

# FOXP1 Promotes Embryonic Neural Stem Cell Differentiation by Repressing Jagged1 Expression

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## SUMMARY

Mutations in *FOXP1* have been linked to neurodevelopmental disorders including intellectual disability and autism; however, the underlying molecular mechanisms remain ill-defined. Here, we demonstrate with RNA and chromatin immunoprecipitation sequencing that FOXP1 directly regulates genes controlling neurogenesis. We show that FOXP1 is expressed in embryonic neural stem cells (NSCs), and modulation of FOXP1 expression affects both neuron and astrocyte differentiation. Using a murine model of cortical development, FOXP1-knockdown *in utero* was found to reduce NSC differentiation and migration during corticogenesis. Furthermore, transplantation of FOXP1-knockdown NSCs in neonatal mice after hypoxia-ischemia challenge demonstrated that FOXP1 is also required for neuronal differentiation and functionality *in vivo*. FOXP1 was found to repress the expression of Notch pathway genes including the Notch-ligand Jagged1, resulting in inhibition of Notch signaling. Finally, blockade of Jagged1 in FOXP1-knockdown NSCs rescued neuronal differentiation *in vitro*. Together, these data support a role for FOXP1 in regulating embryonic NSC differentiation by modulating Notch signaling.

## INTRODUCTION

Neural stem cells (NSCs) are multipotent progenitor cells found in the developing and adult brain in specialized niches of the subgranular zone of the hippocampal dentate gyrus and the subventricular zone (SVZ). During cortical development, the radial glial cells are considered embryonic NSCs acting as neural progenitor/stem cells at the ventricular zone (VZ) and, while migrating toward the cortical plate (CP), differentiate giving origin to neurons, astrocytes, and oligodendrocytes (Fishell and Kriegstein, 2003; Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009). NSCs represent a promising candidate for transplantation in the treatment of pathophysiological conditions of the CNS such as Alzheimer disease, multiple sclerosis, cerebral stroke, or hypoxic-ischemic encephalopathy (Bacigaluppi et al., 2016; Blurton-Jones et al., 2009; Braccioli et al., 2016; Daadi et al., 2010; Martino and Pluchino, 2006; Park et al., 2002). Regulation of NSC differentiation includes involvement of the Notch signaling pathway, which has been widely described as a key factor promoting neural progenitor maintenance and modulation of NSC fate decision in both embryonic and adult NSCs (Ables et al., 2011; Louvi and Artavanis-Tsakonas, 2006). Forkhead box p1 (FOXP1) is a transcription factor belonging to the forkhead family of transcription factors and has been implicated in the development of the heart, lung, esophagus, and immune system, as well as in cancer (Bacon

and Rappold, 2012). During embryonic neural development, FOXP1 has been demonstrated to promote neuronal migration and morphogenesis, as well as differentiation of medium spiny neurons (Li et al., 2015; Precious et al., 2016). Mutations in *FOXP1* have been linked to neurodevelopmental disorders, including speech defects, intellectual disability, and autism (Hamdan et al., 2010; Horn et al., 2010; Le Fevre et al., 2013; Lozano et al., 2015; Palumbo et al., 2013; Sollis et al., 2016). In addition, FOXP1 has recently been shown to regulate a network of autism-associated genes in the hippocampus and striatum, and heterozygous *FOXP1*<sup>+/-</sup> mice exhibit vocal communication defects. However, the role of FOXP1 in NSCs has not been addressed (Araujo et al., 2015). A recent study from Bacon et al. (2015) showed that recombinase Cre-mediated deletion of *FOXP1* in Nestin-positive cells causes autism-like behavior and gross morphological defects in the striatum, detected from early postnatal age onwards. However, the question as to whether FOXP1 regulates embryonic neural progenitor differentiation remains open. In this study, we have investigated the role of FOXP1 in regulating embryonic NSC differentiation. Through both *in vitro* and *in vivo* analyses combined with global transcriptional profiling, here we identify FOXP1 as a driver of NSC differentiation toward astrocytes and neurons. Furthermore, we define FOXP1-mediated repression of *Jagged1*, a key ligand of the Notch pathway, as being a requirement for NSC differentiation. Taken together, these findings highlight FOXP1 as



a pivotal transcription factor in regulating embryonic NSC differentiation, defining a mechanism for its role in neurogenesis.

## RESULTS

### Genome-wide Analysis of FOXP1 DNA Binding in NSCs

While previous studies have implicated FOXP1 in neurogenesis and the pathogenesis of autism, speech defects, and other intellectual disabilities, little is known about the molecular mechanisms underlying these effects. Here, we sought to investigate the role of this transcription factor in NSC functionality. To this end, FOXP1 chromatin immunoprecipitation from NSCs followed by high-throughput sequencing (ChIP-seq) was performed. FOXP1-bound loci were successfully identified as exemplified by peaks mapped at the region of the *Bik* gene, which we previously characterized as a FOXP1 target in human colon carcinoma cells (van Boxtel et al., 2013) (Figure 1A). Analysis of the genomic distribution of FOXP1-binding sites showed that binding events are enriched at promoter regions, 5'-UTRs and transcription start sites (TSS) when compared with random genomic regions (Figure 1B). In addition, binding events were found centrally enriched around the TSS (Figure 1C). *De novo* motif discovery was performed and identified the Forkhead motif, in addition to other co-occurring motifs, including SOX3, NR2E1, and RFX2 (Figure 1E). The FOXP1/Forkhead-consensus DNA-binding motif was found centrally enriched within peaks, further confirming sequence-specific binding (Figure 1D).

To identify which genes are associated with FOXP1-bound regions, the genes with the TSS within 1 kilobase (kb) from Forkhead-motif-centered peaks were selected for gene ontology (GO) analysis. FOXP1-bound genes showed a significant association with abnormalities in the CNS, abnormalities of higher mental function, and cognitive impairment (Figure 1F). This is in line with previous studies supporting association of *FOXP1* mutations with autism, intellectual disability, and speech defects (Horn et al., 2010). Taken together, these data show that FOXP1 associates with the promoter regions of genes involved in diseases of the CNS.

### FOXP1 Regulates Neurogenesis and Notch Signaling Pathway Genes

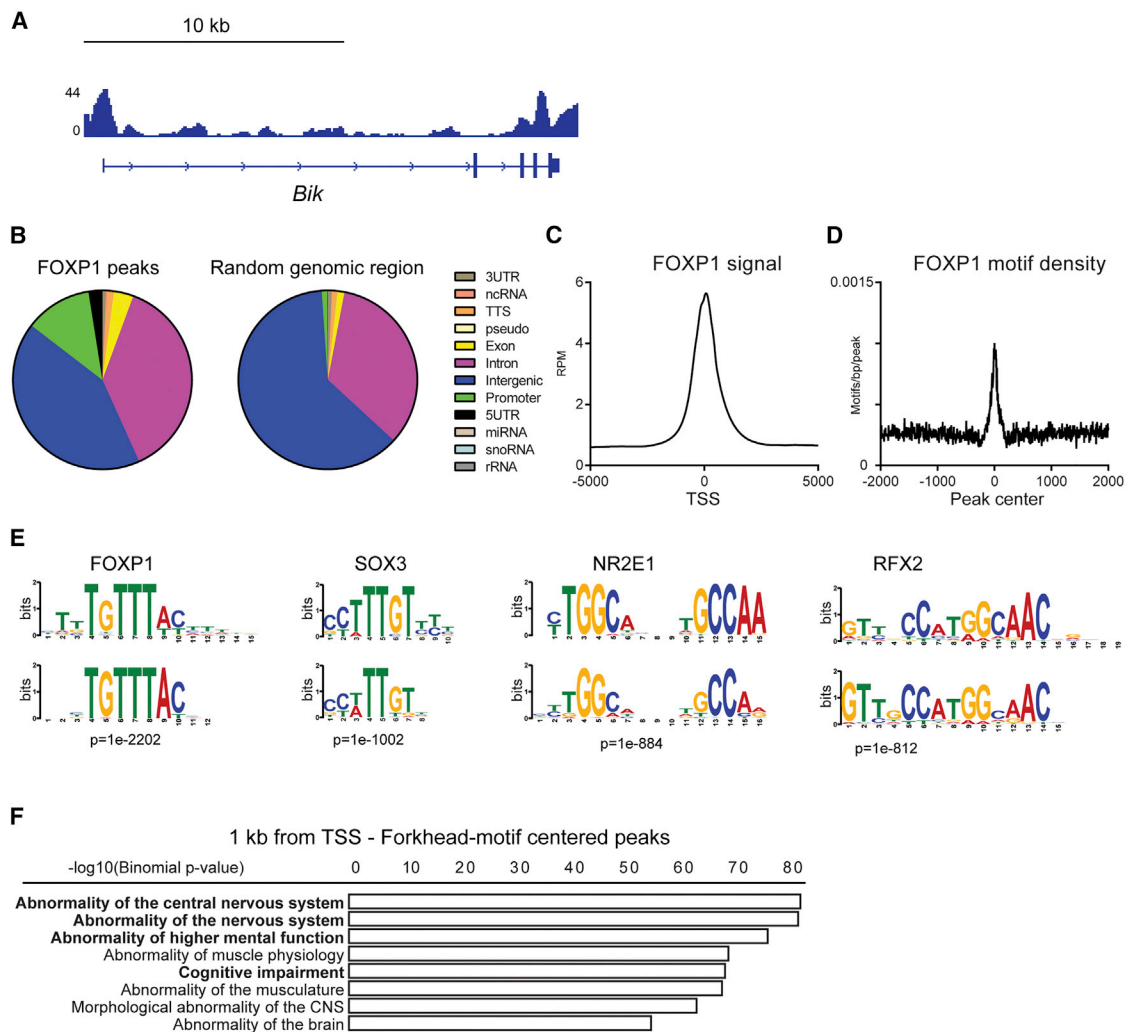
To identify genes that are transcriptionally regulated by FOXP1, shRNA-mediated FOXP1 knockdown (KD) was utilized to deplete NSCs of FOXP1. We then evaluated the effect of FOXP1 KD on transcript expression in NSCs by RNA sequencing (RNA-seq). We confirmed that *Foxp1* mRNA levels were reduced after FOXP1 KD (Figure 2A). Analysis

