

Transcriptional Regulation by FOXP1, FOXP2, and FOXP4 Dimerization

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Abstract *FOXP1*, *FOXP2*, and *FOXP4* are three members of the *FOXP* gene subfamily of transcription factors involved in the development of the central nervous system. Previous studies have shown that the transcriptional activity of FOXP1/2/4 is regulated by homo- and heterodimerization. However, their transcriptional gene targets in the developing brain are still largely unknown. FOXP2 regulates the expression of many genes important in embryonic development, including WNT and Notch signaling pathways. In this study, we investigate whether dimerization of FOXP1/2/4 leads to differential expression of ten known FOXP2 target genes (*CER1*, *SFRP4*, *WISP2*, *PRICKLE1*, *NCOR2*, *SNW1*, *NEUROD2*, *PAX3*, *EFNB3*, and *SLIT1*). FOXP1/2/4 open-reading frames were stably transfected into HEK293 cells, and the expression level of these FOXP2 target genes was quantified using real-time polymerase chain reaction. Our results revealed that the specific combination of FOXP1/2/4 dimers regulates transcription of various FOXP2 target genes involved in early neuronal development.

Keywords FOXP proteins · *FOXP2*-regulated genes · Neurodevelopment · Autism

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Introduction

The *FOXP* subfamily is a member of the *Forkhead box (FOX)* gene family of transcription factors that comprises four members: *FOXP1*, *FOXP2*, *FOXP3*, and *FOXP4*. FOXP1, FOXP2, and FOXP4 are abundantly expressed throughout the developing brain (Teramitsu et al. 2004; Takahashi et al. 2008a, b, 2009; Konopka et al. 2009, whereas FOXP3 is predominantly expressed in the immune system (Takahashi et al. 2009). The emerging evidence shows that the FOXP family of transcription factors are associated with language disorders and may play an important role in the neuropathology of neurodevelopmental disorders such as autism spectrum (Bowers and Konopka 2012). *FOXP2* gene have been linked to an inherited form of language and speech disorders (Lai et al. 2001; MacDermot et al. 2005; Vernes et al. 2008; Fisher and Scharff 2009). Furthermore, *FOXP1* has been recently linked to autism (Hamdan et al. 2010; Horn et al. 2010; O'Roak et al. 2011; Talkowski et al. 2012).

Transcriptional activity of FOXP1, FOXP2, and FOXP4 is regulated by tissue-specific homo- and heterodimerization via a zinc finger and a leucine zipper motif (Li et al. 2004). The neural expression pattern of FOXP1, FOXP2, and FOXP4 partly overlaps (Takahashi et al. 2009), but the combination of specific dimers and their transcriptional targets in the developing brain are still unknown. To date, only FOXP2 target genes have been identified. Studies in human fetal brain samples and neuronal cell models have found that the expression of a majority of *FOXP2*-regulated genes was repressed (Spiteri et al. 2007; Vernes et al. 2007; Konopka et al. 2009). These genes appear to have putative roles in the nervous system such as modulation of synaptic plasticity, neurodevelopment, or neural transmission. Given that the transcriptional activity of FOXP1/2/4 proteins requires dimerization, the specific combination of homodimers and heterodimers may modulate the transcription of specific target genes

that may consequently play an important role in the developing nervous system.

In this study, ten previously identified neural targets of FOXP2 were chosen (*Cerberus 1 (CER1)*, *Secreted frizzled-related protein 4 (SFRP4)*, *WNT1 inducible signaling pathway protein 2 (WISP2)*, *Prickle homolog 1 (PRICKLE1)*, *Nuclear receptor co-repressor 2 (NCOR2)*, *SNW domain containing 1 (SNW1)*, *Neurogenic differentiation factor 2 (NEUROD2)*, *Paired box 3 (PAX3)*, *Ephrin-B3 (EFNB3)*, and *Slit homolog 1 (SLIT1)*) to determine whether different dimers of FOXP1/2/4 proteins will lead to altered expression of the target genes. *CER1*, *SFRP4*, *WISP2*, and *PRICKLE1* participate in the WNT signaling pathway, and *NCOR2* and *SNW1* belong to the Notch signaling pathway. Both WNT and Notch signaling pathways are essential for regulation of embryonic development. Moreover, *NEUROD2*, *PAX3*, *EFNB3*, and *SLIT1* are involved in the development of the central nervous system (CNS). We hypothesize that the formation of different FOXP1/2/4 dimers will lead to differential expression of these target genes. Uncovering the transcriptional regulatory events of FOXP proteins will help characterize their mechanisms of action that might be occurring in the developing brain.

Materials and Methods

Molecular Cloning of *Homo sapiens* FOXP1, FOXP2, and FOXP4 cDNA

The *FOXP1* (GenBank: BC152752; encodes 677 amino acids), *FOXP2* (GenBank: BC143867, encodes 714 amino acids), and *FOXP4* (GenBank: BC052803, encodes 678 amino acids) cDNA were obtained from the DNASU Plasmid Repository or the Mammalian Gene Collection (MGC). The entire open-reading frames were amplified by PCR using forward and reverse primers incorporating *KpnI* and *XhoI* restriction sites (Table 1). Following PCR amplification and restriction digest with *KpnI* and *XhoI*, the FOXP1 and FOXP2 fragments were ligated with T4 DNA ligase into the *KpnI/XhoI* cloning sites of the mammalian expression vector pcDNA3-Flag and pcDNA3.1/*myc*-His (A) (Invitrogen), respectively. The FOXP4 fragment was ligated into the *KpnI/XhoI* cloning sites of both pcDNA3-Flag and pcDNA3/*myc*-His (A) expression vectors. The resulting FOXP1/pcDNA3-Flag, FOXP2/pcDNA3.1/*myc*-His, FOXP4/pcDNA3-Flag, and FOXP4/pcDNA3.1/*myc*-His constructs were fully sequenced at the York University Core Molecular Facility. The sequences were confirmed using the multiple-sequence alignment tool NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). All primers were designed using the Primer3 program (<http://frodo.wi.mit.edu/primer3/>) and produced by Sigma-Aldrich.

Cell Culture

HEK293 cells were obtained from the American Tissue Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10 % fetal bovine serum (FBS), in a 37 °C incubator containing 5 % CO₂. One day prior to transfection, cells were plated in six-well plates (10 cm²/well) and allowed to reach 80–90 % confluency at the time of transfection. Lipofectamine™ 2000 (Invitrogen) method was used for transfection of HEK293 cells according to the manufacturer's instructions. Four micrograms of each FOXP DNA construct was used per well for co-transfection or single transfection. Based on the Geneticin (G418) resistance test, result on untransfected HEK293 cells, 400 µg/mL G418 was applied 30 h posttransfection for selecting cells transfected with appropriate FOXP1/2/4 constructs. The cells were then fed every 2–3 days with medium containing increasing concentration of G418 (400–1,000 mg/mL). Single cell clones were generated by diluting cells in a 96-well plate and expanded in medium with 1,000 µg/mL G418 for analysis.

