PERSPECTIVE

Forkhead box protein P1, a key player in neuronal development?

Forkhead box protein P1 (FOXP1) is a transcription factor belonging to the forkhead box (FOX) proteins, a family of transcriptional regulators sharing a highly conserved forkhead DNA-binding domain (Bacon and Rappold, 2012). Previous reports have proposed a role for FOXP1 in functionally regulating the central nervous system (CNS), while mutations in *FOXP1* have been implicated in cognitive abnormalities (Bacon and Rappold, 2012). FOXP1 has been shown to promote differentiation of medium spiny neurons and to induce neuronal migration during embryonic neuronal development in the mouse cortex (Li et al., 2015; Precious et al., 2016). Mutations in *FOXP1* have been linked to various neurodevelopmental diseases, including autism, intellectual disabilities and speech defects (Hamdan et al., 2010; Horn et al., 2010). Furthermore, Bacon and colleagues demonstrated that Cre-mediated *Foxp1* deletion in Nestin-positive cells induces autism-like behavior in mice, and defects in morphology of the striatum upon postnatal development (Bacon et al., 2015). In line with these findings, heterozygous *Foxp1*$^{+/-}$ mice have been reported to show deficits in vocal communication as well as a deregulation of autism-associated genes in the hippocampus and striatum (Araujo et al., 2015). Taken together, this body of evidence indicates an important role of FOXP1 in regulating CNS development, however the molecular mechanisms underlying neuronal FOXP1 function have remained unclear.

Several recent studies have started to shed light on the role of FOXP1 during neural development (Braccioli et al., 2017; Rocca et al., 2017; Usui et al., 2017) The first study, authored by our group, defines a novel role for FOXP1 in promoting mouse embryonic neural stem cell (NSC) differentiation by repressing Jagged1 (*JAG1*) expression (Braccioli et al., 2017). In this study, RNA-sequencing and chromatin-immunoprecipitation (ChIP)-sequencing were utilized to show that FOXP1 binds to the promoter region of genes in NSCs that are associated with diseases of the CNS, as well as regulating the expression of genes associated with neurogenesis. Through gene-set enrichment analysis (GSEA), it was observed that FOXP1 represses the expression of genes associated with Notch signaling pathway in NSCs. These observations led us to investigate whether FOXP1 can regulate NSC differentiation. Short-hairpin RNA (shRNA)-mediated depletion and overexpression revealed that FOXP1 promotes NSC differentiation in vitro towards both neurons and astrocytes, but not to oligodendrocytes. Consistently, FOXP1 is found to repress the maintenance of progenitor-like characteristics. These observations were validated *in vivo* using two different models. First, *in utero* electroporation of FOXP1-directed shRNAs demonstrated that FOXP1 is required for radial glia development by promoting both neuronal migration and intermediate progenitor differentiation during cortical development. Moreover, through intracranial transplantation of FOXP1-depleted NSCs in a hypoxic-ischemic (HI) brain damage model, FOXP1 was found to promote neuronal differentiation of transplanted NSCs *in vivo*. Additionally, FOXP1 was found to be required for improvement of sensorimotor function mediated by NSC transplantation upon HI. FOXP1 was subsequently found to inhibit expression of the Notch ligand JAG1 by binding to its promoter, and this resulted in inhibition of the Notch pathway in NSCs both *in vitro* and in the developing cortex. Treating FOXP1-depleted NSCs with an anti-JAG1 blocking antibody was found to rescue the reduction of neural differentiation caused by FOXP1 depletion. Taken together, these findings support a role for FOXP1 as a key inducer of embryonic NSC differentiation by repressing JAG1 expression. Confirming this hypothesis, FOXP1 depletion resulted in increased expression of hairy and enhancer of split-1 (HES1), a downstream effector of the Notch pathway in NSCs *in vitro* and increased levels of the activated Notch intracellular domain in the developing cortex. FOXP1 was also found to regulate a subgroup of genes related to autism-spectrum diseases (ASD) in NSCs, validating the notion that FOXP1 plays a fundamental role in autism (Hamdan et al., 2010; Araujo et al., 2015; Bacon et al., 2015). However, several issues underlying the mechanism by which FOXP1 regulates NSC differentiation remain unclear. For example, it remains unclear whether FOXP1 is promoting both migration and differentiation of NSCs separately during cortical development, or whether defects in neuronal differentiation are themselves responsible for the reduced migratory capacity of NSCs. While there are distinct FOXP1 isoforms in NSCs (FOXP1A and FOXP1C), it was not possible to discriminate the individual role of these isoforms in promoting NSC differentiation since the shRNAs utilized to target FOXP1 target both FOXP1A and FOXP1C which are translated from the same mRNA, but from differential starting codons. To investigate this issue, it would be necessary to generate mice carrying a mutation in the alternative start codon preventing FOXP1C translation. It would be also interesting to determine which co-factors interact with or activate FOXP1 in NSCs in order to repress JAG1 and promote NSC differentiation. In our study, we observed that overexpression of FOXP1 leads to increased neuronal differentiation. Therefore, ectopic expression of FOXP1 in NSCs improves the capacity of NSCs to generate neurons, potentially increasing the regenerative capacity of transplanted NSCs upon brain damage. For example, it would be relevant to investigate whether transplantation of FOXP1-overexpressing NSCs upon HI could induce a more efficient generation of neurons when compared to regular NSCs, and if this could lead to increased improvements in functional and anatomical impairments after HI, compared to regular NSC treatment.

