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ORIGINAL ARTICLE

Proteomic analysis of FOXP proteins reveals interactions between cortical transcription factors associated with neurodevelopmental disorders

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Abstract

FOXP transcription factors play important roles in neurodevelopment, but little is known about how their transcriptional activity is regulated. FOXP proteins cooperatively regulate gene expression by forming homo- and hetero-dimers with each other. Physical associations with other transcription factors might also modulate the functions of FOXP proteins. However, few FOXP-interacting transcription factors have been identified so far. Therefore, we sought to discover additional transcription factors that interact with the brain-expressed FOXP proteins, FOXP1, FOXP2 and FOXP4, through affinity-purifications of protein complexes followed by mass spectrometry. We identified seven novel FOXP-interacting transcription factors (NR2F1, NR2F2, SATB1, SATB2, SOX5, YY1 and ZMYM2), five of which have well-estabslished roles in cortical development. Accordingly, we found that these transcription factors are co-expressed with FoxP2 in the deep layers of the cerebral cortex and also in the Purkinje cells of the cerebellum, suggesting that they may cooperate with the FoxPs to regulate neural gene expression *in vivo*. Moreover, we demonstrated that etiological mutations of FOXP1 and FOXP2, known to cause neurodevelopmental disorders, severely disrupted the interactions with FOXP-interacting transcription factors. Additionally, we pinpointed specific regions within FOXP2 sequence involved in mediating these interactions. Thus, by expanding the FOXP interactome we have uncovered part of a broader neural transcription factor network involved in cortical development, providing novel molecular insights into the transcriptional architecture underlying brain development and neurodevelopmental disorders.

Introduction

Transcription factors have emerged as a key class of genes disrupted in monogenic forms of neurodevelopmental disorders such as intellectual disability (ID) and autism spectrum disorder (ASD), consistent with the precise temporal and spatial control of gene expression that underpins neurodevelopmental processes (1). Large-scale next-generation DNA sequencing studies have been particularly successful in identifying monogenic ID/

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ASD-related disorders caused by high-penetrance *de novo* mutations in transcription factor genes (2–4).

Among the many families of human transcription factors, the FOXP subfamily of forkhead box proteins is notable for the neurodevelopmental phenotypes which have been associated with their disruption (5,6). The FOXP family includes four proteins: FOXP1, FOXP2, FOXP3 and FOXP4 (7). FOXP1, FOXP2 and FOXP4 exhibit 55–65% sequence identity and show overlapping expression in the developing brain, as well as in other organs (7,8). FOXP3 is structurally divergent and its expression is limited to T lymphocytes (9). FOXP3 disruption causes an immunological disorder, IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked, OMIM #304790) syndrome, while mutations of FOXP1, FOXP2 and FOXP4 have each been linked to distinct neurodevelopmental disorders. Heterozygous disruptions of FOXP1 cause a broad neurodevelopmental syndrome, which includes global developmental delay and ID, frequently accompanied by features of autism and impaired speech and language abilities (OMIM #613670) (10–14). All pathogenic variants observed to date have occurred *de novo*, consistent with the severe phenotype of the disorder (14). Heterozygous disruptions of FOXP2 cause a rare monogenic form of speech and language impairment including childhood apraxia of speech as a core feature (OMIM #602081) (15–21). Unlike FOXP1-associated disorder, general cognitive functions may be within the normal range, and both inherited and *de novo* cases have been reported (22). Although the FOXP2-related disorder is less severe than that resulting from FOXP1 disruption, individuals with FOXP2 and FOXP1 mutations display overlapping features such as language impairment, suggesting that the syndromes may involve disruption of similar molecular and cellular networks (23).

Disorders relating to FOXP4 disruption had not been reported until recently, when Charng et al. described a homozygous frameshift variant in a child from a consanguineous family affected by developmental delay and malformations in the larynx and the heart (6). It is interesting that this, the only currently suspected case of FOXP4-related disorder, involves a recessive mutation, in contrast to the dominant disorders described for FOXP1 and FOXP2 disruptions. The potential complete absence of FOXP4 protein in this child may also explain why the disorder appears to have broader effects in organs other than the brain. Indeed, all three neurally-expressed FOXP proteins also have roles in the development of other organs, but their neurodevelopmental functions appear to show more dosage sensitivity than roles in non-neural tissues (24–27). Although FOXP4 is the strongest candidate causal gene in the case identified by Charng and colleagues, additional observations of FOXP4 disruption are necessary to confirm its etiological role, particularly since variants in two other genes were also found in a homozygous state in the child and heterozygous state in the parents (6).

