within 5 kilobase (kb) from peaks were further selected for gene-ontology (GO) analysis. SOX4-bound genes showed a significant association with abnormality of the brain, morphological abnormality of the central nervous system and cognitive impairment (Fig. 1G). These observations are in agreement with a previous report indicating that transgenic SOX4 expression within the CNS, mediated by the glial fibrillary acidic protein (GFAP) promoter, induces architectural cerebellar defects and ataxia (Hoser et al., 2007). Taken together, these data show that in NSCs SOX4 is binding to promoter regions of genes regulating CNS development.

2.2. SOX4 regulates neurodevelopment associated genes in NSCs

In order to identify the genes transcriptionally modulated by SOX4, we first generated short-hairpin RNAs (shRNA)-mediated SOX4 knockdown (KD) NSCs. Analysis of SOX4 mRNA and protein expression revealed successful KD (Figs. 2A-B and S1A-B). Next, we performed RNA-sequencing (RNA-seq) on SOX4 KD NSCs which confirmed successful knockdown of SOX4 mRNA (Fig. 2C). We confirmed the specificity of the shRNAs targeting SOX4 by analyzing the expression of the other members of the SoxC family: both *Sox11* and *Sox12* were not found reduced in the RNA-seq data upon SOX4 KD (Fig. S1C). Analysis of RNA-seq data showed that 389 genes were significantly induced and 226 genes were significantly repressed by SOX4 (Fig. 2D and E). To identify the genes that are regulated by SOX4 in a direct manner in NSCs, the set of differentially expressed genes was overlapped with the set of genes whose TSS is within 5 kb from peaks derived from the ChIP-seq analysis. Of the SOX4-modulated genes, 136 were found to be both induced and bound by SOX4, whilst 130 were found to be repressed and bound by SOX4 (Fig. 2E and F). To again focus on biological processes regulated by SOX4, we performed gene ontology (GO)-term analysis using the set of SOX4-bound and regulated target genes. We observed the most significant association with CNS development; furthermore we observed a significant association with Notch signaling pathway (Figs. 2G and S1D). In order to outline which CNS developmental processes were regulated by SOX4, a weighted gene-set enrichment analysis (GSEA) was performed using the RNA-seq dataset ranked by log-fold change. This analysis revealed a significant enrichment of genes that are induced during oligodendrocyte differentiation for genes repressed by SOX4 in the RNA-seq dataset (Fig. 2H). We further observed both a significant enrichment of genes associated with a pro-neural phenotype in glioblastoma and of astrocyte marker genes for genes induced by SOX4 in the RNA-seq dataset (Fig. 2I and J). Taken together, these data indicate that SOX4 can regulate a subset of genes involved in CNS development and Notch signaling; these observation suggest that SOX4 represses genes that are involved in oligodendrogenesis while inducing genes associated with neural and astrocyte differentiation. These findings are in line with reports showing SOX4 and SOX11 as promoting neural in adult NSCs (Mu et al., 2012).

2.3. SOX4 inhibits oligodendrocyte differentiation of NSCs

Since we observed that SOX4 repressed genes involved in oligodendrogenesis, we first investigated SOX4 expression levels during oligodendrocyte differentiation in NSCs. To this end, we induced oligodendrocyte specification of NSCs by removing basic-fibroblast growth factor (bFGF) and epidermal growth factor (EGF) and supplementing triiodothyronine (T3) to the differentiation medium (Fig. 3A) (Baas et al., 1997). SOX4 expression levels were assessed daily, and a gradual decrease in SOX4 protein was detected between day 0 and day 4 (Figs. 3B, S2B–C). Surprisingly, the expression levels of *Sox4* mRNA did not match with the protein levels throughout differentiation (Fig. S2A), suggesting a regulation of SOX4 at post-translational level. Taken together, these observations suggest that SOX4 is downregulated during oligodendrocyte differentiation of NSCs *in vitro*.

To understand whether SOX4 acts as a repressor of oligodendrocyte differentiation, we wished to determine whether modulation of SOX4 expression altered oligodendrocyte differentiation in NSCs. To this end we differentiated SOX4 KD NSCs towards oligodendrocytes. After 5 days of differentiation, SOX4 KD increased the percentage of 2′–3′ cyclic nucleotide 3′phosphodiesterase (CNPase) + -oligodendrocytes, whilst the percentage of Olig2 + -oligodendrocyte precursor cells remained unaffected when compared to control (Fig. 3C-E). This suggest that SOX4 inhibits the maturation of NSCs from Olig2 + -precursor cells into CNPase + -cells during oligodendrocyte differentiation. In order to