of RNA-seq data showed that upon FOXP1 KD, 472 genes were found to be significantly induced by FOXP1 and 617 genes to be repressed by FOXP1 (Figure 2B). To identify which genes were likely directly regulated by FOXP1 in NSCs, the set of differentially expressed genes was overlapped with the set of genes whose TSS is within 25 kb from Forkhead-motif-centered peaks derived from the ChIP-seq analysis. Of the FOXP1-regulated genes, 210 were found to be both induced and bound by FOXP1, whereas 274 were found to be repressed and bound by FOXP1 (Figures 2C and 2D).

To gain further insight into the biological processes potentially regulated by FOXP1, GO term analysis was performed using the subset of FOXP1-bound and transcriptionally regulated target genes. Significant association was observed with neurogenesis, regulation of synapse organization, and nervous system development processes (Figure 2E). Interestingly, also an association with the Notch signaling pathway was found (Figure 2E). Taken together, our data indicate that FOXP1 regulates neurogenesis-specific genes, repressing genes of the Notch pathway.

### FOXP1 Promotes Embryonic Neural Stem Cell Differentiation toward Astrocytes and Neurons *In Vitro*

Since our NGS data indicate that FOXP1 regulates neurogenesis-specific genes, we wished to determine whether FOXP1 can regulate NSC differentiation. To this end, we first determined whether FOXP1 expression was regulated during murine NSC differentiation. NSCs derived from the prefrontal cortex of E14.5 CD-1 embryos were differentiated *in vitro* for 10 days by growth-factor withdrawal (as described in Supplemental Experimental Procedures). FOXP1 expression levels were assessed every 2 days, and a significant increase in *Foxp1* mRNA was observed after 2 days of differentiation that was sustained for at least 6 days (Figure 3A). Consistently, FOXP1A (79 kDa) and FOXP1C (50 kDa) protein isoforms both showed a significant increase during differentiation of the NSCs (Figures 3B and 3C). This increase in FOXP1 levels during the early phase of differentiation indicates a potential functional role for FOXP1 in regulating NSC lineage choices. To evaluate this further, two independent shRNAs targeting FOXP1 were utilized to deplete NSCs of both FOXP1A and FOXP1C isoforms (Figure 3D). Firstly, to assess whether FOXP1 KD affects NSC proliferation, the percentage of Ki67-positive cells were analyzed in the presence of epidermal growth factor and basic fibroblast growth factor, which promote NSC proliferation, and no differences were observed (Figures S1A and S1B). Secondly, to determine whether FOXP1 KD impairs differentiation, NSCs were differentiated for 5 days toward neurons, astrocytes, and oligodendrocytes. Differentiation was quantified by



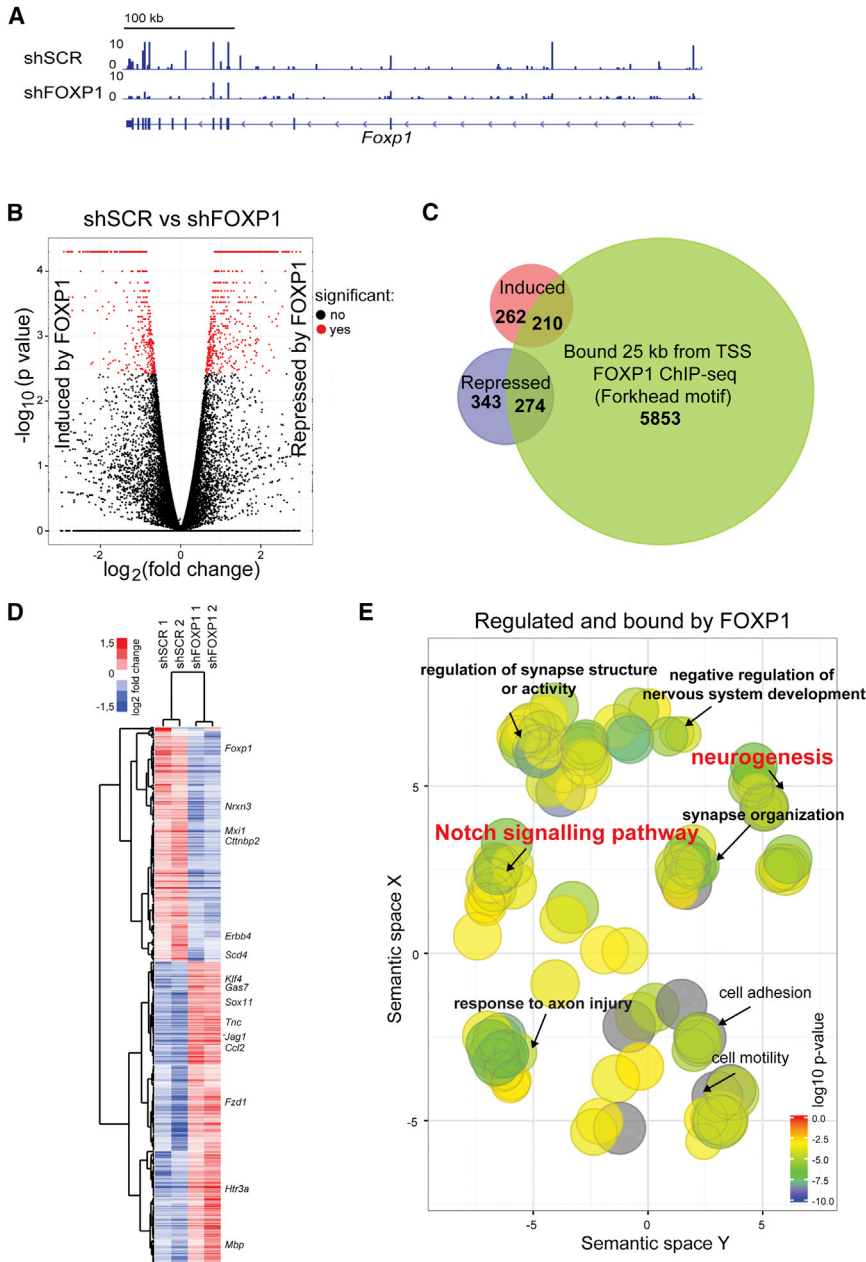
**Figure 1. Genome-wide Analysis of FOXP1 DNA-Binding in NSCs**

ChIP-seq for FOXP1 was performed on chromatin isolated from cultured NSCs as described in [Experimental Procedures](#).