The precise mechanisms by which FOXP family members regulate transcription are just beginning to be explored. Several studies have investigated FOXP2 and FOXP1 target genes in the brain (28–31) but genes regulated by FOXP4 remain to be discovered. An important mechanism in regulation of gene expression is that transcription factors act in a combinatorial fashion, and thus can generate the complex patterns of gene expression underlying development of the brain and other systems using a limited set of transcription factors (1,32). The genes regulated by the FOXP proteins in specific cell types are therefore likely to depend on the co-expression of other transcription factors. Indeed, the transcriptional activity of FOXP1, FOXP2 and FOXP4 is regulated by their ability to form homo- and hetero-dimers with each other through their leucine zipper domain (26). The three proteins show partially overlapping patterns of expression in the brain, such that different combinations of FOXP homo and hetero-dimers may regulate distinct target genes (33–36).

In addition to dimerization between FOXP family members, a small number of interactions between FOXP proteins and other transcription factors have been reported. FOXP1 and FOXP2 interact with the neural transcription factor TBR1, rare mutations of which cause ASD accompanied by language deficits (37). Notably, this interaction can be disrupted by etiological mutations in either FOXP2 or TBR1 (37). Other reported FOXP-interacting transcription factors include GATAD2B and NKX2.1 (38,39). We hypothesized that there may be further interactions between FOXPs and other transcription factors with relevance to physiological developmental processes and neurodevelopmental disorders. We therefore sought to identify transcription factors which may cooperate with FOXP proteins in regulating gene expression during neurodevelopment, by applying a mass spectrometry approach. We identified seven novel FOXP-interacting transcription factors: NR2F1, NR2F2, SATB1, SATB2, SOX5, YY1 and ZMYM2. Several of these novel interactors have well-established roles in the development of the nervous system and/or are associated with neurodevelopmental disorders. The interactions with these binding partners involve different regions of the FOXP2 polypeptide, and are variably affected by different pathogenic variants in FOXP1 and FOXP2. Thus our findings provide new clues to the molecular function of the FOXP proteins, and point to a network of transcription factors involved in cortical development, disruption of which manifests as neurodevelopmental disorder.

Results

Identification of FOXP-interacting transcription factors

To identify transcription factors that may cooperate with FOXP proteins to regulate gene expression, we generated HEK293 cell lines stably expressing FOXP1, FOXP2 or FOXP4 fused to an N-terminal FLAG tag. HEK293 cells endogenously express all three FOXP proteins as well as many other neural genes (40,41). FLAG affinity-purified protein complexes from these cell lines were analysed by mass spectrometry (Fig. 1A and B). Three independent experiments were performed for FOXP1 and FOXP2, and two experiments for FOXP4. After filtering out non-specific interactors, a total of 381 putative FOXP-interacting proteins were identified, with substantial overlap between the three FOXP proteins (Fig. 1C, Supplementary Material, Table S1). For each FOXP protein, the other two FOXPs were among the interacting proteins identified in each experiment, reflecting heterodimerization between FOXP family members (26) (Supplementary Material, Table S1). Moreover, the set of putative FOXP-interacting proteins included previously reported interaction partners such as CTBP1, CTBP2 and GATAD2B, confirming that the affinity-purification procedure retrieved physiologically relevant FOXP-interacting proteins (20,38) (Supplementary Material, Table S1).

The transcription factors among the putative FOXP-interacting proteins were identified, resulting in a set of 28 proteins (Fig. 1C, Table 1, Supplementary Material, Table S2). To prioritize potential interacting transcription factors of most interest for further investigation, we excluded previously reported FOXP interactors, and then selected proteins that were present in two or more replicates, or that had a known