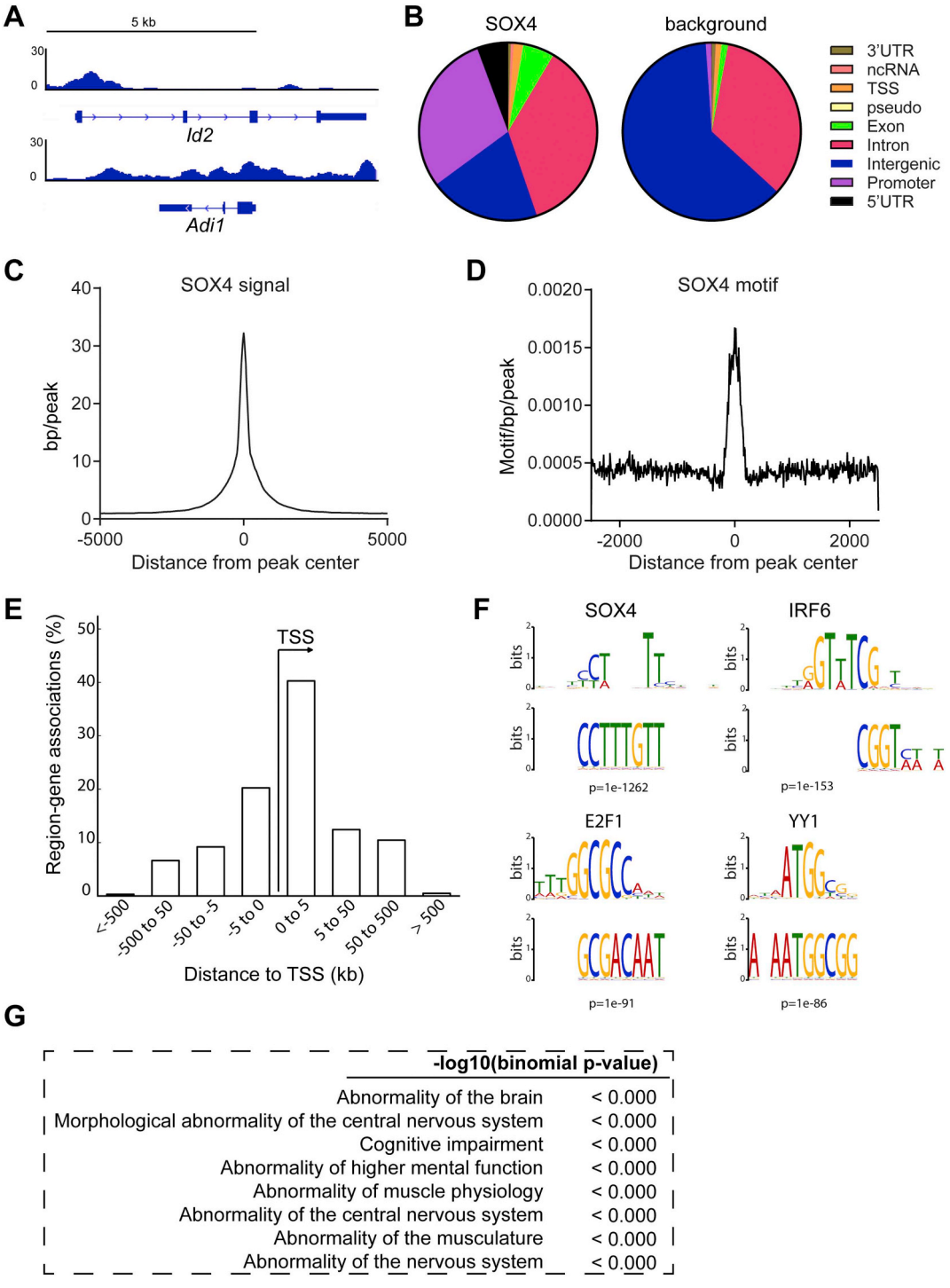

Fig. 1. Genome-wide analysis of SOX4 chromatin-binding in NSCs.

investigate whether this directly, SOX4 was ectopically expressed in NSCs utilizing a doxycycline (dox)-inducible system. Upon 24 h dox treatment, SOX4 overexpression (OE) was clearly detected, but not in the control NSCs (Figs. 3F and S2D). Upon 5 days of differentiation, SOX4 expression reduced the percentage of CNPase+ oligodendrocytes, whilst the percentage of Olig2+-oligodendrocyte precursor cells remained unaltered when compared to control (Fig. 3G-I). These data confirm that SOX4 represses the differentiation of Olig2+-precursor cells into CNPase+-cells during oligodendrocyte differentiation. Taken together, these observations show that SOX4 inhibits oligodendrocyte formation during NSCs differentiation.

2.4. SOX4 inhibits oligodendrocyte differentiation by inducing Hes5 mRNA expression

In order to understand the mechanism by which SOX4 regulates NSC oligodendrocyte differentiation, the genes of the Notch signaling pathway were further analyzed. The Notch signaling pathway was identified in our GO-term analysis (Fig. 2G) and is fundamental for the regulation of NSC differentiation (Ables et al., 2011; Louvi and Artavanis-Tsakonas, 2006). Amongst the genes identified, the transcription factor *Hes5* was found to be induced by SOX4 in the RNA-seq data (Fig. 4A). Moreover, we observed a binding site of SOX4 on the