Western Blot Analysis

Western blot analysis was used in order to determine whether transfected HEK293 cells expressed FOXP1/2/4 proteins. Cell lysates were extracted and prepared as previously described (Vallipuram et al. 2009). The nitrocellulose membranes were incubated overnight at 4 °C with mouse monoclonal anti-*c-myc* (1:200; Santa Cruz Biotechnology) or mouse monoclonal anti-FLAG M2 (1:1,000; Sigma-Aldrich), followed by goat polyclonal secondary antibody mouse IgG-H&L HRP (1:1,000; Abcam) for 1 h at room temperature. The membranes were washed with 1× Tris-buffered saline containing 0.1 % Tween 20 (TBS-T) and incubated with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare). The bound antibodies were detected by Geliance 600 Imaging System (PerkinElmer).

Immunoprecipitation

HEK 293 cells cotransfected with two FOXP genes (FOXP1-Flag/FOXP2-*myc*, FOXP1-Flag/FOXP4-*myc*, or FOXP2-*myc*/FOXP4-Flag, as indicated above) were used for co-immunoprecipitation (Co-IP) assay. Cells were collected using ice-cold nonionic detergent lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, and 5 µL/mL protease inhibitor), and then centrifuged at 14,000× *g* for 10 min. For *c-myc* fusion protein immunoprecipitation (IP), the lysates were incubated with mouse monoclonal anti-*c-myc*

Table 1 Primer sequences for *FOXP1*, *FOXP2*, and *FOXP4* cDNA cloning and sequencing

Gene	Designation vector	Primer sequences (5'→3')	Amplicon size (base pair)
<i>FOXP1</i>	pcDNA3-flag	F: GCTT <u>GGTACCG</u> ATGATGCAAGAATCTGGGACT R: GCCG <u>CTCGAG</u> CCTCCATGTCCTCGTTTACTGG I: AACACAGGCAACAATCACA	2,036
<i>FOXP2</i>	pcDNA3.1/ <i>myc</i> -His A	F: GCTT <u>GGTACCAT</u> GATGACTCCCCAGGTGATCA R: CCG <u>CTCGAG</u> TTCAGATCTTCAGATAAAGGCTC I: TTCCTCCTCGACTACCTCCT	2,205
<i>FOXP4</i>	pcDNA3.1/ <i>myc</i> -His A	F: GCTT <u>GGTACCG</u> AATGATGGTGGAATCTGCCTCG R: CCG <u>CTCGAG</u> GGACAGTTCTTCTCCCGGCA I: CACCGCTACCTCGTTTGC	2,061
<i>FOXP4</i>	pcDNA3-flag	F: GCTT <u>GGTACCG</u> ATGATGGTGGAATCTGCCTCG R: CGT <u>A</u> <u>CTCGAG</u> CGGACAGTTCTTCTCCCGGCA I: CACCGCTACCTCGTTTGC	2,064

F forward primer, R reverse primer, I internal primer. Restriction site sequences for *KpnI* (GGTACC) and *XhoI* (CTCGAG) are underlined

antibody-9E10 (2 µg; Santa Cruz Biotechnology) at 4 °C for 2–4 h, followed by overnight incubation at 4 °C with IgG Sepharose 6 Fast Flow (20 µL; GE Healthcare) pre-equilibrated in the lysis buffer. For Flag fusion protein IP, the lysates were incubated with anti-Flag M2 affinity gel (Sigma-Aldrich) overnight at 4 °C. Following the overnight incubation, the beads were washed four times with ice-cold lysis buffer and the immunoprecipitates were eluted in Laemmli Sample Buffer (Bio-Rad) by boiling. The protein samples were subjected to SDS-PAGE (10 %) and immunoblotting, using mouse monoclonal anti-c-*myc* antibody (1:200; Santa Cruz Biotechnology) for Flag fusion protein or mouse monoclonal anti-FLAG M2 (1:1,000; Sigma-Aldrich) for c-*myc* fusion protein, respectively. The blots were subsequently treated with goat polyclonal secondary antibody Mouse IgG-H&L HRP (1:1,000; Abcam) and detected as described in method of “Western Blot Analysis.”

Immunocytochemistry

Immunocytochemistry was done to confirm the stable expression of the transfected *FOXP* genes in cells. HEK293 cells expressing the indicated *FOXP1*, *FOXP2*, and *FOXP4* homo- or heterodimers were seeded in 35 mm dishes with cover slips previously treated with poly-D-lysine hydrobromide. The cells were fixed with 1× phosphate-buffered saline (PBS) containing 4 % paraformaldehyde for 20 min at 4 °C and permeabilized with 0.5 % Triton® X-100 in 1× PBS for 10 min and blocked in PBS with 0.5 % BSA, Fraction V (Sigma-Aldrich) for 20 min at room temperature. Mouse monoclonal anti-*myc* (1:200; Santa Cruz Biotechnology) or rabbit polyclonal anti-DDDDK tag (1:400; Abcam) primary antibodies were used for 1 h at room temperature. The cells were washed with PBS containing 0.3 % Triton® X-100 (PBS-T) and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:300; Jackson ImmunoResearch Laboratories) and Texas

Red (TR) dye-conjugated anti-mouse IgG (1:300; Jackson ImmunoResearch Laboratories) secondary antibodies. The cells were subsequently washed with PBS-T and incubated with 4'-6-diamidino-2-phenylindole (DAPI; 0.05 %) for 20 min. The coverslips were mounted onto microscope slides with Vectashield Mounting Medium (Vector Laboratories) and visualized using a Nikon Eclipse 80i microscope.

Reverse Transcription and Quantitative Real-Time Polymerase Chain Reaction

Total RNA from transfected HEK293 cells was isolated using the NucleoSpin RNA/Protein kit (Machery-Nagel). Reverse transcription was performed with M-MuLV Reverse Transcriptase (New England Biolabs) in accordance with the manufacturer's instructions. One microgram of RNA was used in a reaction with 10× RT buffer, Oligo d(T)18 mRNA Primer, dNTP mix (2.5 mM each), and M-MuLV Reverse Transcriptase (200 U/µL). This mixture was incubated at 42 °C for 1 h and then at 90 °C for 10 min to inactivate the enzyme. The expression level of target genes in response to various *FOXP1/2/4* dimer combinations was determined using the 7500 Fast Real-Time PCR System with SYBR Green reagent (Applied Biosystems). The quantitative real-time polymerase chain reaction (qRT-PCR) reaction mixture included SYBR Green reagent, 2 µM each of forward and reverse primers, and cDNA template. Primers specific for candidate genes (Table 2) were designed using Primer Express 3.0 (Applied Biosystems) and synthesized by Sigma-Aldrich. The comparative Ct method was used to analyze the data as we previously described (Weingarten et al. 2012). In brief, Raw Ct values were normalized to the geometric means of the housekeeping genes hypoxanthine phosphoribosyl transferase (*HPRT*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) to obtain the Δ Ct values. The Δ Ct values of each gene were subtracted from a calibrator