- (A) Visualization of the FOXP1 ChIP-seq profile within the genomic region surrounding the *Bik* loci.
- (B) Genomic distribution of FOXP1 binding sites in annotated regions compared with background genomic sites.
- (C) Average profile plot of FOXP1. RPM, reads per million.
- (D) Motif-distribution analysis of FOXP1 motif.
- (E) Motif-enrichment analysis of FOXP1 using *de novo* motif discovery.
- (F) Gene ontology analysis using GREAT showing the genes associated with CNS diseases.

measuring lineage-specific marker expression by immunofluorescence:  $\beta$ III-tubulin, glia fibrillary acidic protein (GFAP), and neural/glial antigen 2 (NG2), respectively ([Eng and Ghirmikar, 1994](#); [Memberg and Hall, 1995](#); [Polito and Reynolds, 2005](#)). FOXP1 KD significantly reduced the percentage of  $\beta$ III-tubulin- and GFAP-positive cells ([Figures 3E, 3G, and 3H](#)), while the percentage of NG2-positive cells was unaffected when compared with control ([Figures S1C and S1D](#)). In addition, FOXP1 KD reduced the percentage of astrocyte markers S100B- and Aquaporin4-(AQP4) positive cells ([Figures S1E–S1H](#)) ([Raponi et al., 2007](#); [Xiao and](#)

[Hu, 2014](#)). Consistently, FOXP1 KD increased the expression (per cell) of the stem cell/progenitor marker Nestin when compared with control ([Figures 3F and 3I](#)) ([Lendahl et al., 1990](#)). This indicates that reduction in neuronal and astrocyte differentiation observed after FOXP1 KD is accompanied by maintenance of progenitor-like characteristics. Conversely, ectopic expression of FOXP1 ([Figure 3J](#)) increased the percentage of  $\beta$ III-tubulin- and GFAP-positive cells when compared with control ([Figures 3K–3M](#)). Consistently, FOXP1 overexpression increased expression of S100B and AQP4, while it decreased the expression of



**Figure 2. FOXP1 Regulates Expression of Neurogenesis-Related Notch Signaling Pathway Genes**

RNA-seq was performed on FOXP1 KD NSCs. Putative targets were identified by overlapping the expression data with the ChIP-seq dataset.

(A) Visualization of RNA-seq reads around the genomic locus of *Foxp1* in control and KD conditions.

(B) Volcano plot representing differentially expressed genes in FOXP1 KD NSCs compared with control.

(C) Venn diagram showing overlap between FOXP1-bound and -regulated genes.

(D) Heatmap showing the expression of FOXP1-bound and -regulated genes.

(E) Gene ontology analysis using REVIGO showing the genes associated with neurogenesis and the Notch signaling pathway.

See also [Figure S3](#).

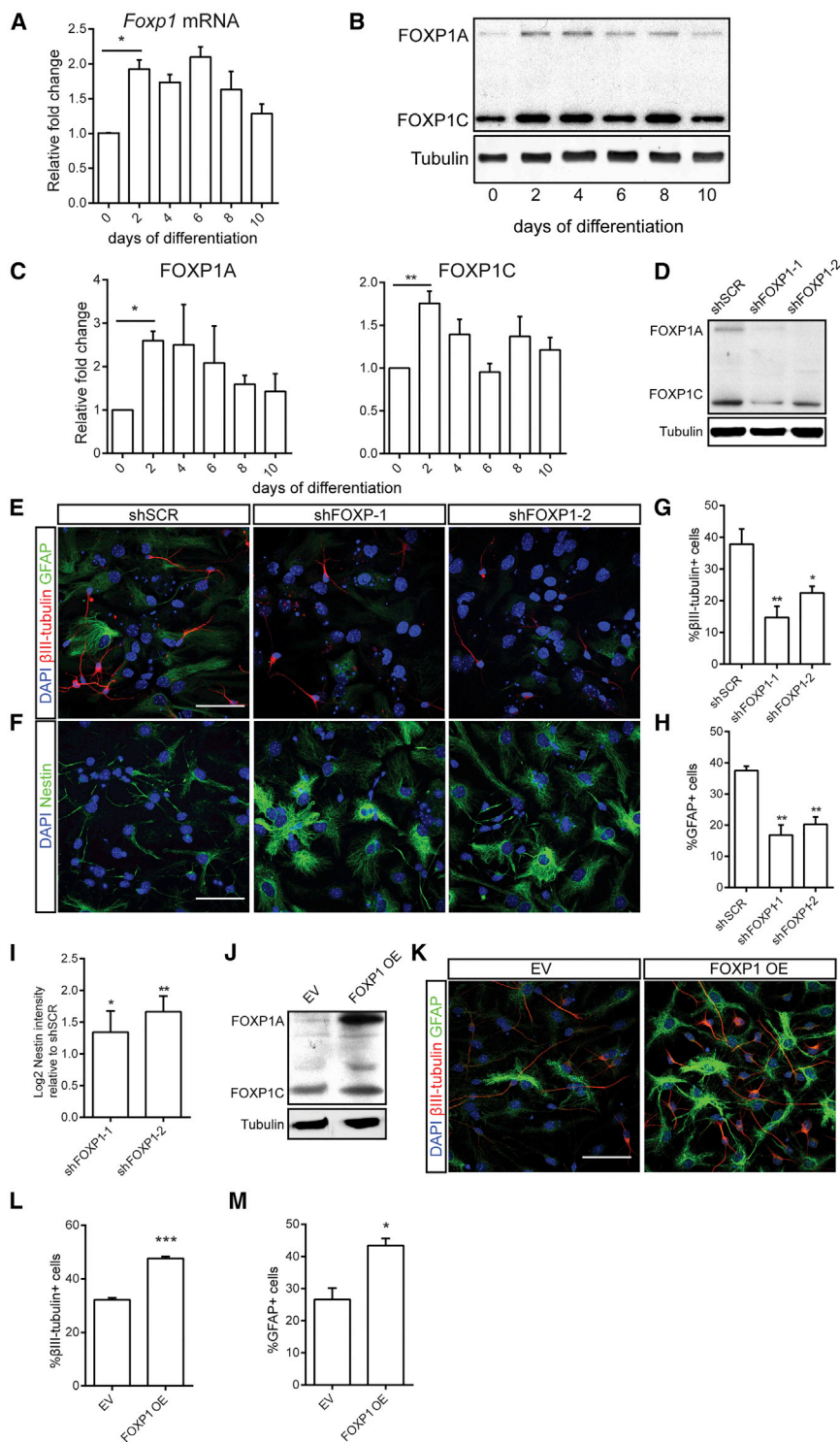
the neural progenitor/stem cell marker SOX2 and the intermediate progenitor maker TBR2 (Ellis et al., 2004; Englund et al., 2005) (Figures S1I–S1P). Taken together, these data demonstrate that FOXP1 is required for NSC differentiation *in vitro*, specifically to both neuronal and astrocyte lineages but not toward oligodendrocytes.

### FOXP1 Is Required for NSC Migration and Differentiation during Cortical Development

During embryonic development of the cortex, NSCs residing in the VZ differentiate into neurons migrating to

the more superficial CP, where they establish functional connections (Kriegstein and Alvarez-Buylla, 2009). To assess whether FOXP1 is expressed by NSCs in the VZ, cortices from day 12 (E12) and 14 (E14) embryos were stained both for the neural precursor/neural stem cell marker SOX2 and FOXP1. As expected, at both E12 and E14, SOX2 expression was mainly localized at VZ (Figure 4A). Interestingly, FOXP1/SOX2-positive cells were found at the VZ of both E12 and E14 cortices (Figure 4A). In addition, FOXP1 was found to be expressed in the CP, consistent with previous reports describing a role for





**Figure 3. FOXP1 Regulates NSC Differentiation *In Vitro***

(A) NSCs were differentiated for 10 days and RNA and protein were collected. Measure of *Foxp1* mRNA levels during differentiation (n = 3 independent experiments) \*p < 0.05. (B) Representative western blot showing the expression of FOXP1A and FOXP1C isoforms.

(C) Quantification of (B). n = 3 independent experiments. \*p < 0.05, \*\*p < 0.01.

(D) Representative western blot showing efficient knockdown of FOXP1A and FOXP1C with two independent shRNAs.

(E) FOXP1 KD NSCs were differentiated for 5 days. Representative confocal images showing the expression of the neuronal marker  $\beta$ III-tubulin (red) and the astrocyte marker GFAP (green). DAPI co-stained nuclei in blue. Bar, 50  $\mu$ m.

(F) Confocal images showing the expression of the neural progenitor marker Nestin (green). DAPI co-stained nuclei in blue. Bar, 50  $\mu$ m.

(G) Quantification of the number of  $\beta$ III-tubulin<sup>+</sup> cells in (E). \*p < 0.05, \*\*p < 0.01.

(H) Quantification of the number of GFAP<sup>+</sup> cells in (E). n = 3 independent experiments. \*\*p < 0.01.

(I) Quantification of (F). n = 3 independent experiments. \*p < 0.05, \*\*p < 0.01.

(J) Representative western blot showing the overexpression of FOXP1.