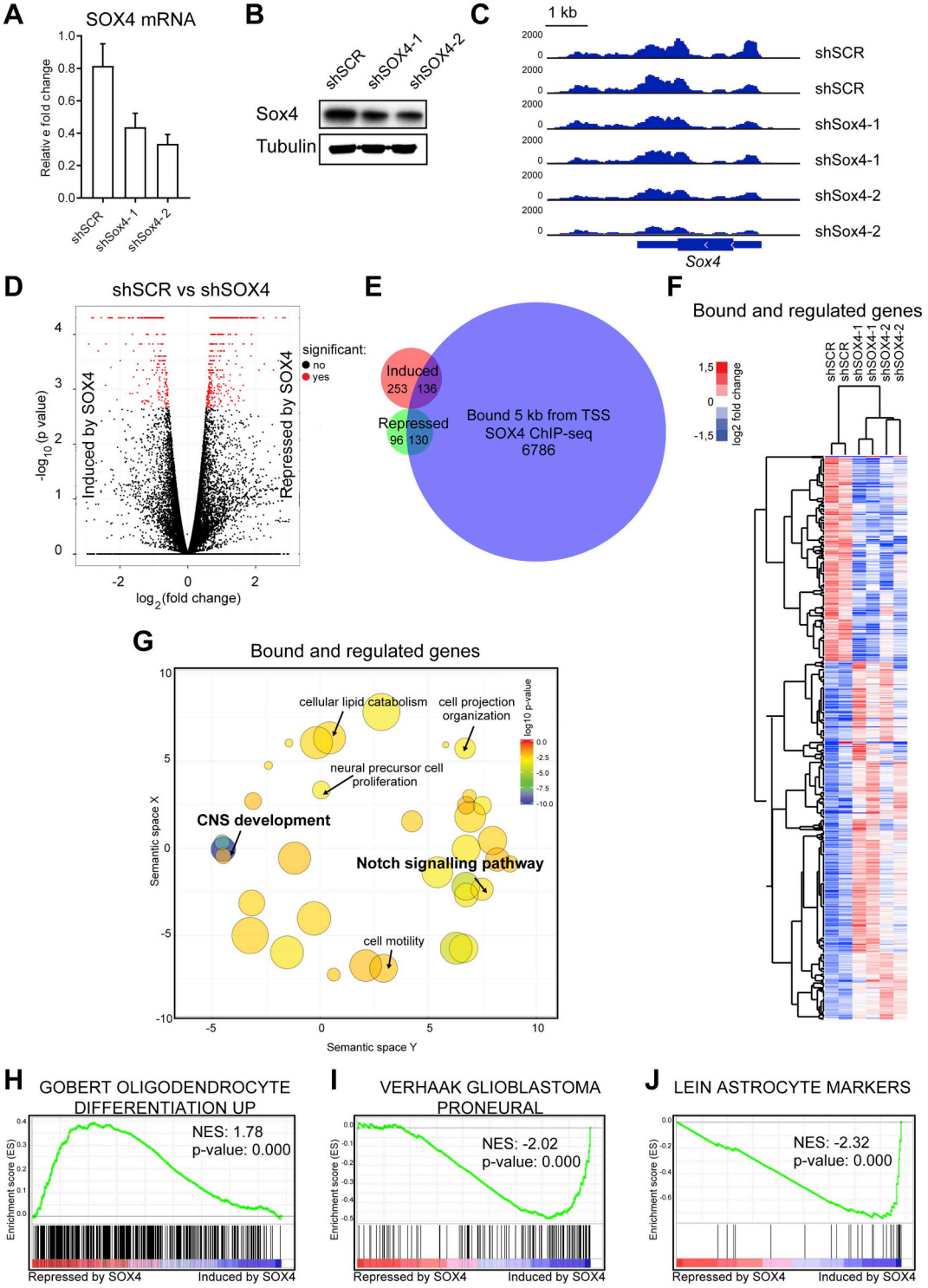

Fig. 2. SOX4 regulates expression of CNS development related genes and represses oligodendrocyte differentiation genes.
RNA-seq was performed on SOX4 KD NSCs. Putative targets were identified by overlapping the expression data with the ChIP-seq dataset. (A) Measure of SOX4 mRNA levels upon SOX4 KD with 2 independent shRNAs (n = 3). (B) Representative western blot showing efficient knockdown of SOX4. Full lenght blots are presented in Supplementary Fig. S1A. (C) Visualization of RNA-seq reads around the genomic locus of *SOX4* in control and KD conditions. (D) Volcano-plot representing differentially expressed genes in SOX4 KD NSCs compared to control. (E) Venn-diagram showing overlap between SOX4-bound and -regulated genes. (F) Heat-map showing the expression of SOX4-bound and -regulated genes. (G) Gene ontology analysis using REVIGO showing the genes associated with CNS development and Notch signaling pathway. (H) Gene-set enrichment analysis (GSEA) representing the enrichment of oligodendrocyte differentiation genes in the RNA-seq expression dataset ranked on log fold change after SOX4 KD. (I) GSEA representing the negative enrichment of pro-neural and (J) astrocyte differentiation genes in the RNA-seq expression dataset ranked on log fold change after SOX4 KD.

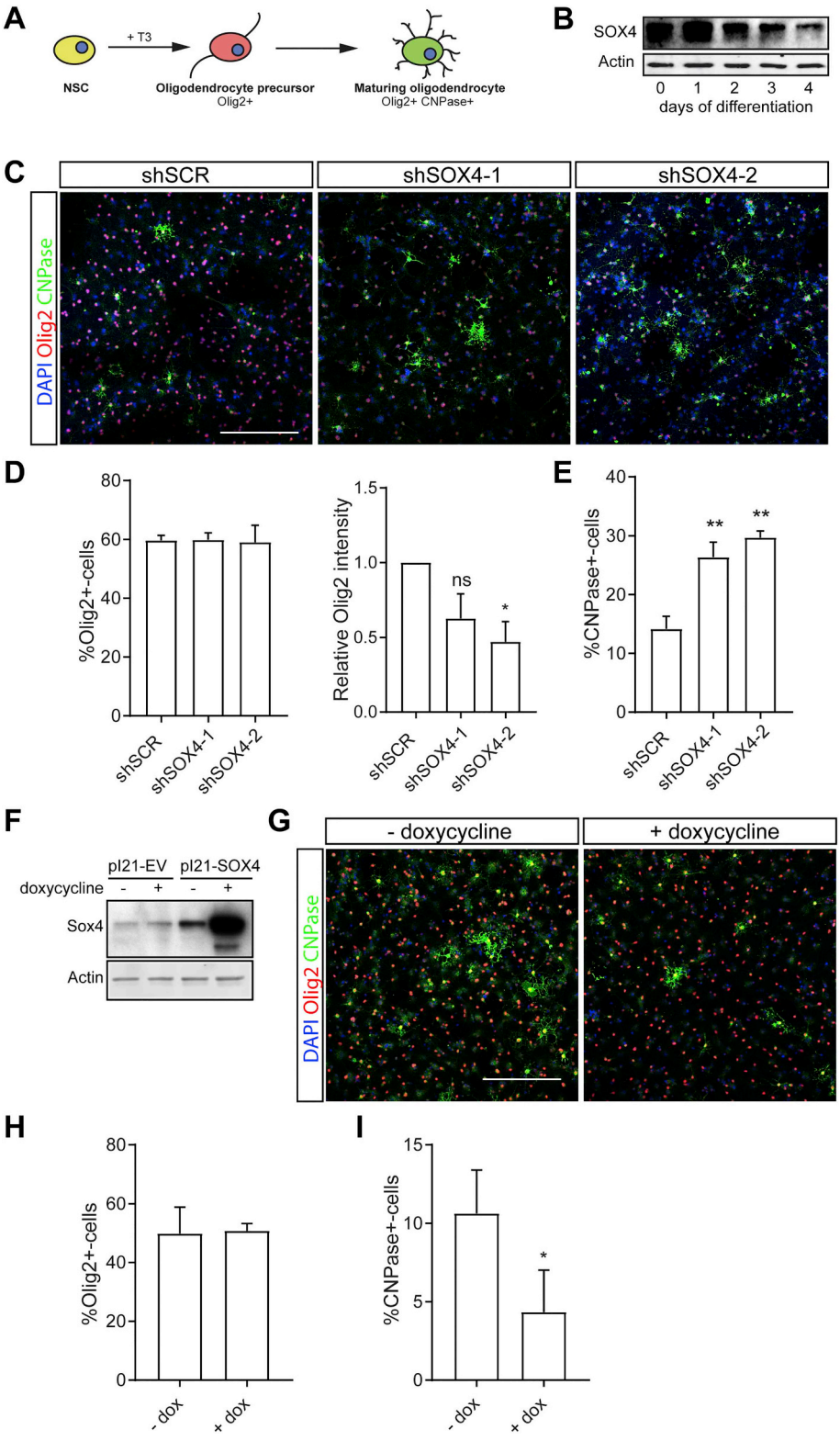

Fig. 3. SOX4 represses oligodendrocyte differentiation of NSCs *in vitro*.
NSCs were differentiated for 4 days in presence of T3. (A) Schematic description of oligodendrocyte markers used in the study. (B) Representative western blot showing SOX4 levels during 4 days of oligodendrocyte differentiation induced by T3. Full lenght blots are presented in Supplementary Fig. S2B. (C) SOX4 KD were differentiated for 4 days in presence of T3. Representative confocal images showing the expression of the oligodendrocyte markers Olig2 (red) and CNPase (green). DAPI co-stained nuclei in blue (bar = 200 μm). (D) and (E): quantification of (C) (n = 3), ** = $p < .01$. SOX4 OE NSCs were differentiated for 4 days in presence of T3. (F) Representative western blot showing conditional SOX4 overexpression in presence of doxycycline. (G) Representative confocal images showing the expression of the oligodendrocyte markers Olig2 (red) and CNPase (green). DAPI co-stained nuclei in blue (bar = 200 μm). (H) and (I): quantification of (G) (n = 3), * = $p < .05$. Dox: doxycycline.