Table 2 Primer sequences for downstream genes of *FOXP2* for qRT-PCR

Gene	Primer sequences (5'→3')	Amplicon size (base pair)
<i>CER1</i>	F: CAGAGTTCTCTTCCCCCGTACT R: TTCTCCTCAGCTTCTCATGGT	82
<i>NCOR2</i>	F: TCAGCGGAGTGAAGCAGGAG R: TCGATGCTGGCTGAGGAGAT	93
<i>NEUROD2</i>	F: CAAGCGGCCAGACCTAGTGT R: CTGCGACAGACCCTTGCA	55
<i>PAX3</i>	F: GAGAGAACCCGGGCATGTT R: CCGCGTCTTGAGTAATTTGTC	57
<i>SNW1</i>	F: GGCAGAAGCCCTCTACATTGC R: ACTTGGGCACGCATTCC	63
<i>SFRP4</i>	F: CCCGGAGGATGTTAAGTGGAT R: GTCAACATCAAGAGGCCTTTCC	70
<i>WISP2</i>	F: GGTCGCAGTCCACAAAACAG R: CACCGTGTCCCCATTCC	57
<i>PRICKLE1</i>	F: TCACTGTGGCAGGCACCAT R: CTCAGCTTCTGTGCACTCATCAG	91
<i>EFNB3</i>	F: TCGGCGAATAAGAGGTCCA R: GTCCCCGATCTGAGGGTACA	60
<i>SLIT1</i>	F: GGAGGCCACTGGGATGTTTA R: CCATCTTCAATTTCTGACACCTTGT	84

F forward primer, *R* reverse primer

(Δ Ct of the gene from cells transfected with a corresponding vector only). The obtained values ($\Delta\Delta$ Ct) were then normalized to the value derived from untransfected cells. For example, the Δ Ct of FOXP1-Flag sample, derived from HEK293 cells transfected with FOXP1/pcDNA3-Flag, was first compared with a calibrator derived from HEK293 cells transfected with the pcDNA3-Flag vector only and then normalized with the Δ Ct of untransfected cells. The double transfection samples were compared with the average of both empty vector controls. Three separate passages of each cell line were used as biological replicates. Reported relative quantification (RQ) ratios are the mean of comparisons of three biological replicates. Statistical significance was determined by performing a one-way analysis of variance (ANOVA) followed by post hoc analyses with Student's *t*-test. Differences were considered to be statistically significant at $p < 0.05$.

Results

FOXP1/2/4 Protein Expression in HEK293 Cells

HEK293 cells were stably transfected with 3'-Flag-tagged FOXP1 (FOXP1-Flag), 3'-myc-His-tagged FOXP2 (FOXP2-Myc), 3'-Flag-tagged FOXP4 (FOXP4-Flag), or 3'-myc-His-

tagged FOXP4 (FOXP4-Myc) alone or in combination. The protein expression of FOXP1-Flag and FOXP4-Flag was confirmed with Western blot analysis using anti-Flag antibody. We detected a band at a predicated size of 78 kDa, which was not seen in the untransfected HEK293 cells or cells that were transfected with pcDNA3-Flag empty vector (Fig. 1a). The protein expression of FOXP2-Myc and FOXP4-Myc was confirmed using anti-Myc antibody. We detected an 82-kDa band for the FOXP2 and a 78-kDa band of FOXP4 protein but as expected no band was observed in untransfected HEK293 cells or cells transfected with the pcDNA3.1/*myc*-His empty vector (Fig. 1b). The co-expression of FOXP1-Flag with FOXP2-Myc, FOXP1-Flag with FOXP4-Myc, and FOXP2-Myc with FOXP4-Flag in HEK293 cells was verified with respective antibodies as well (Fig. 1a, b).

Dimerization of FOXP1, FOXP2, and FOXP4

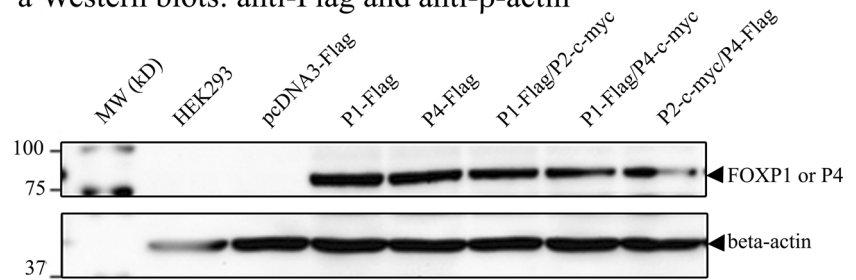
Previous studies have already demonstrated that FOXP1, FOXP2, and FOXP4 proteins homodimerize with themselves and heterodimerize with each other in transfected HEK293 cells (Li et al. 2004). We performed coimmunoprecipitation assays to confirm that FOXP1, FOXP2, and FOXP4 heterodimerize in the HEK293 cells expressing FOXP1-Flag with FOXP2-Myc, FOXP1-Flag with FOXP4-Myc, or FOXP2-Myc with FOXP4-Flag. Cell lysates containing FOXP2-Myc and FOXP4-Myc were immunoprecipitated with anti-*c-myc* antibody, and the interacting FOXP1-Flag was detected with mouse anti-FLAG M2 (Fig. 1c, left). FOXP4-Flag was immunoprecipitated with mouse anti-FLAG M2, and the interacting FOXP2-Myc was detected with anti-*c-myc* (Fig. 1c, right). Although the presence of homodimers in each cotransfected cell line cannot be ruled out, our results show that all three FOXP1, FOXP2, and FOXP4 proteins heterodimerize with each other, which was consistent with the previous findings.