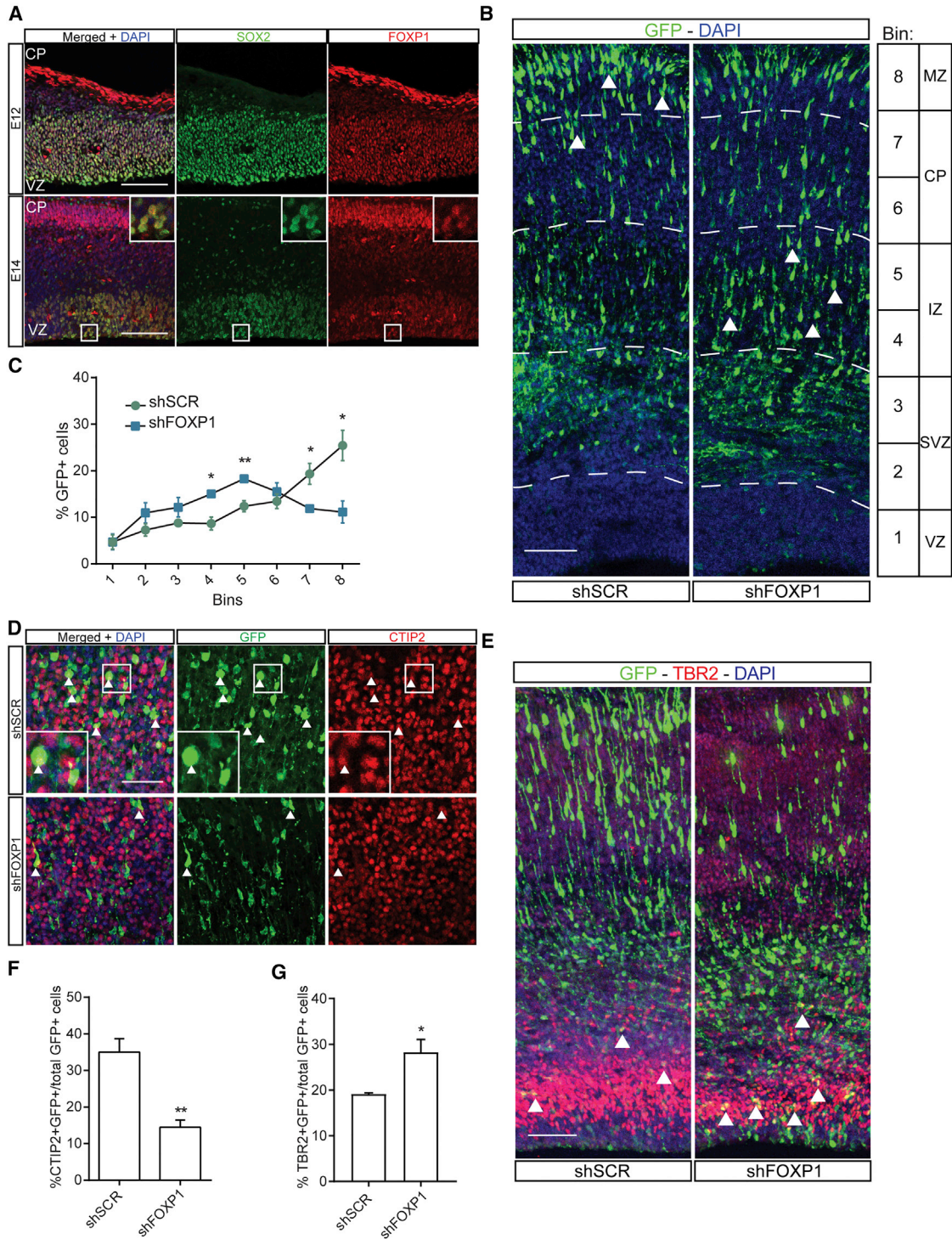
(K) FOXP1 overexpressing NSCs were differentiated for 5 days. Representative confocal images showing the expression of the neuronal marker  $\beta$ III-tubulin (red) and the astrocyte marker GFAP (green). DAPI co-stained nuclei in blue. Bar, 50  $\mu$ m.

(L) Quantification of the number of  $\beta$ III-tubulin<sup>+</sup> cells in (K). \*\*\*p < 0.001.

(M) Quantification of the number of GFAP<sup>+</sup> cells in (K). n = 3 independent experiments. \*p < 0.05. Error bars represent SEM. See also Figure S1.

FOXP1 in differentiated neurons (Araujo et al., 2015; Precious et al., 2016). To evaluate whether FOXP1 KD affects NSC differentiation during cortical development, E14.5 embryos were electroporated *in utero* with shRNA

vectors together with GFP in order to transduce the NSCs present at the VZ, followed by immunohistochemical analysis of neurons derived from these progenitors. At E17.5, the majority of GFP-positive cells were found at the CP.



#### Figure 4. FOXP1 Is Required for Radial Glia Development during Embryogenesis

(A) E12 and E14 motor cortices were stained for FOXP1 and SOX2 to assess the expression of FOXP1 by NSCs during development. Z stack confocal images showing co-expression of FOXP1 (red) and SOX2 (green) at the VZ both at E12 and E14. Expression of FOXP1 can be detected also at the CP. Inserts show magnification of the boxed area. DAPI co-stained nuclei in blue. Bar, 100  $\mu$ m.

(B) E14.5 cortices were electroporated *in utero* with shRNAs against FOXP1 in combination with GFP. Animals were terminated at E17.5. Representative z stack confocal images of the motor cortex showing GFP<sup>+</sup> neurons (green), originating from the VZ. FOXP1 KD induces

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In contrast, FOXP1 KD showed an increase of GFP-positive cells at the intermediate zone (IZ), and a reduction of GFP-positive cells at the CP/marginal zone (MZ) when compared with control (Figures 4B and 4C). These data indicate that FOXP1 KD reduces migration of the differentiating neurons deriving from the VZ, as reported previously (Li et al., 2015). To assess whether this phenotype was accompanied by reduced differentiation, we analyzed the expression of CTIP2, a transcription factor expressed by a subtype of post-mitotic cortical neurons during development, and TBR2, a transcription factor expressed by intermediate progenitors (Englund et al., 2005; Leone et al., 2008). Upon FOXP1 KD, reduction of the percentage of CTIP2<sup>+</sup>/GFP<sup>+</sup>-cells was observed compared with shSCR control (Figures 4D and 4F), indicating that FOXP1 is required for the differentiation of NSCs toward post-mitotic cortical neurons. Moreover, FOXP1 KD induced an increased percentage of GFP<sup>+</sup>/TBR2<sup>+</sup>-cells compared with control, indicating accumulation of GFP<sup>+</sup>-cells in the intermediate progenitor population (Figures 4E and 4G). Consistently, FOXP1 OE decreased expression of TBR2 upon NSC differentiation *in vitro* (Figures S1K and S1L). Together, these data indicate that FOXP1 promotes intermediate progenitor differentiation. In conclusion, these observations indicate that FOXP1 is required for differentiation of NSCs during corticogenesis.

### FOXP1 Is Required for *In Vivo* Functionality of NSCs in a Hypoxic-Ischemic Brain Damage Model

NSC transplantation has been developed as a treatment for various CNS injuries. To investigate whether FOXP1 is required for the functionality of NSCs upon transplantation to treat brain injury, FOXP1-depleted NSCs genetically labeled with dsRed were transplanted in a murine model of neonatal hypoxic-ischemic (HI) brain injury. As we have previously described, this model induces unilateral damage to the hippocampus, neocortex, and striatum, resulting in sensorimotor impairment (Braccioli et al., 2016; van der Kooij et al., 2010). Nine days after birth, animals underwent right carotid artery occlusion followed by systemic hypoxia (as described in Experimental Procedures) to induce a unilateral brain lesion. At 10 days after HI,

FOXP1 KD NSCs were transplanted intracranially at the lesion site in the ipsilateral hippocampus, and the animals were killed 5 days later to assess the fate of the transplanted NSCs (Figure 5A). Another group of animals survived until day 28 to assess the effect of FOXP1 KD NSC transplantation on motor behavior.

We have previously demonstrated that transplanted NSCs in HI animals localize around the ipsilateral hippocampus and differentiate toward the neuronal lineage by expressing the neuroblast marker doublecortin (DCX) as soon as 3 days after transplantation (Braccioli et al., 2016). Expression of GFAP in the transplanted cells is absent at this time, indicating no differentiation of the transplanted NSCs toward the astrocyte lineage (Braccioli et al., 2016). Figure 5B shows that FOXP1 KD NSCs were still detectable in the hippocampal area at 5 days after transplantation; however, there was a significantly reduced percentage of DCX-dsRed-positive NSCs when compared with control NSCs (shSCR NSCs) (Figures 5B and 5C). These data support the notion that FOXP1 is required for neuronal differentiation of (transplanted) NSCs *in vivo* as well.