promoter region of *Hes5* in the ChIP-seq dataset (Fig. 4A). To validate SOX4 binding to the promoter region of *Hes5*, we analyzed the promoter sequence of *Hes5* for predicted SOX4 binding sites with ContTra v2 (Broos et al., 2011). This led to the identification of 3 putative binding sites for SOX4 (Fig. 4A). To investigate the binding between SOX4 and these predicted binding sites, we performed an oligonucleotide pull-down assay by incubating cell lysates of HEK293T cells with biotinylated oligonucleotides (dsOligo) containing the putative SOX4 binding sites or their mutated version (MUT) (see Methods, Table 2). We observed binding between SOX4 and each of the 3 dsOligo, and the binding was significantly abolished by mutating SOX4 binding site in dsOligo 1 and dsOligo 3 (Figs. 4B and S3A–B). Additionally, we observed that SOX4 binding was stronger with dsOligo 3, suggesting a stronger interaction between SOX4 and this region (Figs. 4B and S3A–B). Next, we confirmed the regulation of *Hes5* mRNA expression by SOX4 in NSCs by knocking down SOX4 using two

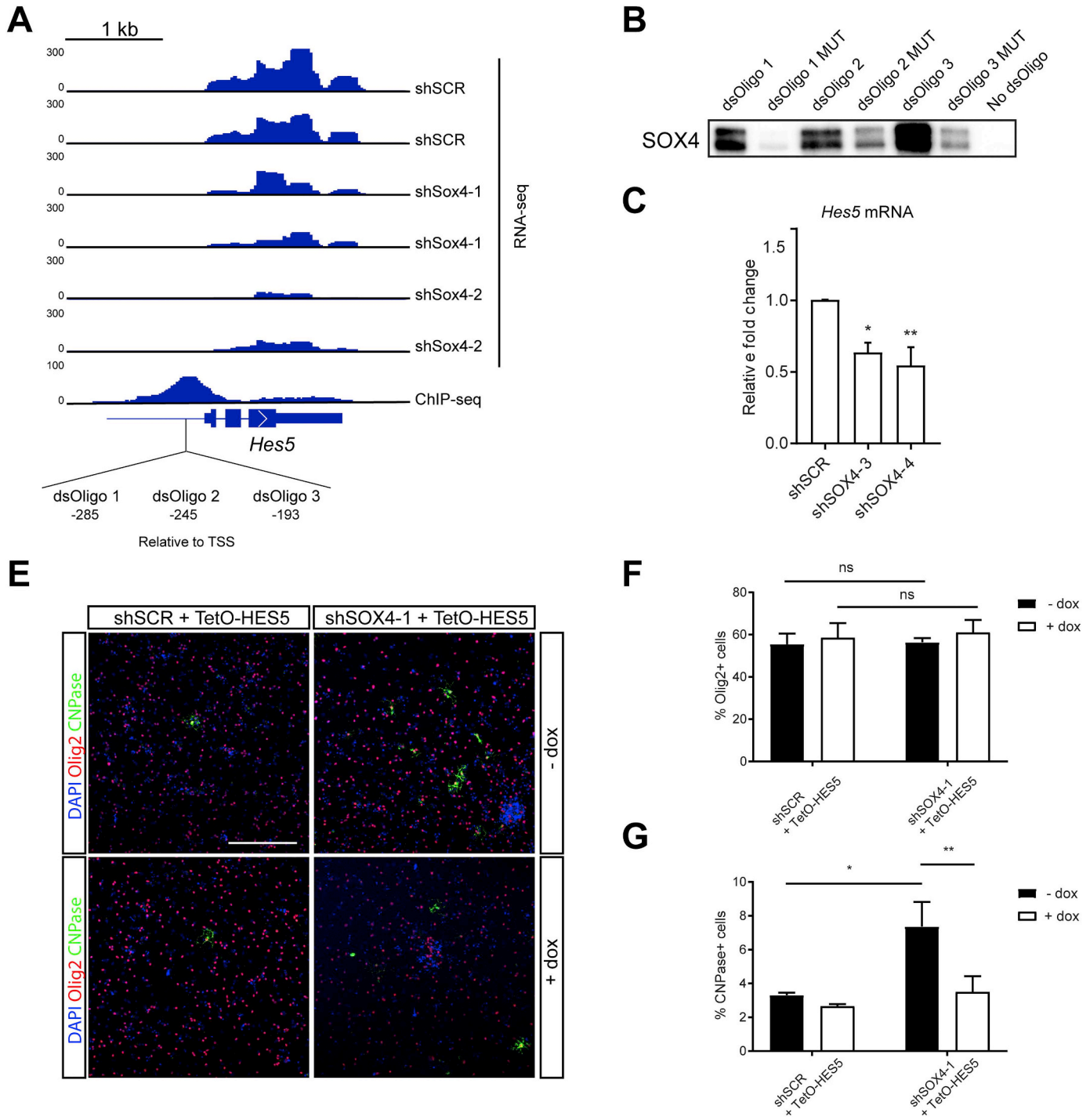

Fig. 4. SOX4 represses oligodendrocyte differentiation of NSCs by directly inducing *Hes5 expression.*
(A) Visualization of RNA-seq reads and SOX4 ChIP-seq profile around the genomic locus of *Hes5* in control and KD conditions. The scheme underneath indicates the regions where the dsOligo probes containing SOX4 binding sites used in (B) were designed based on predicted SOX4 binding sites (see Methods). Positions relative to *Hes5* TSS indicate positions of predicted SOX4 binding sites. (B) Lysate of HEK293T cells overexpressing SOX4 was incubated with byotinilated dsOligo probes described in (A) or the respective mutated versions. Representative blot showing binding of SOX4 to the probes. As a negative control no dsOligos were used. Full lenght blot is presented in Supplementary Fig. S3A. (C) Measure of *Hes5* mRNA levels upon SOX4 KD (n = 5) * = p < .05, ** = p < .01. (D) Conditional *Hes5* overexpression in SOX4 KD NSCs during oligodendrocyte differentiation in presence of doxycycline. Representative confocal images showing the expression of oligodendrocyte markers Olig2 (red) and CNPase (green). DAPI co-stained nuclei in blue. (E) and (F): quantification of (D) (n = 3), * = p < .05; ** = p < .01. Dox: doxycycline.