Subcellular Localization of FOXP Proteins

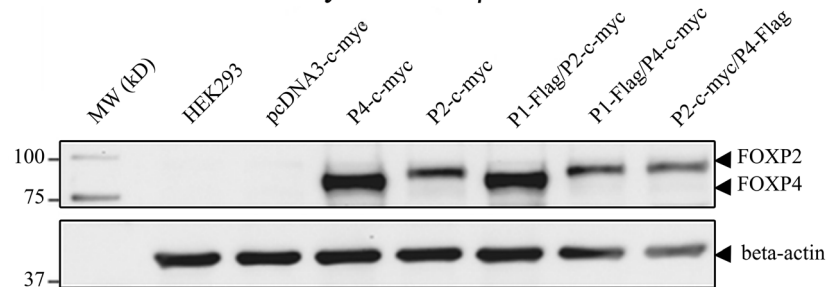
In order to confirm the protein expression and the subcellular localization of FOXP1, FOXP2, and FOXP4, immunocytochemistry was performed on HEK293 cells stably expressing FOXP1-Flag, FOXP2-Myc, FOXP4-Flag, or FOXP4-Myc alone or in combination. The subcellular localization of FOXP proteins was determined using anti-Flag or anti-Myc antibodies and detected with FITC- and Texas Red-conjugated secondary antibodies. The results show that the tagged FOXP1, FOXP2, and FOXP4 expressed in the nucleus, which was confirmed using DAPI (Fig. 2a, b). Moreover, cells cotransfected with two constructs (FOXP1-FOXP2, FOXP1-FOXP4, and FOXP2-FOXP4) show co-localization of the corresponding FOXP proteins (Fig. 2c).

Fig. 1 Expression of FOXP1/2/4 protein in HEK293 cells using Western blot analysis. **a** Immunoblotting of cells transfected with FOXP-FLAG-tagged constructs with anti-Flag M2 antibody. **b** Immunoblotting of cells transfected with FOXP-*c-myc*-tagged constructs with anti-*c-myc* antibody. FOXP1, FOXP4 proteins of 78 kD, and FOXP2 of 82 kD were detected. Untransfected cells and cells transfected with corresponding pcDNA3.1-FLAG or pcDNA3.1-*c-myc* vectors were also analyzed. Immunomembranes were re-probed with anti- β -actin antibody as equal loading control. **c** Interaction between FOXP1/2/4 proteins in co-transfected HEK293 cells. FOXP1-*c-myc* / FOXP2-Flag and FOXP1-*c-myc* / P4-Flag-expressing cell lysates were immunoprecipitated (IP) using anti-*c-myc* and immunoblotted (IB) with anti-Flag M2 for detection of FOXP1 (left). FOXP2-*c-myc*/FOXP4-Flag cell lysates were immunoprecipitated with anti-Flag M2 and immunoblotted with anti-*c-myc* antibodies for detection of FOXP2 (right)

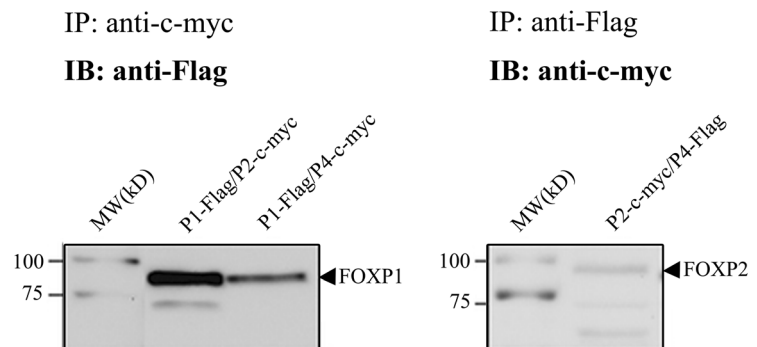
a Western blots: anti-Flag and anti- β -actin



b Western blots: anti-myc and anti- β -actin



c Co-immunoprecipitation



Quantification of the Expression Level of FOXP2 Target Genes

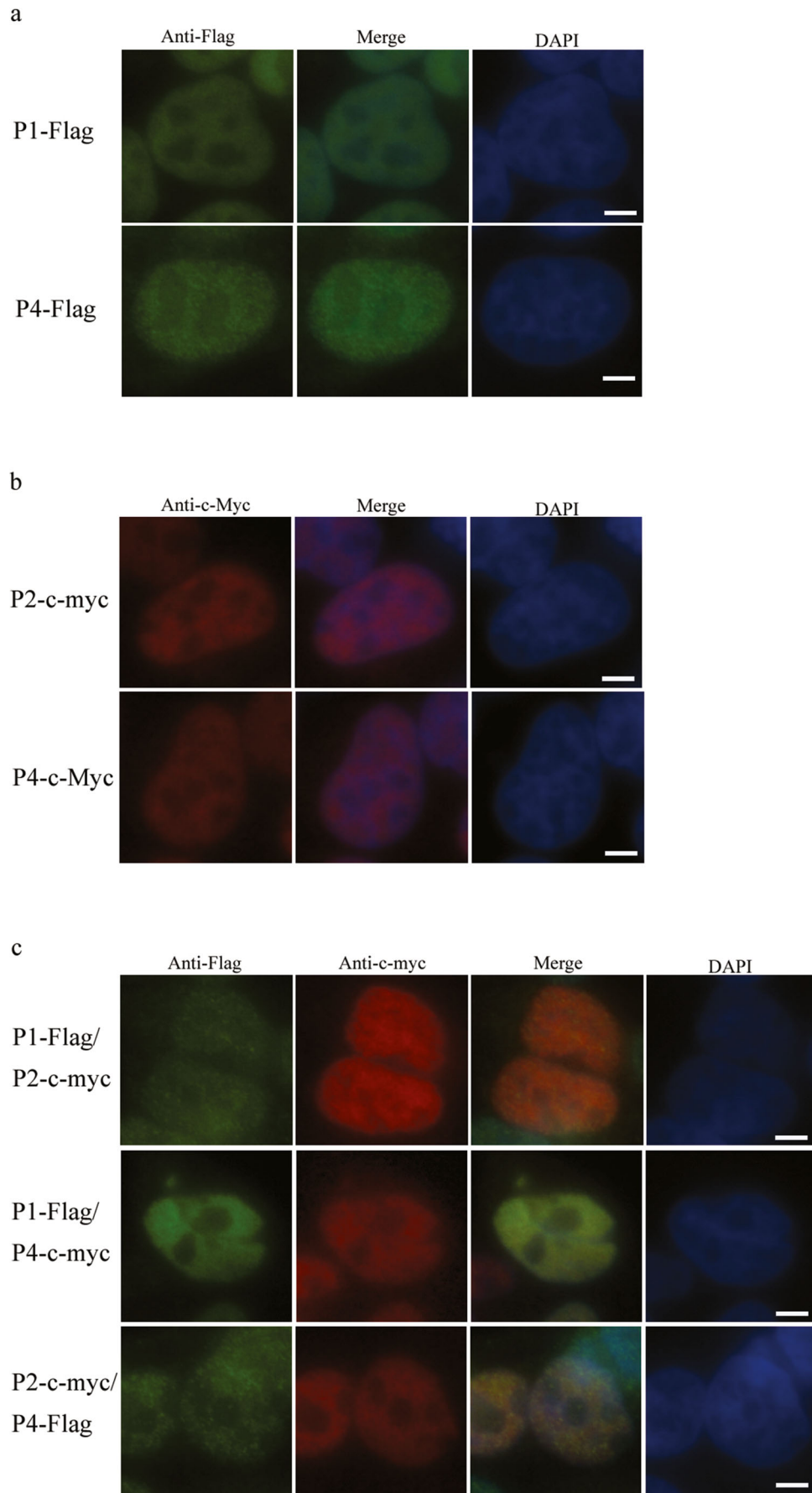
Quantitative real-time polymerase chain reaction was used to assess gene expression differences due to the combinatorial actions of FOXP1/2/4 proteins on the downstream targets of FOXP2. The downstream targets of FOXP2 (*CER1*, *SFRP4*, *WISP2*, *PRICKLE1*, *NCOR2*, *SNW1*, *NEUROD2*, *PAX3*, *EFNB3*, and *SLIT1*) were chosen based on their role during mouse embryonic development. Using mRNA from transfected HEK293 cell lines, the expression level of the target genes was quantified relative to the geometric means of the housekeeping genes *HPRT* and *GAPDH* and compared with cells transfected with the respective pcDNA3-Flag or pcDNA3.1/*myc*-His empty vectors. To account for any gene