To test the functional consequences of FOXP1 KD in transplanted NSCs after HI, an additional group of animals was assessed for sensorimotor function utilizing the cylinder-rearing test (CRT) at 28 days after HI (18 days after transplantation) (Figure 5A) (Schallert et al., 2000; van der Kooij et al., 2010). We have previously shown that NSC transplantation ameliorates HI-induced motor impairment under these experimental conditions (Braccioli et al., 2016). The control HI animals treated with vehicle (VEH) showed impaired sensorimotor function (i.e., increased preference for the non-impaired forepaw) when compared with sham-control animals (SHAM). Animals treated with control (shSCR) NSCs displayed a potent improved performance when compared with VEH-treated HI animals, which is in line with our previous findings (Braccioli et al., 2016). Consistent with the histological findings in Figures 5B and 5C, FOXP1 KD completely abolished the improvement in motor behavior mediated by NSC transplantation after HI when compared with shSCR control NSCs (Figure 5D). Taken together, these data indicate that FOXP1 promotes differentiation of transplanted NSCs

accumulation of neurons in the IZ and reduction of neurons in the cortical plate (CP)/marginal zone (MZ) (white arrowheads). SVZ, subventricular zone; IZ, intermediate zone. The cortex has been subdivided into 8 bins for quantification. DAPI co-stained nuclei in blue. Bar, 25  $\mu$ m.

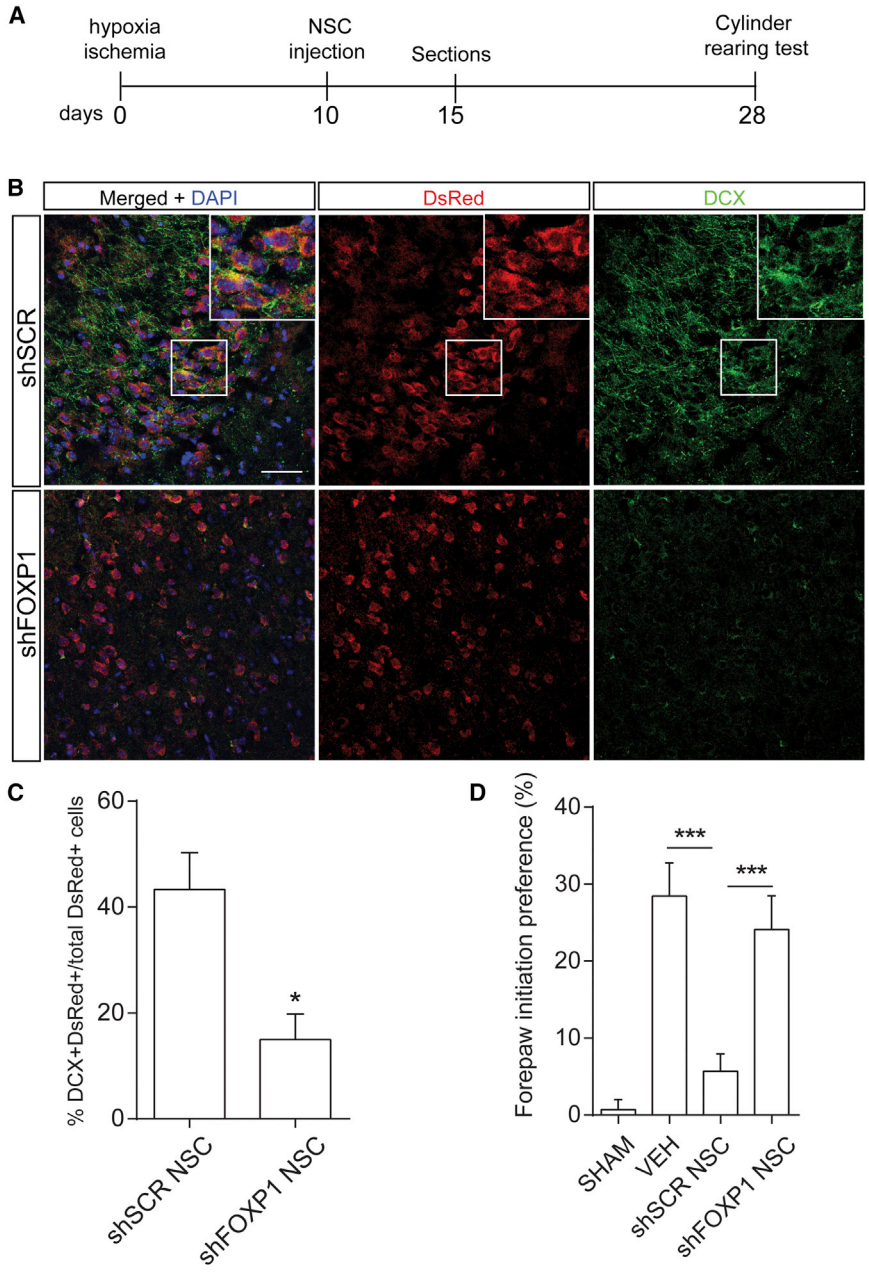
(C) Quantification of (B). Mice per group: shSCR, n = 3; shFOXP1, n = 3. \*p < 0.05, \*\*p < 0.01.

(D) Representative z stack confocal images showing CTIP2<sup>+</sup>/GFP<sup>+</sup>-cells (white arrowheads) in the cortex upon FOXP1 depletion. Insets show magnification of the boxed area. Green, GFP; red, CTIP2. DAPI co-stained nuclei in blue. Bar, 50  $\mu$ m.

(E) Representative z stack confocal images showing TBR2<sup>+</sup>/GFP<sup>+</sup>-cells (white arrowheads) in the cortex upon FOXP1 depletion. Insets show magnification of the boxed area. Green, GFP; red, TBR2. DAPI co-stained nuclei in blue. Bar, 25  $\mu$ m.

(F) Quantification of (D). Mice per group: shSCR, n = 3; shFOXP1, n = 3. \*\*p < 0.01. Error bars represent SEM.

(G) Quantification of (E) (mice per group: shSCR, n = 4; shFOXP1, n = 5). \*p < 0.05.



**Figure 5. FOXP1 Is Required for NSC Differentiation *In Vivo* after Transplantation into HI Mice**

(A) Experimental setup showing the day of sham or HI operation (day 0, postnatal day 9), intracranial NSC treatment (day 10), and the day of termination for histological sections (day 15). In another group of animals, the cylinder-rearing test was performed at day 28.

(B) Representative z stack confocal images of the hippocampus showing the presence of the transplanted dsRed NSCs (red) and the expression of the neuroblast marker DCX (green). Yellow indicates colocalization. DAPI co-stained nuclei in blue. Bar, 50  $\mu$ m. Insets show magnification of the boxed area.

(C) Quantification of double-positive cells in (B). Mice per group: shSCR NSC, n = 3; shFOXP1 NSC, n = 3. \*p < 0.05.

(D) Results of the cylinder-rearing test at 28 days showing that FOXP1 is required for the improvement in motor behavior mediated by NSCs after HI. Mice per group: SHAM, n = 9; VEH, n = 9; shSCR NSC, n = 10; shFOXP1 NSC, n = 8. \*\*\*p < 0.001. Error bars represent SEM.

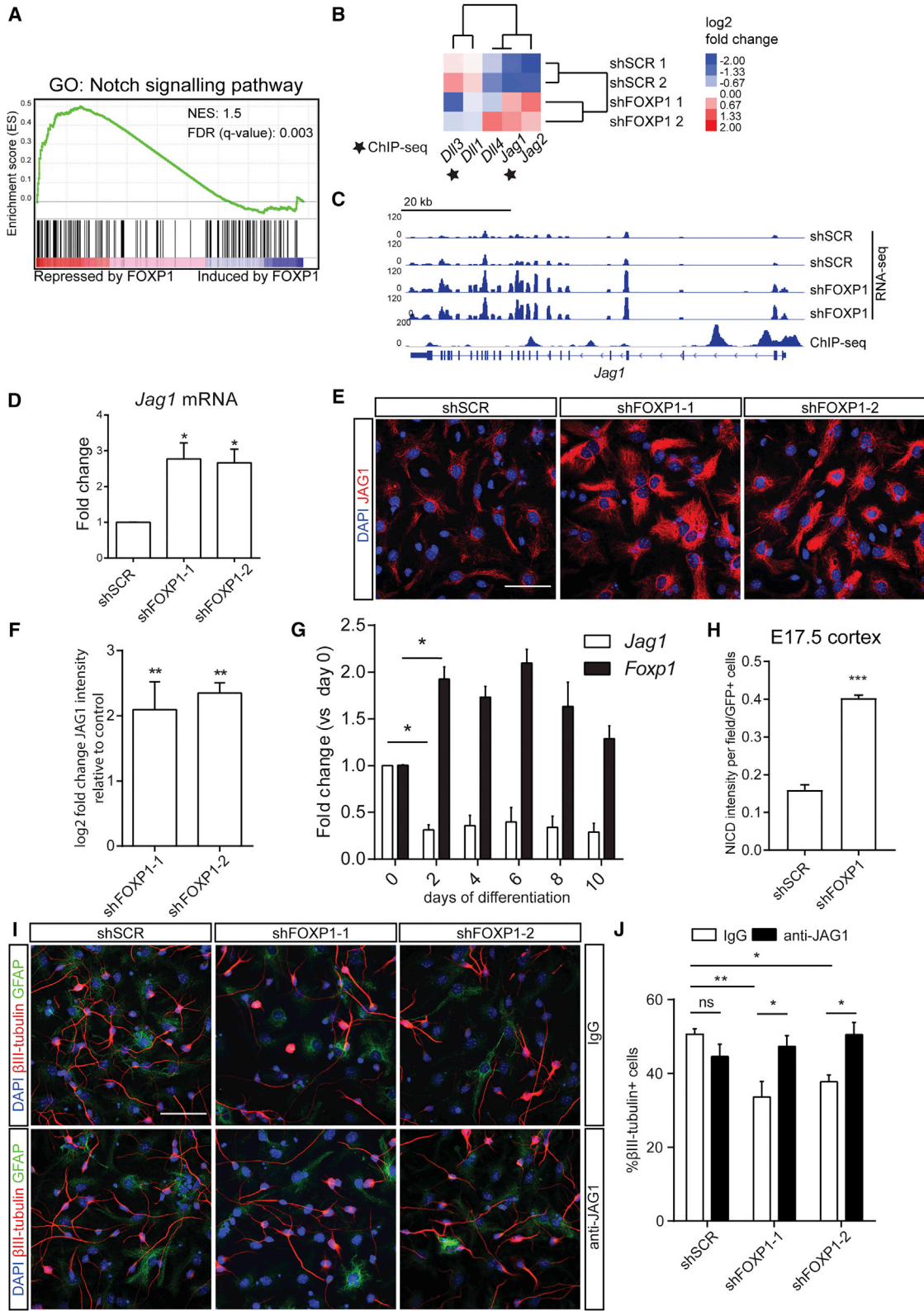
toward the neuronal lineage *in vivo*, and FOXP1 is required for the functional improvement of the animals mediated by NSC transplantation after HI.