independent shRNAs distinct from those utilized in the RNA-seq experiment (Fig. 4C). Taken together, these observations strongly demonstrate that SOX4 regulates *Hes5* mRNA expression by binding to its promoter region. HES5 has been shown to repress oligodendrocyte differentiation and myelination (Kondo and Raff, 2000; Liu et al., 2006). Therefore, SOX4-mediated induction of *Hes5* expression may explain the inhibition of oligodendrogenesis mediated by SOX4 during NSC differentiation. In order to evaluate whether HES5 overexpression could rescue the increase of oligodendrogenesis upon SOX4 depletion, we conditionally overexpressed HES5 in SOX4 KD NSCs during

oligodendrocyte differentiation using a doxycycline inducible lentiviral vector (TetO-HES5). We first validated the induction of *Hes5* mRNA upon doxycycline treatment (Fig. S3B). Next, we induced oligodendrocyte differentiation of shSCR + TetO-HES5 and shSOX4 + TetO-HES5 NSCs for 4 days in presence or absence of doxycycline. We then analyzed the percentage of CNPase+ and Olig2+ cells with confocal microscopy (Fig. 4D). According to previous results (Fig. 3C-E), the precentage of Olig2+ cells remained unchanged along the different conditions while the percentage of CNPase+ cells was increased upon SOX4 KD, compared to control, confirming that SOX4 inhibits oligodendrocyte maturation (Fig. 4D-F). However, upon doxycycline treatment, shSOX4 + TetO-HES5 NSCs showed a significant decrease in the percentage of CNPase+ cells, compared to shSOX4 + TetO untreated NSCs, indicating that *Hes5* acts as an effector of SOX4 and thereby inhibiting oligodendrocyte differentiation of immature oligodendrocyte precursor cells (Fig. 4D and F). Taken together, these observations strongly suggest that SOX4 negatively regulates oligodendrogenesis, at least in part by inducing the expression of *Hes5*.

3. Discussion

Here we show for the first time a role of SOX4 in regulating oligodendrocyte differentiation from murine embryonic NSCs. SOX4 modulates genes involved in CNS development, specifically repressing genes involved in oligodendrocyte differentiation, while inducing genes associated with neural and astrocyte phenotypes. SOX4 expression inhibits oligodendrocyte differentiation of NSCs *in vitro*, and *Hes5*, as a direct target activated by SOX4, mediates inhibition of oligodendrocyte differentiation. These novel observations suggest that SOX4 inhibits NSC differentiation towards oligodendrocytes by inducing Hes5 expression.

SOX4, belonging to the SoxC family of transcription factors, is highly homologous with SOX11, sharing a high level of identity within the high-mobility group (HMG) domain and the C-terminal transactivation domain (TAD) (Dy et al., 2008). Several studies have provided evidence that SOX4 together with SOX11 regulate neurogenesis. Bergsland and colleagues demonstrated how during neural tube development in chicken both SOX4 and SOX11 induce the expression of pro-neural genes, βIII-tubulin and MAP2, suggesting that SOX4 and SOX11 are crucial for the later differentiation steps of neural differentiation (Bergsland et al., 2006). This is mediated by the direct induction of βIII-tubulin expression by SOX4 and SOX11. In our study we identified the genes regulated directly by SOX4 in NSCs. Interestingly, by knocking down SOX4, and without reducing with SOX11 or SOX12 expression (Fig. S1A), we identify multiple genes involved in CNS development. This suggests that SOX4 has an independent role from SOX11 in regulating a set of genes involved in neural development. In agreement with Bergsland and colleagues, GSEA shows that SOX4 induces pro-neural genes (Bergsland et al., 2006), supporting that SOX4 induction of a neural phenotype is conserved amongst chicken and mouse, although βIII-tubulin was not one of the SOX4-regulated genes (data not shown). This suggests that SOX4-mediated gene regulation is context dependent. SOX4 and SOX11 have been shown to promote neural progenitor proliferation and survival in mouse, as inactivation of these SoxC members induces apoptosis (Bhattaram et al., 2010). These effect can be partially explained by the activation of the expression of TEAD2 by SOX4 and SOX11, since TEAD2 is known to promote cell survival during organogenesis (Bhattaram et al., 2010). SOX4 and SOX11 co-expression in the adult mouse hippocampus has been shown to promote neurogenesis from neural precursor cells (Mu et al., 2012). SOX4 and SOX11 directly induce the expression of the neuronal protein doublecortin (DCX) by binding to its promoter (Mu et al., 2012). However, when compared to our datasets, we cannot find TEAD2 nor DCX amongst the targets regulated by SOX4 (data not shown), suggesting once more a strong context specificity. There is evidence that SOX4 and SOX11 play *non*-redundant roles in neuronal development, since they are expressed by different neural populations during corticogenesis in mice (Chen et al., 2015). SOX11 activates the pro-neural gene NeuroD1 in early born neurons; while SOX4 is required for the maintenance of intermediated progenitors by activating Tbrain2 (Chen et al., 2015). This supports our conclusion that SOX4 plays a unique role, independent from SOX11, in regulating NSC differentiation.