expression at the endogenous level, the obtained RQ values were normalized to the level of untransfected HEK293 cells.

Statistically significant differences were found amongst the cells transfected with various combinations of FOXP1/2/4 constructs for eight of the selected target genes (*CER1*, *SFRP4*, *WISP2*, *PRICKLE1*, *NCOR2*, *SNW1*, *NEUROD2*, and *EFNB3*). Figure 3 depicts the RQ of FOXP2 downstream target genes involved in WNT and Notch signaling pathways as well as development of the nervous system.

WNT Signaling Pathway Genes

The expression of *CER1* was repressed by all FOXP1/2/4 combinations. There was a significant effect of FOXP1/2/4 dimerization on *CER1* gene expression ($F(5, 12)=9.87$; $p=0.0006$).



◀ **Fig. 2** Nuclear localization of FOXP1/2/4 proteins in transfected HEK293 cells using immunocytochemistry. **a** FOXP1 and FOXP4 proteins were detected with mouse anti-Flag antibody and FITC-conjugated secondary antisera. **b** FOXP2 and FOXP4 proteins were detected with mouse anti-c-myc antibody and Texas-Red-conjugated secondary antisera. **c** Co-localization of FOXP1-Flag with FOXP2-Myc, FOXP1-Flag with FOXP4-Myc, and FOXP2-Myc with FOXP4-Flag was detected with corresponding antibodies. DAPI-stained nuclei and merged views shows overlap of FOXP with the nucleus. Scale bar=5 μ m

Post hoc comparisons indicated that the RQ ratio for FOXP1 expressing cells (-1.12 ± 0.01 ; mean \pm SEM) was not different than FOXP2 (-2.28 ± 0.35) but was significantly different from FOXP4 expressing cells (-2.73 ± 0.14 ; $p=0.0002$) (Fig. 3a). The RQ ratio for cells transfected with both FOXP1 and FOXP2 (-2.59 ± 0.11) was found to be significantly different from FOXP1 expressing cells ($p=0.0004$) but not different from FOXP2, indicating that FOXP1 does not seem to influence the expression of *CER1* in FOXP1/P2 cells. The expression level of *CER1* in FOXP1/P4 expressing cells (-2.18 ± 0.35) was not statistically significantly different from the cells expressing only FOXP1 or FOXP4. The RQ ratio for FOXP2/P4 expressing cells (-1.28 ± 0.02) was smaller than its corresponding FOXP2 and FOXP4 homodimeric counterparts ($p=0.0064$ and $p=0.0005$, respectively), suggesting that expressing FOXP2/P4 show weaker transcriptional repression than FOXP2 and FOXP4 homodimers (Fig. 3a).

There was a significant effect of FOXP1/2/4 dimerization on *SFRP4* gene expression ($F(5, 12)=24.00$; $p<0.0001$). The expression of *SFRP4* was mainly repressed by various FOXP1/2/4 combinations with the exception of the FOXP4 homodimers that caused transcriptional activation. FOXP1/P2 expressing cells (-2.60 ± 0.06) produced significantly greater transcriptional repression than the homodimeric counterparts ($p=0.0205$ and $p=0.0131$, respectively). FOXP4 homodimers (1.07 ± 0.02) caused transcriptional activation; this was found to be significantly different ($p<0.0001$) from FOXP1 homodimers (Fig. 3a). However, the RQ ratio in FOXP1/P4 co-transfected cells (-1.62 ± 0.10) was not different from FOXP1 homodimers, suggesting that FOXP4 likely does not regulate *SFRP4* expression via FOXP1/P4 heterodimers. The RQ ratio of FOXP4 homodimers was also significantly different from FOXP2 homodimers, as well as FOXP2/P4 expressing cells (-1.80 ± 0.18) at the $p<0.001$ level (Fig. 3a). The RQ ratio of FOXP2 homodimers differed significantly ($p<0.0001$) from FOXP2/P4 expressing cells. Furthermore, FOXP1/2, FOXP1/4, and FOXP2/4 dimers combination cause transcriptional repression of *SFRP4* expression.

The results showed significant differences in *WISP2* expression across FOXP1/2/4 protein combination ($F(5, 12)=9.49$; $p=0.0007$). Similarly to *SFRP4*, FOXP1 homodimers (-1.49 ± 0.08) and FOXP2 homodimers (-2.00 ± 0.27) were responsible for the transcriptional repression of *WISP2*

(Fig. 3a). The RQ ratio of *WISP2* in FOXP1/P2 cell line (-3.76 ± 0.47) was significantly greater than cells expressing FOXP1 or FOXP2 only and shows even further transcriptional repression ($p=0.0029$ and $p=0.0127$, respectively). FOXP1/P4 combination (0.32 ± 0.87) caused a significantly different effect than FOXP1 and FOXP4 alone ($p=0.0116$ and $p=0.0160$, respectively) (Fig. 3a). We also observed that FOXP1/P2 co-transfection are significantly ($p=0.0009$) greater transcriptional repressors of *WISP2* than FOXP2/P4 combination.

The effect of FOXP1/2/4 dimerization on *PRICKLE1* expression was significant ($F(5, 12)=7.65$; $p=0.0019$). FOXP2 homodimers (1.68 ± 0.34) cause transcriptional activation that is significantly different from FOXP1 homodimers (-1.46 ± 0.23 ; $p=0.0003$) and FOXP4 homodimers (-1.22 ± 0.10 ; $p=0.0006$) that both caused transcriptional repression (Fig. 3a). FOXP2/P4 expressing cells show the same level of transcriptional repression of *PRICKLE1* as FOXP4 homodimers. This suggests that FOXP2 likely does not regulate *PRICKLE1* expression through interactions with FOXP4 (Fig. 3a).