**FOXP1 Promotes Neurogenesis by Transcriptionally Repressing Jag1**

Our data clearly identify FOXP1 as a critical mediator of NSC function both *in vitro* and *in vivo*. In our NGS analysis, we identified Notch signaling pathway genes as both bound and regulated by FOXP1 (Figure 2F), suggesting a potential mechanism for control of NSC differentiation (Ables et al.,

2011; Louvi and Artavanis-Tsakonas, 2006). A weighted gene set enrichment analysis (GSEA) probing for enrichment of genes belonging to the GO term Notch signaling pathway in the RNA-seq dataset was performed. This analysis revealed a striking and significant enrichment of Notch signaling genes as being repressed by FOXP1 (Figure 6A). To validate the repression of Notch signaling output by FOXP1, we measured the expression levels of the downstream Notch effectors *Hes1* and *Hes5* mRNA in our RNA-seq dataset (Louvi and Artavanis-Tsakonas, 2006). Upon FOXP1 KD, *Hes1* mRNA expression was increased, while





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*Hes5* expression was reduced (Figure S2B). To validate these findings, we utilized two independent shRNAs targeting FOXP1 in undifferentiated NSCs (Figure S2B). While we confirmed that *Hes1* mRNA levels were consistently upregulated upon FOXP1 KD, *Hes5* levels were not found to be negatively regulated by both shRNAs against FOXP1 for reasons that remain unclear (Figure S2B). In conclusion, these data indicate that FOXP1 negatively regulates the final outcome of Notch signaling. Among the Notch ligands expressed in NSCs, Jagged1 (*Jag1*), Jagged2 (*Jag2*), Delta-like 1 (*Dll1*), Delta-like 3 (*Dll3*), and Delta-like 4 (*Dll4*), only *Dll3* and *Jag1* were bound and differentially regulated by FOXP1 (Figure 6B). *Dll3* was found to be induced by FOXP1 while *Jag1* was found to be repressed by FOXP1 in the RNA-seq data (Figure 6B). To validate the regulation of the Notch ligands by FOXP1, the mRNA expression levels of *Jag1*, *Dll3*, *Jag2*, *Dll4*, and *Dll1* were analyzed in NSCs expressing two independent FOXP1 shRNAs. *Jag1* repression and *Dll3* induction by FOXP1 were confirmed (Figures 6D and S2A). DLL3 is the least characterized of the Notch ligands, deemed to both inhibit Notch signaling in *cis* (Ladi et al., 2005) and to activate the Notch pathway in the developing brain (Zhao et al., 2009). JAG1, however, has been shown to be required for stem cell maintenance in the postnatal SVZ (Nyfeler et al., 2005) and during granular cell neurogenesis in the adult dentate gyrus (Lavado and Oliver, 2014). Moreover, JAG1 inhibits differentiation of adult neural stem cells by promoting NSC quiescence in the SVZ (Ottone et al., 2014). This raises the possibility that FOXP1 promotes NSC differentiation by repressing JAG1 expression. Indeed, highly enriched regions for FOXP1 binding across the promoter region of JAG1 were identified (Figure 6C). The expression of JAG1 protein was also found to be increased in FOXP1-depleted NSCs after 5 days of differentiation when compared with control NSCs, indicating that increased JAG1 levels inhibit differentiation (Figures

6E and 6F). If FOXP1 indeed represses JAG1 expression during NSC differentiation, an increase in FOXP1 levels would correspond to a decrease in JAG1 expression. To evaluate this, *Jag1* and *Foxp1* mRNA expression were measured during NSC differentiation. When compared with undifferentiated NSCs, *Jag1* mRNA was significantly reduced after 2 days of differentiation corresponding to an increase in *Foxp1* mRNA (Figure 6G). In order to investigate the location where the expression of JAG1 is localized during embryonic cortical development, we performed immunostaining for JAG1 in E12–E14 cortical sections. We detected the expression of JAG1 in the cortical region both at E12 and E14, with a more prominent staining in the region of the ventricular/subventricular zone (VZ/SVZ) (Figure S2C). In addition, we found that JAG1 expression co-localized with the expression of the radial glia marker GLAST (Kriegstein and Alvarez-Buylla, 2009) (Figure S2C). These data suggest the importance of JAG1 in regulating radial glia differentiation *in vivo*. However, we did not observe a striking correlation between high levels of JAG1 and the expression of Nestin during NSC differentiation *in vitro* (data not shown), suggesting that JAG1 expression is apparently not specifically associated with a defined neural progenitor phenotype during NSC differentiation *in vitro*. To establish whether FOXP1 represses Notch signaling *in vivo*, E14.5 embryos were electroporated *in utero* with shRNA vectors together with GFP followed by immunohistochemical analysis of activated Notch intracellular domain (NICD) with an antibody recognizing the activated form of Notch cleaved at the residue Valine1744 (Mumm et al., 2000). FOXP1 KD induced an increase in NICD expression in the cortical regions containing GFP-positive cells compared with control (Figures 6H and S2D). This observation further validates our *in vitro* data, supporting that FOXP1 is negatively regulating Notch activation by repressing JAG1 expression *in vivo*.

### Figure 6. FOXP1-Mediated Expression of Jag1 Is Required for Neuronal Differentiation of NSCs

- (A) Gene set enrichment analysis representing the enrichment of Notch signaling pathway genes in the RNA-seq expression dataset ranked on log<sub>2</sub> fold change after FOXP1 KD.
- (B) Heatmap showing the expression of Notch pathway ligands.
- (C) Visualization of RNA-seq reads and FOXP1 ChIP-seq profile around the genomic locus of *Jag1* in control and KD conditions.
- (D) Measure of *Jag1* mRNA levels upon FOXP1 KD. n = 3 independent experiments. \*p < 0.05.
- (E) Confocal images showing the expression of JAG1 after 5 days of differentiation (red). DAPI co-stained nuclei in blue. Bar, 50 μm.
- (F) Quantification of (E). n = 3 independent experiments. \*\*p < 0.01.
- (G) NSCs were differentiated for 10 days. Measure of *Foxp1* and *Jag1* mRNA level. n = 3 independent experiments. \*p < 0.05.
- (H) E14.5 cortices were electroporated *in utero* with shRNAs against FOXP1 in combination with GFP. Animals were terminated at E17.5. Quantification of Figure S2D (mice per group: shSCR, n = 3; shFOXP1, n = 3). \*\*\*p < 0.001. Error bars represent SEM.
- (I) FOXP1 KD NSCs were differentiated for 5 days in the presence of the anti-JAG1 blocking antibody. Representative confocal images showing expression of the neuronal marker βIII-tubulin (red) and the astrocyte marker GFAP (green). DAPI co-stained nuclei in blue. Bar, 50 μm.
- (J) Quantification of (I). n = 3 independent experiments. \*p < 0.05, \*\*p < 0.01, ns, non-significant. Error bars represent SEM. See also Figure S2.