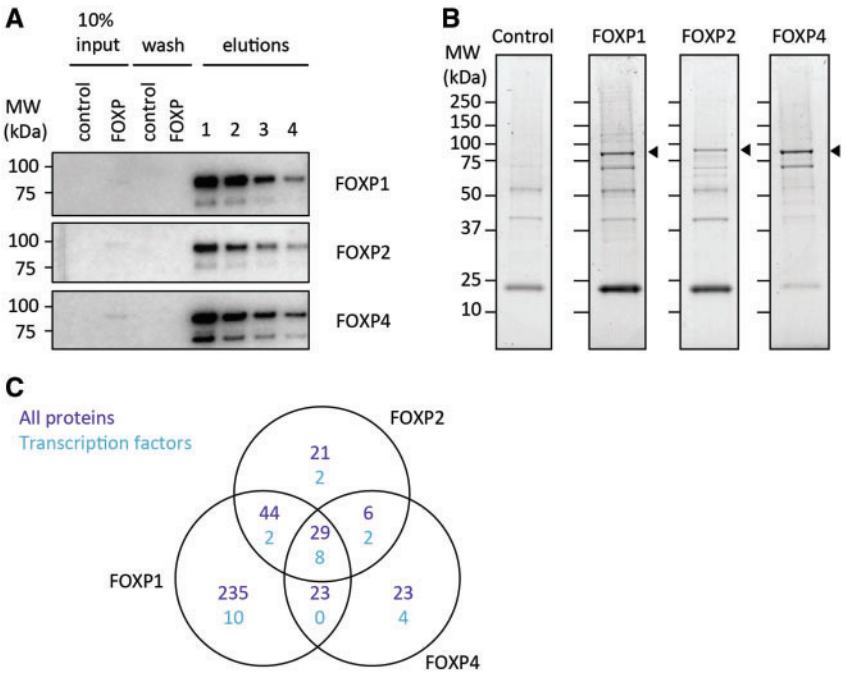

Figure 1. Identification of FOXP1-, FOXP2- and FOXP4-interacting proteins. (**A**) Affinity purifications of FOXP protein complexes using HEK293 cell lines stably expressing FLAG-FOXP1 (top panel), -FOXP2 (middle panel) and -FOXP4 (bottom panel) protein complexes. FLAG-tagged species were isolated using a FLAG affinity-purification strategy. Western blots of total lysate (10% Input), washed proteins (wash), and affinity-purified material (elutions) were probed with an anti-FLAG antibody. (**B**) Coomassie-stained SDS-polyacrylamide gels of the affinity purifications of FOXP1, FOXP2, FOXP4 and control purification. Arrowheads indicate bands corresponding to FOXP1, FOXP2 or FOXP4 for each IP. (**C**) Venn diagram showing the overlaps between the proteins (purple) or transcription factors (light blue) identified in each FOXP purification.

involvement in neurodevelopmental disorder. Because the FOXP proteins have high sequence similarity and are likely to have shared interactors, the appearance of a protein in two experiments with different FOXPs was treated as a replicate. We also included transcription factors if two or more members of the same family of transcription factors were observed (Supplementary Material, Table S2). This selection resulted in a filtered list of 12 putative FOXP interactors for follow up.

Validation of interactions between FOXPs and transcription factors

To validate the selected putative FOXP-interacting transcription factors, we used a Bioluminescence resonance energy transfer (BRET) assay. The BRET assay allows protein–protein interactions to be observed in live cells, and its effectiveness has previously been shown for successfully confirming interactions between FOXPs and other proteins (14,20,37,42,43). In the BRET assay, cells are transfected with a protein of interest fused to *Renilla* luciferase, and a candidate interaction partner fused to yellow fluorescent protein (YFP). An interaction between the two proteins under investigation can bring the luciferase and YFP moieties into sufficient proximity for resonance energy transfer to occur, causing a shift in the wavelength of the emitted light.

We generated YFP-fusion proteins for the 12 putative FOXP interactors, and tested them for interaction with luciferase-fusions of FOXP1, FOXP2 and FOXP4 using the BRET assay. We detected interactions between FOXP proteins and seven of the 12 putative interactors: SOX5, SATB1, SATB2, NR2F1, NR2F2, YY1 and ZMYM2 (Fig. 2A and B). In some cases a specific FOXP-cofactor interaction was detected in the BRET assay that was not identified in the mass spectrometry screen. For example, NR2F1, NR2F2 and SATB1 (identified through FOXP4 interactome screening) interacted with FOXP1 and FOXP2 in the BRET but had not been detected by mass spectrometry for those two FOXP proteins (Fig. 2B). This is likely due to the greater sensitivity of the targeted BRET investigations; these proteins may not have been detected among the complex mixture of proteins isolated by affinity purification. Transfection of cells with YFP-fusions of the interactors validated using the BRET assay and mCherry-FOXP2 showed that all seven interactors exhibited nuclear localization that completely overlapped with FOXP2 (Fig. 2C). ZMYM2 and SOX5 formed nuclear speckles when expressed as YFP-fusions, which caused partial redistribution of co-expressed FOXP2, consistent with the presence of a physical interaction between ZMYM2/SOX5 and FOXP2 (Fig. 2C).