SOX4 and SOX11 have been shown to be expressed by oligodendrocyte precursors (Kuhlbrodt et al., 1998). Potzner et al. demonstrated that sustained expression of a SOX4 transgene induced by the myelin basic protein (MBP) promoter region, which induces SOX4 expression during oligodendrocyte differentiation, inhibiting myelination in the whole CNS (Potzner et al., 2007). SOX4 also inhibits differentiation of Schwann cell, which myelinate the axons of peripheral nerves (Bartesaghi et al., 2015). Bartesaghi and colleagues showed that transgenic expression of SOX4 in Schwann cells through the promoter of the myelin protein zero (MPZ) gene and the enhancer of MBP inhibits myelination in a mouse model of Charcot–Marie–Tooth 4C disease (Bartesaghi et al., 2015). These observations suggest that SOX4 negatively regulates oligodendrocyte differentiation and prevents myelination. Accordingly, we identify SOX4 as a negative regulator of oligodendrocyte differentiation of NSCs. Olig2 is an early marker of oligodendrocyte progenitors which is expressed by oligodendrocyte progenitors, while CNPase is a myelin associated protein that is expressed in later stages during oligodendrocyte differentiation compared to Olig2 (Miron et al., 2011). Interestingly, we observe that SOX4 does not influence the percentage of Olig2 + −cells but represses the number of CNPase+ − cells during NSC differentiation towards oligodendrocyte. However, a decrease in Olig2 intensity per cell was found upon SOX4 KD (Fig. 3D), although the meaning of this reduction is unclear.

Taken together, these data suggest that SOX4 does not influence the fate specification of NSCs towards the oligodendrocyte lineage, but rather their differentiation towards more mature oligodendrocytes. SOX4 could inhibit oligodendrocyte differentiation either by inhibiting the expression of genes positively regulating this process, or inducing the expression of negative regulators (Kondo and Raff, 2000; Liu et al., 2006). The expression of HES5, an inhibitor of oligodendrogenesis and myelination, decreases during oligodendrocyte precursor cell differentiation and overexpression of HES5 inhibits oligodendrocyte differentiation (Kondo and Raff, 2000; Wu et al., 2003). Furthermore, *Hes5*$^{-/-}$ mice have shown increased myelination as shown by an increase of the myelin associated proteins proteolipid protein (PLP), ceramide galactosyl transferase (CGT), MBP, and CNPase expression (Liu et al., 2006). Specifically, HES5 has been demonstrated to directly repress the activity of promoter regions of CGT and MBP (Liu et al., 2006). Taken together these data support the notion that HES5 is a repressor of oligodendrocyte differentiation. We demonstrated that SOX4 directly binds to *Hes5* promoter and increases its mRNA expression, and we have shown that the induction of *Hes5* expression by SOX4 mediates the repression of oligodendrocyte differentiation of NSCs by SOX4. *Hes5* has been described, together with *Hes1*, to promote NSC maintenance during embryogenesis in mice (Ohtsuka et al., 1999). While our data clearly indicate that SOX4 induces *Hes5* expression in NSCs, we found no evidence of SOX4 affecting *Hes1* levels in our RNA-seq dataset (data not shown). This is in contrast with previous observations suggesting that SOX4 depletion in biliary cells leads to reduced expression of *Hes1*, suggesting that SOX4 acts in a cell- specific manner (Poncy et al., 2015). In contrast to our findings, Kuwajima et al. recently provided preliminary data suggested that SOX4 directly represses *Hes5* expression in retinal ganglion cells (RGC) in mice. However, this data needs to be validated in relevant cell types since the only evidence for *Hes5* repression was provided by utilizing a *Hes5* promoter-luciferase assay in HEK293 cells in combination with SOX4 overexpression (Kuwajima et al., 2017). However, if correct, this finding would be another example of the context-dependent role of SOX4. Usui and colleagues showed that in retinal cells inhibition of the Notch pathway by means of

the γ-secretase inhibitor DAPT induced SOX4 expression; moreover, ectopic activation of the Notch pathway by expression of Notch intracellular domain (NICD) suppressed SOX4 expression (Usui et al., 2013). These observations indicate that while SOX4 acts downstream of the Notch pathway by inducing *Hes5* expression in NSCs, it might be inhibited by Notch signaling activation, suggesting a possible feed-back mechanism. Interestingly, a member of the SoxB family, SOX21, has been described to promote hippocampal neurogenesis in adult mice by repressing *Hes5* expression (Matsuda et al., 2012). It would be interesting to investigate whether SOX21-mediated repression of *Hes5* expression promotes oligodendrogenesis, in contrast to SOX4. In conclusion these observations suggest that SOX4 represses NSC differentiation towards oligodendrocytes by inducing the expression of *Hes5*, shedding light on a novel regulator of oligodendrocyte differentiation.

4. Methods

4.1. qPCR

RNA was isolated using the RNAeasy kit (Qiagen, Hiden, Germany) according to manufacturers' instruction. Reverse transcriptase reaction for the generation of cDNA was performed using iScript cDNA synthesis kit (Bio-rad, Hercules, CA) followed by real-time quantification with the LightCycler 96 Real-Time PCR System (Roche Life Sciences, Penzberg, Germany) using SYBR Green Supermix (Bio-Rad) for cDNA application following manufacturers' protocol. Relative expression was calculated with the ΔΔCt method using hypoxanthine-guanine phosphoribosyltransferase (Hprt) to normalize. See Table 1 for the list of primers used.

4.2. Cell culture

HEK293T were cultured in DMEM medium (Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum plus antibiotics. NSCs derived from embryonic day 14.5 CD-1 mice were purchased from R&D Systems, Minneapolis, MN. NSCs were grown as neurospheres in DMEM:F12 medium (Thermo Scientific) supplemented with 50× B-27 (Thermo scientific) with 20 ng/mL EGF and 20 ng/mL bFGF (both Peprotech, Rocky Hill, NJ) plus antibiotics (Thermo Scientific). For experiments NSCs were cultured on 35 mm glasses coated with poli-L-ornithine (10 μ/mL, Sigma Aldrich) and Laminin (1 μ/mL, Sigma Aldrich) and let adhere for 48 h. For the differentiation assay, NSC differentiation was induced by growth factor withdrawal and addition of 50 ng/mL of triiodothyronine (T3) (Sigma Aldrich). Cells were fixed 5 days after in formaldehyde (FA) 4%. Lentiviral particles were generated as previously described (Vervoort et al., 2013b). NSCs were transduced twice in adherence on plates coated with poli-L-ornithine (10 μ/mL) Sigma Aldrich) and Laminin (5 μ/mL, Sigma Aldrich). The following shRNAs vectors were used: shSOX4-1 (TRCN0000012078), shSOX4-2 (TRCN0000012080), shSOX4-3 (TRCN0000234110), shSOX4-4 (TRCN0000012081) and shSCR (SHC002), all from Sigma Aldrich. The cells were selected with puromycin (0.5 μg/mL). For the overexpression experiment the coding region of murine SOX4 was amplified from pcDNA3.1-FLAG-SOX4 (Beekman et al., 2012) with the addition of an N-terminal hemagglutinin (HA) tag peptide, cloned into pDONR221 (Thermo Fisher Scientific) with Gateway BP Clonase II Enzyme mix (Thermo Fisher Scientific) and then into the lentiviral vector pINDUCER21 (ORF-EG) (from Stephen Elledge & Thomas Westbrook) (Addgene plasmid # 46948) (Yang et al., 2011) with Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific) according to manufacturer instructions. For HES5 overexpression, we utilized the lentiviral vector TetO-FUW-HES5, which was a gift from Rudolf Jaenisch (Addgene plasmid # 61536) (Cassady et al., 2014). Overexpression was induced with the addition of 0.5 μg/mL of doxycycline (Sigma-Aldrich) to the culture medium.