Notch Signaling Pathway Genes

The results show that the transcriptional outcomes of *NCOR2* differed significantly across the six FOXP1/2/4 protein combinations ($F(5, 12)=32.17$; $p<0.0001$) but appeared to be mainly upregulated. FOXP1/P2 combination (3.79 ± 0.41) produced a significantly higher upregulation of *NCOR2* than its homodimeric counterparts ($p=0.0018$ and $p=0.0026$, respectively) (Fig. 3b). Cells co-expressing FOXP1/P4 (-1.24 ± 0.16), which caused transcriptional repression, had a significantly different effect on *NCOR2* expression than FOXP1 and FOXP4 homodimers ($p=0.0014$ and $p=0.0143$, respectively), which resulted in transcriptional activation. FOXP2 and FOXP4 homodimers led to *NCOR2* upregulation with no significant difference; although comparisons with FOXP2/P4 combination (5.79 ± 0.55) revealed a significantly larger RQ ratio ($p<0.0001$ and $p<0.0001$, respectively) (Fig. 3b).

The results revealed significant differences in *SNWI* expression between FOXP1/2/4 protein combinations ($F(5, 12)=190.85$, $p<0.0001$).

The expression level of *SNWI* by FOXP1 homodimers (1.22 ± 0.08) was significantly upregulated ($p<0.0001$) as compared with FOXP2 homodimers (-1.33 ± 0.13) and FOXP1/P2 expressing cells (-1.55 ± 0.04) that both led to transcriptional repression (Fig. 3b). Therefore, it seems unlikely that FOXP1 regulates the expression of *SNWI* in concert with FOXP2. Similarly, the expression level of *SNWI* mediated by FOXP4 homodimers was found to be significantly upregulated ($p<0.0001$) when compared with FOXP2 only and FOXP2/P4 combinations (-1.18 ± 0.02) both causing transcriptional repression (Fig. 3b). Thus, FOXP4 is also unlikely to heterodimerize with FOXP2 to modulate the expression of

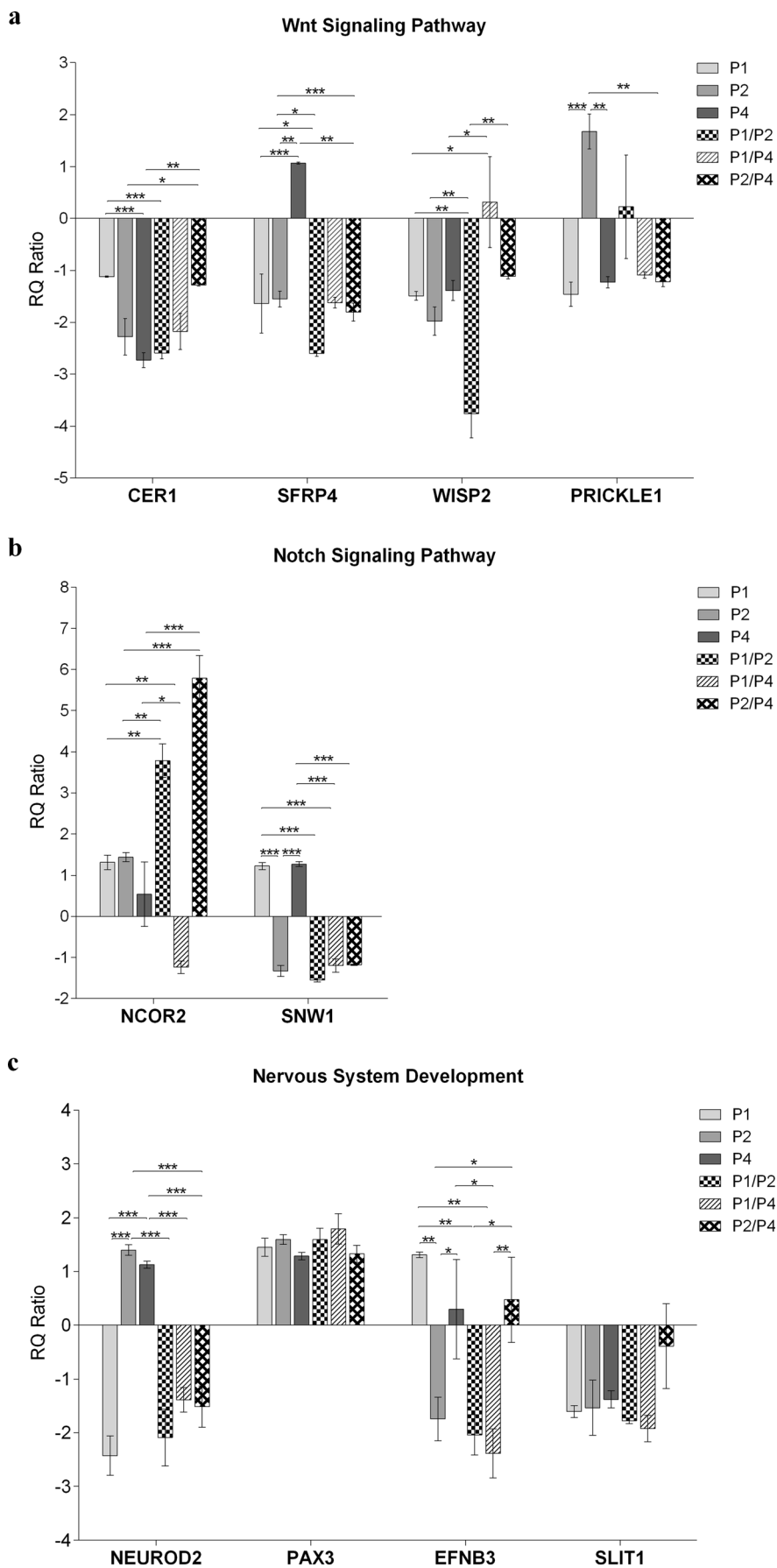


Fig. 3 Quantitative real-time polymerase chain reaction of *FOXP2* target genes in FOXP1/P2/P3 stably transfected HEK293 cells. HEK293 cells were transfected with FOXP1 (*P1*), FOXP2 (*P2*), and FOXP4 (*P4*) or double-transfected with FOXP1/P2 (*P1/P2*), FOXP1/P4 (*P1/P4*), and FOXP2/P4 (*P2/P4*), respectively. **a** WNT signaling pathway genes (*CER1*, *SFRP4*, *WISP2*, and *PRICKLE1*); **b** Notch signaling pathway genes (*NCOR2* and *SNW1*); and **c** genes involved in the development of the nervous system (*NEUROD2*, *PAX3*, *EFNB3*, and *SLIT1*). Expression changes (*y*-axis) are presented as the mean of RQ ratios from three independent transfection experiments. Error bars, \pm SEM. The paired Student's *t* test was performed to determine whether there is statistical significance between the compared FOXP-transfected cell lines. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$ —statistical significance between the compared samples

SNW1. Lastly, *SNW1* expression in FOXP1/P4 expressing cells (-1.20 ± 0.16) was significantly downregulated ($p < 0.0001$) as compared with both FOXP1 and FOXP4 homodimers that caused upregulation instead (Fig. 3b).