A second study by Konopka and colleagues has tackled the question as to whether FOXP1 plays a role in the neocortex during postnatal development in mice, as this stage is relevant for ASD (Usui et al., 2017). To this end, they generated a conditional FOXP1 knockout by crossing Emx1-Cre mice, expressing the Cre-recombinase specifically in radial glia, with *Foxp1*$^{flox/flox}$ mice. This led to deletion of *Foxp1* in both the cortex and the hippocampus and caused abnormalities in vocal communication during postnatal development. Additionally, it was observed that FOXP1 depletion caused alterations in brain structure and architecture of the postnatal neocortex, as indicated by reduction in neocortical size and altered localization of neurons in the deep cortical layers. Furthermore, analysis of gene expression changes in the postnatal cortex upon FOXP1 depletion revealed that FOXP1 regulates the expression of genes involved in synapse formation and neuronal development. A subset of genes identified was associated with ASD and regulated by FOXP1, an observation that provides additional weight to the idea that *FOXP1* is a relevant gene involved in the pathogenesis of ASD. In exploring the mechanisms regulating FOXP1 activity during neuronal development, FOXP1 was found to be sumoylated, and levels of sumoylated-FOXP1 decreased from embryonic to postnatal development. By generation of a sumoylation-deficient mutant of FOXP1 (K636R), it was demonstrated that FOXP1 sumoylation is necessary to promote neurite outgrowth of mouse and human cortical neurons *in vitro*, and neuronal migration in mouse embryonic cortex *in vivo*. Sumoylation of FOXP1 was also found to prevent the interaction of FOXP1 with members of the nucleosome remodeling deacetylase (NuRD) chromatin remodeling complex, including histone deacetylases (HDAC) 1/2 and metastasis associated proteins (MTA) 1/2. However, while sumoylated-FOXP1 was shown to promote neurite outgrowth and neuronal migration, it remains unclear whether either this sumoylation as such, or the association of FOXP1 with the NuRD complex, is directly required for FOXP1-mediated transcriptional regulation. Rocca et al. (2017) also provide evidence that FOXP1 can be sumoylated in rat cells (in this case the sumoylated residue is K670) and that this modification promotes dendritic outgrowth in embryonic rat cortical neurons. The authors suggest that sumoylation of FOXP1 enhances binding of FOXP1 to the transcriptional co-repressor C-terminal-binding protein 1 (CTBP1). In this study, it was also shown that sumoylation at K670 is required for FOXP1-mediated transcriptional repression of the simian virus (SV)40 promoter in human embryonic kidney cells (HEK)293T, implicating that this modification could be important in regulating the transcriptional targets of FOXP1 (Rocca et al., 2017) (**Figure 1**). Taken together, these observations indicate that FOXP1 requires sumoylation to interact with CTBP1, thereby repressing transcription of its target genes. However, it would be relevant to investigate whether the expression of relevant target genes is affected in sumoylation-deficient FOXP1 mutants during embryonic and postnatal cortical development. Furthermore, two different FOXP1 isoforms were identified in the postnatal cortex, FOXP1A and FOXP1D. It would be interesting to investigate whether both isoforms (together with FOXP1C) become sumoylated, and how this modification affects the transcriptional output.

Both our study and study of Usui and colleagues described above indicate that FOXP1 regulates ASD-related genes. It is now important to evaluate whether these ASD-related FOXP1 target genes are conserved between embryonic and postnatal development. To this end, we compared the results of our study with the results obtained by Konopka's group: we overlapped the genes associated with ASD and those differentially regulated by FOXP1 found by Usui et al. (2017) and targeted by FOXP1 from our study. Interestingly, we identified a significant overlap of three ASD-associated genes (*Cttnbp2*, *Pcdh15* and *Cdh10*), besides *Foxp1* itself, that were downregulated both in NSCs upon FOXP1 knockdown in our dataset and in the cortex at postnatal day(P)0 upon