Table 1
List of qPCR primers used.

Primer	Sequence
FW-SOX4	CCAGTTCTTGCACGCTGTTT
REV-SOX4	TGTTGCAAGGTAGGAAGCCA
FW-HES5	GAGAAAAACCGACTGCGGAAG
REV-HES5	GCGAAGGCTTTGCTGTGTTT
FW-HPRT	CCTAAGATGAGCGCAAGTTGAA
REV-HPRT	CCACAGGACTAGAACACCTGCTAA

FW: Forward primer.
REV: Reverse primer.

4.3. Western blot

Cells were lysed directly in the plate with Laemmli buffer (0.12 mol/L Tris-HCL (pH 6.8), 4% SDS and 20% glycerol). Protein concentration was measured with the Lowry assay. 40 μg of each sample were analyzed by sodium dodecil sulfate polyacrilammide gel (SDS-PAGE) and transferred by electrophoresis onto polyvinylidene difluoride membrane (Millipore). The membranes were blocked using 5% milk protein in TBST (0.3% Tween, 10 mM Tris pH 8 and 150 mM NaCl in H_2O) and probed with anti-SOX4 (Diagenode, Liège, Belgium, CS-129-100, 1:3000), anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, sc16160, 1:2000), and anti-tubulin (Sigma Aldrich, St. Louis, MS, T5168, 1:50000). Signal was detected using Amersham ECL Western Blotting Detection Reagent (Amersham, Little Chalfont, United Kingdom) or LI-COR Odyssey Imager (LI-COR Biosciences, Lincoln, NE).

4.4. Oligonucleotide pull-down assay

For the oligonucleotide pull-down assay three paris of biotinylated oligonucleotides were designed based on 3 putative binding sites for SOX4 in the promoter region of *Hes5* identified using ConTra v2 (http://bioit.dmbr.ugent.be/contrav2/index.php) (Broos et al., 2011) (Table 2). 2×10^6 HEK293T cells were plated in 10 cm Petri dish and transfected with pcDNA3.1-FLAG-Sox4 using PEI. 48 h later cells were lysed in 500 uL or RIPA buffer (50 mM TrisCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40 (US Biological, Massachusetts, USA), 1% sodium deoxycholate, 0.1% SDS) and 1% HALT Protease Inhibitor Cocktail (Thermo Scientific, Rockford, USA). Samples were incubatd at 4 °C for 15 min and centrifuged at 4 °C for 10 min at full speed. The nuclear extract obtained was used to assay DNA-protein interactions. Streptavidin conjugated beads (Thermo Scientific) were washed in 1 mL of RIPA, centrifuged at 6000 RPM for 30 s three times and at the end resuspended in 300 μL of PBS. Beads were coupled to primers, each mix containing 20 μL of annealed primers, 30 μL of washed streptavidin agarose beads, 15 μL of FBS and 90 μL of PBS. The mixes were incubated for 1 h at RT, centrifuged at 6000 RPM for 1 min at 4 °C. Three washes in PBS were performed and then samples were resuspended in cold binding buffer (500 μL/tube) (10 mM EPES, 10 mM KCL, 0.1 mM EDTA, 80 mM NaCl, 0.5% NP-40, 2 mM DTT, 1% HALT, 1 μL of 0.5 μg salmon sperm DNA per 50 μL of binding buffer). For each condition 40 μL of cell lysate was added and the mixes incubated in rotation for 2 h at 4 °C, centrifuged at 6000 RPM for 1 min at 4 °C. Then, samples were washed 3 times with cold PBS 1% HALT. For each condition 40 μL sample buffer was added, centrifuged at 6000 RPM for 1 min at 4 °C and the supernatant was transferred in a new tube. Samples were then analyzed with Western Blot and probed with anti-SOX4 antibody (Diagenode, CS-129-100, 1:3000).

4.5. Immunofluorescence

For differentiation experiments, cells were fixed in 4% FA and stained with rabbit anti-Olig2 (Millipore, Bedford, MA), mouse anti-CNPase (Abcam, Cambridge, UK), followed by incubation with donkey anti-rabbit AlexaFluor-555 or goat anti-mouse AlexaFluor-488 (both

Table 2
List of biotynilated oligonucletides used in pull-down assay. SOX4 putative (and mutated) binding sites are indicated in bold.

Oligonucleotide	Sequence
dsOligo 1 FW	5'/Biotin/CGTTCCCACAGCCCGG**ACATTGTG**CCGCGCGGCCCACCTGCTC
dsOligo 1 REV	GAGCAGGTGGGCCGCGCGGCACAATGTCCGGGCTGTGGGAACG
dsOligo 1 MUT FW	5'/Biotin/CGTTCCCACAGCCCGG**CACGGAGT**CCGCGCGGCCCACCTGCTC
dsOligo 1 MUT REV	GAGCAGGTGGGCCGCGCGGCACAATGTCCGGGCTGTGGGAACG
dsOligo 2 FW	5'/Biotin/GGGGAGCGAC**CATTGTG**CCCGCGCCAATTCACAGGCAATTTAG
dsOligo 2 REV	CTAAATTGCCTGTGAATTGGCGCGGGCACAATGGTCGCTCCCC
dsOligo 2 MUT FW	5'/Biotin/GGGGAGCGAC**TCGGACA**CCCGCGCCAATTCACAGGCAATTTAG
dsOligo 2 MUT REV	CTAAATTGCCTGTGAATTGGCGCGGGTGTCCGAGTCGCTCCCC
dsOligo 3 FW	5'/Biotin/TTAGCGTGCGCTAATGGGCCGGC**GCCTTTGTGCGGC**CGGCGCC
dsOligo 3 REV	GGCGCCGGCCGCACAAAGGCGCCGGCCCATTAGCGCACGCTAA
dsOligo 3 MUT FW	5'/Biotin/TTAGCGTGCGCTAATGGGCCGGA**ATTGGCACAATTA**CGGCGCC
dsOligo 3 MUT REV	GGCGCCGTAATTGTGCCAATTCCGGCCCATTAGCGCACGCTAA

Thermo Scientifics, 1:250). Nuclei were counterstained with 4′,6-Diamidino-2-Phenylindole (DAPI). Fluorescent confocal images were captured with an AxioCam MRm (Carl Zeiss, Sliedrecht, The Netherlands) on a LSM 700 confocal microscope (Carl Zeiss). The number of Olig2- and CNPase-positive cells were quantified by counting cells in 8 different fields of the culture dish.