Gene Involved in the Development of the Nervous System

Statistical analysis showed significant differences ($F(5, 12) = 26.25$; $p < 0.0001$) across FOXP1/2/4 protein combinations on *NEUROD2* expression. The RQ ratios of FOXP1 (-2.43 ± 0.37) and FOXP2 homodimers (1.40 ± 0.10) were significantly ($p < 0.0001$) different. FOXP1 homodimers caused downregulation, FOXP2 homodimers led to upregulation of *NEUROD2* (Fig. 3c). The RQ ratio of FOXP1/P2 combination (-2.10 ± 0.53) was significantly ($p < 0.0001$) different from FOXP2 only expressing cells but was nearly equivalent to that of FOXP1. Thus, it does not seem likely that the regulation of *NEUROD2* involves FOXP1/P2 heterodimers. The RQ ratio of FOXP4 homodimers (1.13 ± 0.06) was significantly different from FOXP1 only and FOXP1/P4 combination (-1.39 ± 0.23 ; $p < 0.0001$ and $p = 0.0001$, respectively) (Fig. 3c). While FOXP4 homodimers led to *NEUROD2* upregulation, FOXP1 only and FOXP1/P4 expressing cells caused *NEUROD2* downregulation. In addition, the RQ ratio of FOXP1 homodimers was not significantly different from that of FOXP1/P4 combination. This demonstrates that FOXP4 is not likely to influence *NEUROD2* expression through interactions with FOXP1. The RQ ratio of FOXP2/P4 combination (-1.51 ± 0.38) was significantly different from that of both FOXP2 and FOXP4 homodimers at the $p < 0.0001$ level (Fig. 3c). Even though FOXP2 and FOXP4 homodimers caused transcriptional activation, the co-expression of FOXP2 and FOXP4 brought about the transcriptional repression of *NEUROD2*.

The expression of *PAX3* was uniformly upregulated by FOXP1/2/4 dimers (Fig. 3c). No significant difference was found between FOXP1/2/4 dimeric combinations in *PAX3* expression ($F(5, 12) = 1.14$; $p = 0.39$).

Statistical analysis revealed significant differences in *EFNB3* expression across FOXP1/2/4 protein combinations

($F(5, 12) = 7.32$; $p = 0.0023$). The RQ ratio of FOXP1 homodimers (1.31 ± 0.05) was significantly different from FOXP2 homodimers (-1.74 ± 0.4 ; $p = 0.0028$) and FOXP1/P2 combination (-2.04 ± 0.37 ; $p = 0.0014$) that both suppressed the expression (Fig. 3c). The RQ ratio of cells co-expressing FOXP1/P4 (-2.39 ± 0.46) had a significantly repressed as compared with FOXP1 and FOXP4 alone (0.30 ± 0.92 ; $p = 0.0007$ and $p = 0.0064$, respectively) that both caused transcriptional activation. *EFNB3* expression is differentially regulated between FOXP2 and FOXP4 homodimers ($p = 0.0275$) (Fig. 3c). The RQ ratio of FOXP2/P4 combination (0.47 ± 0.79) was found to be significantly ($p = 0.0185$) different from FOXP2 but not FOXP4 cells. The expression of *EFNB3* in cells co-expressing FOXP2/P4 was found to be significantly different from FOXP1/P2 or FOXP1/P4 combination ($p = 0.0094$ and $p = 0.0043$, respectively).

The expression of *SLIT1* was unanimously downregulated by all FOXP1/2/4 combinations (Fig. 3c). There was no significant effect of FOXP1/2/4 dimerization on *SLIT1* expression ($F(5, 12) = 1.82$; $p = 0.18$).

In summary, this study was able to show the functional regulation of FOXP1/2/4 over downstream FOXP2 targets in a cell-based model system. The findings suggest that the relative levels and interacting of FOXP2 and its co-factors, FOXP1 and FOXP4, modulate its ability to act as a transcriptional activator or repressor. Furthermore, this study provides evidence of the complexity of regulatory mechanisms mediated by FOXP1/2/4.

Discussion

Previous studies have shown that homo- and heterodimerization of the FOXP1/2/4 proteins is required for DNA binding (Li et al. 2004). Although transcriptional targets of each FOXP dimers in the brain remain to be determined, our current findings provide further evidence for transcriptional regulation of these proteins. We investigated whether the expression of ten previously identified FOXP2 target genes (*CER1*, *SFRP4*, *WISP2*, *PRICKLE1*, *NCOR2*, *SNW1*, *NEUROD2*, *PAX3*, *EFNB3*, and *SLIT1*) is regulated by homo- and hetero-dimers of FOXP proteins. Quantitative assessments of mRNA expression were performed on a gene-by-gene basis in stable HEK293 cell lines transfected with FOXP1, FOXP2, and FOXP4 in all combinations. We confirmed that in FOXP1/2, FOXP1/4, and FOXP2/4 cells express heterodimers. However, these cells likely also have corresponding homodimers. Six of the genes tested (*CER1*, *SFRP4*, *WISP2*, *SNW1*, *EFNB3*, and *SLIT1*) demonstrated reduced expression in the presence of FOXP2 homodimers and four genes (*PRICKLE1*, *NCOR2*, *NEUROD2*, and *PAX3*) displayed increased transcription. The results indicate that

FOXP2 has dual functionality, acting to repress or activate gene expression, which is concordant with previous findings. Eight genes (*CER1*, *SFRP4*, *WISP2*, *PRICKLE1*, *NCOR2*, *SNW1*, *NEUROD2*, and *EFNB3*) displayed significant effects of altered gene expression due to FOXP1/2/4 interaction. The ability to dimerize in different combinations could be a way in which transcriptional regulation is fine tuned in a cell-specific manner. Our study demonstrated that preferred FOXP1/2/4 dimer combinations for transcriptional activation or repression differed on an individual gene basis in HEK293 cell line expressing various combinations of FOXP1/2/4 dimers. A prior study found that neurite outgrowth and synaptic plasticity are the most prominent biological themes associated with FOXP2 function in the embryonic nervous system (Vernes et al. 2011). Our findings represent a starting point into the functional characterization of FOXP1/2/4 transcriptional regulation of various genes during neuronal development.