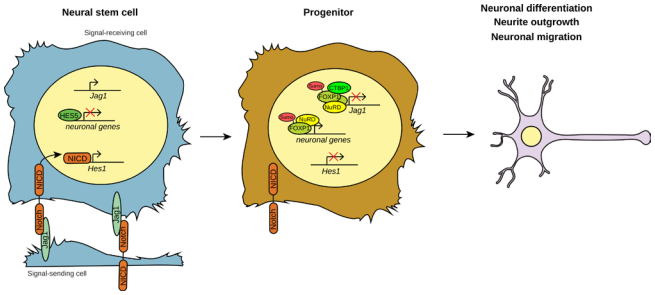

Figure 1 Schematic model illustrating the role of forkhead box protein P1 (FOXP1) in neurogenesis.
Left: In neural stem cells (NSCs) Jagged1 (JAG1) expression induces Notch activation and stem cell maintenance, partly by repression of transcription of neuronal genes by the downstream Notch effector enhancer of split-1 (HES1). During neuronal progenitor differentiation (middle), FOXP1 is expressed and sumoylated (Sumo). This leads FOXP1 to interact with the nucleosome remodeling deacetylase (NuRD) complex and C-terminal-binding protein 1 (CTBP1), binding to the promoter region of JAG1 to repress its expression. Reduced level of JAG1 subsequently leads to reduction in Notch signaling, hence allowing expression of neuronal genes. Simultaneously, sumoylated FOXP1 interacts with NuRD and binds to the promoter of neuronal genes, thereby inducing their expression. These events eventually lead to neuronal differentiation, neurite outgrowth and neuronal migration (right).

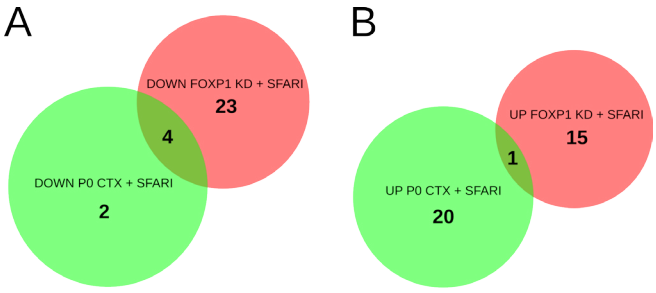

Figure 2 Overlap between autism-spectrum diseases (ASD)-associated genes and genes regulated by forkhead box protein P1 (FOXP1).
The differentially expressed genes identified by Braccioli et al. (2017) upon FOXP1 knockdown (KD; red) in neural stem cells were overlapped with the list of ASD-associated genes from the Simons Foundation Autism Research Initiative (SFARI) dataset used by Usui et al. (2017). This gene-set was then overlapped with the ASD-associated genes differentially expressed in the cortex at P0 described in the study of Usui et al. (2017) (green). (A) Down-regulated genes; (B) upregulated genes.

Foxp1 deletion in the study (Usui et al., 2017) (**Figure 2A**). Conversely, while we found only 1 gene (*Sparcl1*) that was upregulated both in NSCs upon FOXP1 knockdown and in the cortex at P0 upon *Foxp1* deletion (**Figure 2B**). It would be interesting to address the function of these genes in relation to FOXP1 activity as they could underlie a conserved mechanism of regulation in both embryonic and postnatal neurogenesis. During adulthood, neurogenesis continues to occur in specialized niches in the CNS such as the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Kriegstein and Alvarez-Buylla, 2009). However, it is not known whether FOXP1 plays a role in this process. Conditional deletion of FOXP1 in the adult neural progenitor compartment would help resolve the question whether FOXP1 promotes adult NSC differentiation and neuronal migration. In this adult setting, it would be interesting to investigate whether FOXP1 sumoylation is also required to promote neural development as well as to inhibit Notch pathway by repressing JAG1 expression.

Moreover, it would be relevant to investigate the interactome of FOXP1, in order to identify neurodevelopmental proteins that might cohoperate with FOXP1 in regulating neurogenesis. To this end, a recent study published by Estruch et al. (2018) indicates that FOXP1 interacts with SOX5, SATB2, SATB1, NR2F1 and NR2F2, which have been linked to neurodevelopment.

In conclusion, these studies show that FOXP1 is necessary to promote neuronal migration and differentiation during embryonic and postnatal brain development. Additionally, sumoylation of FOXP1 promotes neuronal migration and neurite outgrowth, as well as inhibits FOXP1 interaction with the NuRD complex (**Figure 1**). Since the evidence presented by Usui et al. (2017) and Rocca et al. (2017) indicates that sumoylation regulates FOXP1 transcriptional activity and its function in promoting neurite outgrowth and neuronal migration, it would be relevant to study whether inducing or inhibiting FOXP1 sumoylation in NSCs could alter the regenerative capacity of NSC transplantation upon HI brain damage. *Vice versa*, increasing the amount of sumoylated FOXP1 in NSCs might be a strategy to promote neurogenesis and neuronal maturation. Finally, FOXP1 promotes embryonic neural differentiation by inhibiting the Notch pathway, at least in part by repressing JAG1 expression (**Figure 1**). Moreover, in both embryonic and postnatal brain development, FOXP1 regulates a subset of ASD-associated genes. Taken together, these observations indicate a relevant role for FOXP1 in promoting neural development both during embryogenesis and postnatally, and confirm FOXP1 as a key gene involved in the etiology of ASD.

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