If an increase in JAG1 expression in FOXP1 KD NSCs is responsible for reduced NSC differentiation, inhibition of Notch signaling by a  $\gamma$ -secretase inhibitor would act to rescue this phenotype. To explore this, we differentiated FOXP1 KD NSCs for 5 days in the presence of the  $\gamma$ -secretase inhibitor DAPT (Dovey et al., 2001). As observed in the earlier experiments shown in Figures 1E, 1G, and 1H, FOXP1 depletion reduced both  $\beta$ III-tubulin- and GFAP-positive cells after differentiation (Figures S2F–S2H). Upon treatment with DAPT, the number of  $\beta$ III-tubulin-positive cells deriving from FOXP1 KD NSCs was restored to the level of control NSCs treated with DAPT, compared with FOXP1-depleted NSCs treated with DMSO (Figures S2F–S2H). However, DAPT treatment induced an increase of  $\beta$ III-tubulin-positive cells in all conditions compared with DMSO-treated cells, accompanied by a drastic reduction of GFAP-positive cells (Figures S2F–S2H). These data are consistent with previous reports indicating that DAPT enhances neuronal differentiation of embryonic stem cells (Crawford and Roelink, 2007). Taken together, these observations indicate that inhibition of  $\gamma$ -secretase by DAPT rescues the neuronal differentiation potential of FOXP1 KD NSCs, further confirming the role of FOXP1 in negatively regulating the Notch pathway.

In order to validate a specific role for JAG1, we sought to investigate whether specifically blocking the interaction between JAG1 and Notch receptor would act to rescue the reduction in NSC differentiation caused by FOXP1 KD. FOXP1 KD NSCs were treated with an anti-JAG1 blocking antibody during 5 days of differentiation. As previously observed in our study, FOXP1 depletion reduced both  $\beta$ III-tubulin- and GFAP-positive cells after differentiation (Figures 6I, 6J, and S2E). Strikingly, upon treatment with the anti-JAG1 antibody, the number of  $\beta$ III-tubulin-positive cells deriving from FOXP1 KD NSCs was restored to the level of controls when compared with FOXP1-depleted NSCs treated with the isotype control (Figures 6I and 6J). However, no significant effect was observed in the number of GFAP-positive cells deriving from FOXP1 KD NSCs upon anti-JAG1 treatment (Figure S2E). This indicates that increased JAG1 expression in FOXP1 KD NSCs inhibits differentiation specifically toward neurons.

Taken together, these observations strongly indicate that FOXP1 negatively regulates the Notch pathway through directly repressing JAG1 expression by binding to its promoter, and that this repression is essential for NSC differentiation.

## DISCUSSION

Here, we demonstrated that FOXP1 is a critical regulator of embryonic NSC differentiation based on several observa-

tions. Firstly, we showed how FOXP1 controls the expression of multiple genes regulating neurogenesis by binding to their promoter regions. Next, we demonstrated that FOXP1 is required for *in vitro* differentiation of NSCs toward neurons and astrocytes. Secondly, utilizing two different models, we showed that FOXP1 regulates neuronal differentiation *in vivo*. Namely, we showed that FOXP1 was required for differentiation of neurons originating from the radial glia during embryonic corticogenesis. Moreover, we demonstrated that NSCs transplanted into the hippocampus of neonatal mice with HI brain injury require FOXP1 to become neuroblasts. Furthermore, FOXP1 in NSCs was essential after transplantation to conduct functional recovery in HI-affected mice. Moreover lastly, we showed that FOXP1 negatively regulates the Notch signaling pathway by repressing the expression of *Jag1* through binding to its promoter, and that this repression is necessary for neuronal differentiation of NSCs. These data detail a key role for FOXP1 in regulating the functionality of NSCs and define an underlying molecular mechanism involving regulation of the Notch pathway by targeting *Jag1*.

Recent evidence has suggested that *FOXP1* is a major determinant in several neurodevelopmental diseases such as autism, speech defects, and intellectual disabilities; however, so far this has lacked a detailed mechanistic explanation (Frohlich et al., 2017; Hamdan et al., 2010; Le Fevre et al., 2013; Lozano et al., 2015; Palumbo et al., 2013; Sollis et al., 2016). Importantly, the specific cell types involved in the etiology of these conditions have not been characterized. We propose that FOXP1 has a crucial role in promoting embryonic NSC differentiation and migration. Whether FOXP1 KD is affecting both migration and differentiation of NSCs separately or whether reduced differentiation affects the migratory capacity of the NSCs from the VZ remains unclear. Defects in the regulation of neural progenitors during development are associated with a wide range of neurodevelopmental disorders, including autism (Kaushik and Zarbalis, 2016). To this end, we found that FOXP1 binds near genes linked to CNS diseases. In accordance with a previous study, we observed that FOXP1 can regulate a subset of autism-related genes from the Simons Foundation Autism Research Initiative dataset (SFARI) (<http://sfari.org>) (Araujo et al., 2015; Basu et al., 2009) (Figure S3A). These observations indicate that these disease-associated genes might become deregulated during development due to mutations altering the functionality of FOXP1 or changes in its expression levels (Chien et al., 2013; Lozano et al., 2015). In our ChIP-seq analysis, we found the consensus motives of the neurodevelopmental transcription factors SOX3 and NR2E1/TLX associated with the Forkhead motif (Archer et al., 2011; Wang and Xiong, 2016), indicating a possible interaction between





FOXP1 and these factors to regulate genes involved in neurogenesis. SOX3 inhibits neural progenitor differentiation by preventing premature activation of neuronal genes by competing for binding sites with SOX11 (Bergsland et al., 2011). It is possible that FOXP1 may compete for binding of pro-neural genes with SOX3, thereby inducing differentiation. Similarly, NR2E1/TLX controls the expression of a gene network involved in NSC maintenance (Islam and Zhang, 2015), and FOXP1 may repress their transcription, thereby promoting neurogenesis. We have previously shown that *Foxp1* expression can be transcriptionally induced by FOXO3, with FOXP1 subsequently inhibiting a subset of transcriptional targets activated by FOXO3 through a negative feedback loop (van Boxtel et al., 2013). In NSCs, FOXO3 has been shown to be required for NSC homeostasis by inducing a program of genes that prevents premature differentiation (Renault et al., 2009). Possibly, FOXP1 expression may be induced by FOXO3 also in NSCs where it may then suppress FOXO3-dependent genes that prevent differentiation.

The Notch pathway has a fundamental role in the maintenance of neural progenitors. After activation of the Notch receptor by its ligands, such as JAG1 or DLL1, the Notch intracellular domain (NICD) translocates to the nucleus. There, together with RBPJ, NICD induces the expression of transcriptional inhibitors such as HES1 and HES5. These factors repress the expression of pro-neural genes, thereby preventing differentiation (Gaiano and Fishell, 2002; Kopan and Ilagan, 2009; Louvi and Artavanis-Tsakonas, 2006; Shimojo et al., 2008). As our data indicate, FOXP1 binds directly to the *Jag1* promoter, thereby repressing its transcription. When FOXP1 levels are reduced, the increased expression of JAG1 on the cell surface results in enhanced Notch activation in neighboring cells, as demonstrated by the increased levels of NICD induced by FOXP1 depletion we observed during cortical development. This finally results in increased levels of HES1 and thereby prevention of differentiation. We have shown that FOXP1-mediated *Jag1* repression is a requirement for neuronal differentiation but not for astrocyte specification *in vitro*. Therefore it will be relevant to investigate other FOXP1 targets involved in astrogliogenesis. Previously, it has been shown that expression of JAG1 by endothelial cells in the adult SVZ induces quiescence in NSCs by promoting the expression of stemness/progenitor genes (Ottone et al., 2014). In line with this, we observed increased expression of the neural stem/progenitor marker Nestin after FOXP1 KD and reduction of the expression of the progenitor markers SOX2 and TBR2 upon FOXP1 overexpression *in vitro*. During NSC differentiation, the expression of lineage-specific genes is increased with concomitant inhibition of processes involved in the maintenance of the stem cell/progenitor state such as the Notch pathway (Im-

ayoshi et al., 2013; Shimojo et al., 2008). Strikingly, upon FOXP1 KD, we observed an induction of genes related to neurogenesis (Figure S3B). Taken together, these observations indicate that FOXP1 acts by both inducing the expression of neurogenesis-specific genes as well as by directly repressing *Jag1*.