4.6. Chromatin immunoprecipitation and sequencing

Before the chromatin immunoprecipitation (ChIP) and sequencing, NSCs were grown as neurospheres in complete medium in 6-wells. ChIP was performed as previously described (Van Boxtel et al., 2013). Briefly, crosslink was performed with disuccinimidyl glutarate (DSG) (Thermo Scientifis) for 45 min followed by 30 min incubation with formaldehyde 1%. The reaction was blocked with glycine 100 mM. Shearing was performed using Covaris S2 (Covaris, Woburn, MA) for 8 min at maximum intensity. The sonicated chromatin was incubated O/N at 4 °C in presence of 10 μg of rabbit anti-SOX4 (Diagenode, CS-129-100) coupled to A/G sepharose beads (Santa Cruz Biotechnology). Kapa Hyper Prep Kit (Kapa Biosystems, Wilmington, MA) was used for End-repair, A-tailing and ligation of sequence adaptors. Samples were amplified by PCR and the libraries were size-selected in the 200–500 bp range. Bar-coded libraries were sequenced on Illumina NextSeq500 sequencer as previously described (Peeters et al., 2015) (50 bp, single-end, Utrecht sequencing facility, Utrecht Medical Center, Utrecht, The Netherlands).

4.7. RNA sequencing

Total RNA was extracted from adherent NSCs cultured on 6-well coated plastic plates for 48 h using the RNAeasy Kit (Qiagen). RNA quality was tested on the Bioanalyzer (Agilent, Santa Clara, CA) and sample quality was optimal with RNA integrity number (RIN) higher than 9.0. Sample preparation was performed using Poly(A)Purist MAG Kit (Thermo Scientific) according to manufacturers' instructions. Isolated mRNA was subsequently repurified using mRNA-ONLY Eukaryotic mRNA Isolation Kit (Epicentre (Illumina, Inc.), Madison, WI, USA). Sequencing libraries were prepared using SOLiD Total RNA-Seq Kit (Applied Biosystems Life Technologies) according to the standard protocol recommendations and sequenced on SOLiD Wildfire sequencer to produce 50 bp reads as previously described (Van Boxtel et al., 2013).

4.8. NGS data analysis

For RNA-sequencing analysis sequencing reads were mapped against the reference genome (mm10 assembly) using TopHat v2.0.9 as previously described (Trapnell et al., 2012). Only uniquely mapped reads were selected for further analysis. In brief, guide transcripts were assembled using CuffLinks v2.2.1. Reads were quartile normalized using the –library-norm-method quartile option. Differential gene analysis was performed using CuffDiff. Cluster 3.0 and Java TreeView software v1.1.6 were used for visualization of heatmaps. ChIP-sequencing reads were mapped with Bowtie 2.1.0 against the reference genome (mm10) using default settings (Langmead and Salzberg, 2012). Peaks were called with MACS2 software using the input as a control (Heinz et al., 2010). Mapped fragments were extended according to the average fragment size and converted to TDF files, visualized with IGV tools v2.3.36 and represented as coverage normalized tracks (Robinson et al., 2011). SAMtools was used for manipulation of SAM and BAM files; manipulation of BED file format was performed with BEDtools (Li et al., 2009; Quinlan and Hall, 2010). Motif discovery, peak annotation and generation of histograms were performed using HOMER software. For motif discovery the 200 bp sequence surrounding each peak was examined and motifs with length of 8, 10 and 12 nt were searched. Motifs were identified through *de novo* motif discovery. For functional annotation and SOX4 target identification the software GREAT was used, where genes that presented peaks within 5 kb from the TSS were selected (McLean et al., 2010). Average distances to TSS of peaks were calculated with GREAT. The ToppGene suite database (https://toppgene.cchmc.org/enrichment.jsp) and the REVIGO visualization tool were used for GO-term analysis of differentially expressed and SOX4-bound genes (Chen et al., 2009; Supek et al., 2011).

4.9. Gene set enrichment analysis

For Gene Set Enrichment Analysis (GSEA) pre-ranked analysis was performed with the GSEA software probing for enrichment of curated gene sets (C2) in the RNA-seq dataset ranked by log fold-change (Carbon et al., 2009; Subramanian et al., 2005).

4.10. Statistics

Data are expressed as mean ± SEM and regarded statistically significant if $p < .05$. Data were analyzed using one-way ANOVA with Dunnett's post-test. For the differentiation experiment with SOX4 overexpression and the oligonucleotide pull-down assay data were analyzed using two-tailed paired Student's t-test.

Accession numbers

The ChIP-seq data presented in this study have been deposited in NCBI's Gene Expression Omnibus (GEO) database under GEO: GSE120894 (linked to GSE121174). The RNA-seq data presented in this study have been deposited in NCBI's GEO database under GEO: GSE121173 (linked to GSE121174).

Competing interests

The authors declare no competing interests.

Author contribution

LB and PJC designed the experiments and wrote the manuscript. LB performed the experiments. SJV contributed to Figs. 1-2. GP contributed to Figs. 3-4. CHN provided theoretical and technical input.

ChIP-seq for SOX4 was performed on chromatin isolated from cultured NSCs as described in Methods. (A) Visualization of SOX4 ChIP-seq profile within the genomic region surrounding the *Id2* and *Adi1* loci. (B) Genomic distribution of SOX4 binding sites in annotated regions compared to background genomic sites. (C) Average signal plot of SOX4. (D) Motif-distribution analysis of SOX4 motif. (E) Average distance to TSS plot of SOX4 peaks (F) Motif enrichment analysis of SOX4 using *de novo* motif discovery. (G) Gene ontology analysis using GREAT showing the genes associated with CNS abnormalities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2018.10.005.

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