Our study was able to determine the effects of FOXP1/2/4 dimerization on the transcriptional outcomes of *CER1*, *SFRP4*, *WISP2*, and *PRICKLE1*, which are components of the WNT signaling pathway that have been previously identified as putative targets of FOXP2. WNT signaling participates in multiple developmental events during embryogenesis and also in adult tissue homeostasis. We showed that *CER1* expression is repressed not just by FOXP2 homodimers, but also by FOXP1 and FOXP4 homodimers. The results show that FOXP1 does not influence *CER1* expression through interactions with FOXP2 or FOXP4. FOXP2 and FOXP4 homodimers were strong transcriptional repressors of *CER1* in comparison to FOXP2/P4 combination. The results indicate that *SFRP4* expression was downregulated by FOXP1 and FOXP2 homodimers as well as the presence of both FOXP1 and FOXP2. However, FOXP4 homodimers causes *SFRP4* upregulation and is unlikely to regulate its expression via heterodimerization with FOXP1.

The various FOXP1/2/4 combinations demonstrated different regulatory roles in *WISP2* expression. Even though FOXP1 and FOXP2 homodimers caused downregulation of *WISP2*, FOXP1/P2 combination demonstrated greater ability to suppress *WISP2* expression. Conversely, FOXP1 and FOXP4 homodimers were comparable transcriptional repressors of *WISP2*, but FOXP1/P4 co-expression led to gene levels that more closely resembled the baseline. Our results also suggest that FOXP2 homodimers have differential effects on *PRICKLE1* expression than FOXP1 or FOXP4 homodimers. *PRICKLE1* expression was found to be activated by FOXP2 homodimers but repressed by FOXP1 and FOXP4 homodimers. The results also indicate that FOXP2 does not regulate *PRICKLE1* expression through interactions with FOXP4.

Similar changes in transcriptional outcomes of components of WNT signaling pathway during embryogenesis in response to FOXP1/2/4 dimerization might be responsible for pathologies of the nervous system such as speech and language

impairments. FOXP1/2/4 regulatory mechanisms that go awry during early brain development may result in abnormal regulation of downstream targets that might disrupt a variety of biological processes. For example, Swinkels et al. recently reported delays in speech development in patients with 9p deletion syndrome, of which *CER1* appeared to be a prime candidate for mutation analysis (Swinkels et al. 2008).

The present study revealed the transcriptional outcomes of *NCOR2* and *SNW1*, components of the Notch signaling pathway, in response to FOXP1/2/4 dimerization. Notch-mediated signaling plays seminal roles in cell fate control, influencing differentiation, proliferation, and apoptotic events, at all stages of development (Artavanis-Tsakonas et al. 1999). Our results showed that FOXP2 homodimers led to *NCOR2* upregulation. Furthermore, in FOXP1/P2 and FOXP2/P4 cell lines co-expressing two proteins, we observed higher *NCOR2* levels than cells expressing single proteins only. FOXP1/P4 combination had opposing actions to the homodimeric counterparts and caused the downregulation of *NCOR2* expression. These findings suggest that the relative levels of FOXP1/2/4 proteins, and thus the different combinations of dimers that may be formed, determine its ability to act as an activator or repressor. Cells co-expressing any two proteins FOXP1/2/4 proteins show downregulation of *SNW1*. FOXP2 homodimers lead to downregulation of *SNW1* while FOXP1 and FOXP4 homodimers caused upregulation. The results indicate that FOXP2 is unlikely to dimerize with FOXP1 or FOXP4 in *SNW1* regulation.

Such differential expression of *NCOR2* in the developing brain may have profound consequences. *NCOR2* has been implicated in the regulation of embryonic neural stem cell proliferation and differentiation, and plays a critical role in forebrain development (Jepsen et al. 2007). Interestingly, even as *NCOR2* is identified as a downstream target of FOXP2, a functional interaction between *NCOR2* and FOXP1 has been reported (Jepsen et al. 2008; Wilke et al. 2012). *NCOR2*/FOXP1 protein complexes have been found to regulate a program of gene repression essential to myocardial development. The data indicate that *NCOR2*-mediated regulation may be a common mechanism by which FOXP1 and other FOXP proteins monitor gene expression programs during organogenesis. This suggests that *NCOR2* and FOXP1/2/4 may comprise a functional biological unit required to orchestrate specific programs critical for CNS development.

We also investigated four targets of FOXP2 (*NEUROD2*, *PAX3*, *EFNB3*, and *SLIT1*) that have known roles in CNS development. Our results showed no significant differences in transcriptional outcomes between FOXP1/2/4 combinations in *PAX3* and *SLIT1* expression, which suggests some functional redundancy of FOXP1/2/4/ in gene regulation. However, there was a significant effect of FOXP1/2/4 dimerization on *NEUROD2* and *EFNB3* expression. *NEUROD2* expression was downregulated by FOXP1 homodimers but

upregulated by FOXP2 and FOXP4 homodimers, which indicates that FOXP1 does not influence *NEUROD2* expression through interaction with FOXP2 or FOXP4. Moreover, FOXP2 does dimerize with FOXP4, where FOXP2/P4 expressing cells show transcriptional repression of *NEUROD2*. *NEUROD2* plays an important role in early neuronal differentiation (Franklin et al. 2001; Wilke et al. 2012) It has been shown that *NEUROD2*-null mice show excessive granule cell loss in postnatal cerebellar development and exhibit ataxia, motor deficits, premature death, and reduced seizure threshold (Olson et al. 2001). Thus, it is important to understand how this gene is regulated by FOXP proteins in the human brain.

We showed that FOXP1/2/4 dimerization has also differential effects on *EFNB3* expression. Whereas FOXP2 homodimers caused transcriptional repression, FOXP1 and FOXP4 homodimers were responsible for transcriptional activation. FOXP1/P4 co-expression led to the downregulation of *EFNB3*. The results suggest that FOXP2 is unlikely to influence *EFNB3* expression through heterodimerization. *EFNB2* is a well-validated target of FOXP2 that is differentially regulated between human and chimpanzee *FOXP2* orthologues (Konopka et al. 2009; Vernes et al. 2011). Now, *EFNB3* is proposed to be a target of FOXP2 as well (Spiteri et al. 2007). *EFNB* proteins are expressed in axons and dendrites and function to transduce signals that are important for axon guidance and pruning, presynaptic development, and synaptic plasticity (Xu et al. 2011). Thus, the strong impact of FOXP1/2/4 dimerization on *EFNB3* might reflect major FOXP2 functions that are relevant to the development and maintenance of neuronal networks.

As the expression pattern of FOXP1, FOXP2, and FOXP4 vary (Shu et al. 2001; Lu et al. 2002; Teramitsu et al. 2004), a combination of homo- or heterodimers can regulate tissue-specific gene transcription. While transcriptional targets for FOXP1/2/4 dimers remain to be elucidated, this is the first study showing how dimerization of these proteins can influence the complex mechanisms involved in regulation of FOXP2 target gene expression. Further identification of brain-specific transcriptional targets regulated through FOXP dimerization would be critical for understanding the mechanisms underlying language acquisition and language-related deficits.

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