The detection of the expression of two isoforms of FOXP1, which share the same mRNA (FOXP1A and FOXP1C), raises the question whether there would be an isoform-specific role for FOXP1. FOXP1C, the smaller isoform, is translated starting from a differential start codon and it lacks the Q-rich domain (Shu et al., 2001). The shRNAs we utilized to deplete FOXP1 in our experiments target the mRNA that code for both isoforms. This makes it impossible to investigate the specific role of each isoform in regulating NSC differentiation. FOXP1 has also been shown to both homo- and heterodimerize with FOXP2 and FOXP4 (Shu et al., 2001; Sin et al., 2015). Notably, FOXP1, FOXP2, and FOXP4 expression is simultaneously found in different areas of the songbird and human brain (Chen et al., 2013; Mendoza et al., 2015; Teramitsu et al., 2004). Moreover, FOXP1 and FOXP2 have been shown to regulate shared target in the murine striatum (Araujo et al., 2015). It is possible that the FOXP1C isoform lacks the capacity of dimerization, therefore regulating transcription in a distinct manner to FOXP1A. FOXP1 has been mainly regarded as a transcriptional repressor (Shu et al., 2001; van Boxtel et al., 2013). However, in our own data we observed that the expression of the majority of FOXP1-bound genes were either repressed or activated (data not shown). It could be that in NSCs, FOXP1 plays a role as both an inducer and a repressor of transcription, perhaps with one isoform of FOXP1 activating gene expression and the other one repressing it.

In conclusion, we demonstrate that FOXP1 promotes NSC differentiation both *in vitro* and *in vivo*, and that this occurs at least in part through the repression of *Jag1* by FOXP1. This study sheds light on a regulator of neural development and identifies FOXP1 as a modulator of the Notch pathway. Identifying key regulators of NSC differentiation during development of the brain as well as after cerebral injury might provide future tools to develop novel treatments for neurodevelopmental disorders or neurological diseases that would benefit from enhanced neuroregeneration.

## EXPERIMENTAL PROCEDURES

### qPCR

RNA was isolated using the RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturers' instruction. Reverse transcriptase reaction for the generation of cDNA was performed using an 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 Supermax (Bio-Rad) for cDNA application following the manufacturers' protocol. Relative expression was calculated with the  $\Delta\Delta C_t$  method using beta-2-microtubulin (*B2m*) to normalize. The list of primers used can be found in [Supplemental Experimental Procedures](#).

### 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. Each sample (40  $\mu$ g) was analyzed by SDS-PAGE and transferred by electrophoresis onto polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membranes were blocked using 2% BSA in TBST (0.3% Tween, 10 mM Tris [pH 8], and 150 mM NaCl in H<sub>2</sub>O) and probed with anti-FOXP1 (Cell Signaling Technologies, Danvers, MA, no. 2005, 1:1,000) and anti-Tubulin (Sigma-Aldrich, St. Louis, MO; T5168, 1:50,000). Signal was detected using Amersham ECL Western Blotting Detection Reagent (Little Chalfont, UK).

### Animals

#### *Transplantation of NSCs after Hypoxic-Ischemic Brain Damage*

All experiments were performed in accordance with international guidelines and approved by Experimental Animal Committee Utrecht (DEC-Utrecht, University Utrecht, Utrecht, The Netherlands). For the transplantation experiment, 9-day-old (P9) C57BL/6J mice underwent HI by permanent right carotid artery occlusion under isoflurane anesthesia (4% induction, 1.5% maintenance) followed by 45 min exposure to 10% oxygen ([Nijboer et al., 2008](#)). This procedure results in unilateral damage to the hippocampus, neocortex, and striatum ([van der Kooij et al., 2010](#)). Sham-operated control animals underwent anesthesia and incision only. In total, 44 pups of both genders from 10 different litters were randomly distributed among all experimental groups. No significant gender differences were identified for any of the measured parameters. All analyses were performed in a blinded setup.

At day 10 after induction of HI,  $1 \times 10^5$  NSCs resuspended in 2  $\mu$ L of PBS or vehicle (2  $\mu$ L of PBS) were injected under isoflurane anesthesia at 2 mm caudal to bregma, 1.5 mm right from midline, and 4 mm below the dural surface in order to reach the ipsilateral hippocampus ([Braccioli et al., 2016](#)). Some of the animals were killed on day 15 (5 days post treatment) after HI by overdose of pentobarbital followed by transcardial perfusion with PBS followed by 4% formaldehyde. Brains were collected and post-fixed in 4% formaldehyde.

Some of the animals survived until day 28 after induction of HI and were subjected to the CRT. The CRT was used to assess forelimb use asymmetry as described before ([Schallert et al., 2000](#); [van der Kooij et al., 2010](#)). Briefly, mice were individually placed in a Plexiglas cylinder and observed for 3 min. The forepaw used to contact the cylinder wall during a weight-bearing full rear was scored as left (L; impaired), right (R; non-impaired), or both. Non-impaired (R) paw preference was calculated as follows:  $[(R - L)/(L + R + \text{both})] \times 100\%$  ([van der Kooij et al., 2010](#); [van Velthoven et al., 2010](#)). Inclusion criterion was a minimum of 10 weight-bearing movements in

3 min. Two animals from the shFOXP1 NSC group were excluded as they did not meet the inclusion criterion. The test was performed by a trained observer blinded to treatment.

#### *In Utero Electroporation*

For the *in utero* electroporation experiments, mouse embryos were injected with combinations of shFOXP1-1 and shFOXP1-2 (1:1 molar ratio) or shSCR together with pCAG-GFP. Motor cortices were targeted by electroporation with an ECM 830 Electro-Square-Porator (Harvard Apparatus, Holliston, MA) set to five unipolar pulses of 50 MS at 30 V (950-ms interval). Embryos were placed back into the abdomen, and abdominal muscles and skin were sutured separately. Embryos were collected at E17.5, and brains were fixed in 4% formaldehyde. For the analysis of the expression of FOXP1 in the developing cortex, embryos were collected at E12 and E14 and fixed in 4% formaldehyde.

#### *Chromatin Immunoprecipitation and Sequencing*

Before ChIP and sequencing, NSCs were grown as neurospheres in complete medium in six wells. ChIP was performed as previously described ([van Boxtel et al., 2013](#)). Briefly, crosslink was performed with disuccinimidyl glutarate (DSG) (Thermo Scientific) for 45 min followed by 30 min incubation with formaldehyde 1%. The reaction was blocked with glycine 100 mM. Shearing was performed using Covaries S2 (Covaris, Woburn, MA) for 8 min at maximum intensity. The sonicated chromatin was incubated overnight at 4°C in the presence of 5  $\mu$ g of anti-FOXP1 (ab16645, Abcam) coupled to A/G Sepharose beads (Santa Cruz Biotechnology). A 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 an Illumina NextSeq500 sequencer as previously described ([Peeters et al., 2015](#)) (50 bp, single-end, Utrecht sequencing facility, Utrecht Medical Center, Utrecht, The Netherlands).

#### *RNA Sequencing*

Total RNA was extracted from adherent NSCs cultured on 6-well coated plastic plates for 48 hr 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 higher than 9.0. Sample preparation was performed using a Poly(A)Purist MAG Kit (Thermo Scientific) according to the manufacturer's instructions. Isolated mRNA was subsequently repurified using an mRNA-ONLY Eukaryotic mRNA Isolation Kit (Epicentre Illumina, Madison, WI). Sequencing libraries were prepared using a SOLiD Total RNA-Seq Kit (Applied Biosystems Life Technologies) according to the standard protocol recommendations and sequenced on a SOLiD 5500 Wildfire sequencer to produce 50 bp reads as described previously ([van Boxtel et al., 2013](#)).

#### *Gene Set Enrichment Analysis*

For GSEA, pre-ranked analysis was performed with the GSEA software probing for enrichment of mouse genes belonging to the GO term Notch signaling pathway (GO:0007219) in the RNA-seq dataset ranked by log fold change ([Carbon et al., 2009](#); [Subramanian et al., 2005](#)).



## Statistics

Data are expressed as means  $\pm$  SEM and regarded statistically significant if  $p < 0.05$ . Data were analyzed using one-way ANOVA with Dunnett's post-test. One-way ANOVA with Tukey's post-test was used for the CRT. For the anti-Jag1 blocking antibody experiment, two-way ANOVA with Sidak's and Tukey's post-test were used. For the analysis of GFP-positive cells in the embryos and DCX-dsRed-positive cells in the transplantation experiment, two-tailed, unpaired Student's *t* test was used.

For additional information see [Supplemental Experimental Procedures](#).

## ACCESSION NUMBERS

The ChIP-seq data presented in this study have been deposited in NCBI's Gene Expression Omnibus (GEO) database under GEO: GSE101632 (linked to GSE101633). The RNA-seq data presented in this study have been deposited in NCBI's GEO database under GEO: GSE101605 (linked to GSE101633).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at <https://doi.org/10.1016/j.stemcr.2017.10.012>.

## AUTHOR CONTRIBUTIONS

L.B. and C.H.N. performed the experiments and analyzed the data. L.B., C.H.N., and P.J.C. designed the experiments and wrote the manuscript. S.J.V. contributed to the NGS analysis. Y.A. and R.J.P. contributed with the *in utero* electroporation experiments. C.J.H. provided intellectual input. O.B. provided technical assistance.

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