Five candidate interactors (NFAT5, TFDP1, TP53, ZBTB2 and ZNF687) did not show evidence of interaction in the BRET assay. The lack of interaction in this assay was not due to the candidate interactors being localized outside the nucleus in live cells, as all these proteins showed total or partial nuclear localization, overlapping with that of FOXP2 (Fig. 2C). NFAT5, ZBTB2 and TFDP1 were present in the cytoplasm, as has been reported previously, but still showed overlapping expression with FOXP2 in the nucleus (44,45) (Fig. 2C). The failure to observe an interaction with these proteins in the BRET assay does not exclude that these proteins might be true FOXP interactors. For a signal to be detected in the BRET assay, the luciferase and YFP peptides need to be in close proximity and also must be oriented correctly so that resonance energy transfer may occur. Therefore, certain pairs of interactors may not allow for efficiency resonance transfer (46). In addition, the affinity-purification procedure can purify large complexes of proteins, some of which may interact only indirectly with the bait protein (47,48). Pairs of

Table 1. List of candidate FOXP-interacting transcription factors identified in the mass spectrometry screens

Gene symbol	Function	Associated phenotype
BNC2	Regulation of skin pigmentation	
CDC5L	Regulation of cell cycle	
EMSY	DNA repair	
FOXC1	Embryonic and ocular development	Iris hypoplasia and glaucoma
FOXP1	Brain, heart and lung development	ID with language impairment
FOXP2	Brain, heart and lung development	Severe language impairment
FOXP4	Brain, heart and lung development	Developmental delay[a]
FUBP1	Regulation of cell cycle	
GATAD2B	Brain development	ID
HIC2	Unknown function	
NFAT5	Regulation of inflammatory response	
NR2F1	Brain and heart development	ID with optic atrophy
NR2F2	Brain and heart development	Congenital heart defects
RFX1	Unknown function	
SATB1	Regulation of immune system and brain development	
SATB2	Brain and bone development	ID with language impairment
SOX13	T-cell development	
SOX5	Brain and bone development	ID with language impairment
TFDP1	Regulation of cell cycle	ID with language impairment[a]
TOX	T-cell development	
TP53	Regulation of cell cycle	Li-Fraumeni syndrome
YY1	Embryonic development	ID
ZBTB2	Regulation of cell cycle	
ZBTB39	Unknown function	
ZFHX4	Brain and muscle development	Ptosis
ZMYM2	Immune system regulation	
ZNF148	Unknown function	ID with language impairment
ZNF687	Bone development	Paget disease of bone

[a]Tentative association.

proteins that interact indirectly are less likely to produce a signal in the BRET assay because the interaction-mediating proteins are not overexpressed, and the distance between the protein pair may also be too great for efficient energy transfer. Therefore, NFAT5, TFDP1, TP53, ZBTB2 and ZNF687 may have been isolated by affinity purification due to indirect interaction with the FOXP bait protein.

Neural transcription factors interact with distinct sites within FOXP proteins

We next sought to determine which FOXP regions are involved in the interactions with each of the transcription factors validated using the BRET assay. Here, we focused on FOXP2, building on our prior experience with mapping interaction sites within this particular protein. Specifically, we performed further BRET assays using a series of synthetic variants of FOXP2 isoform I (NM_014491.3, NP_055306.1) truncated at the N- or C-terminus, which have been employed previously to map the binding sites of FOXP2 interaction partners (Fig. 3A) (20,37,43). For SOX5 and ZMYM2, an N-terminal FOXP2 fragment containing residues 1–258 of isoform I retained the ability to interact, while the counterpart C-terminal fragment (residues 259–715) did not, indicating that the critical binding determinants for SOX5 and ZMYM2 lie within the N-terminal 258 residues of FOXP2 (Fig. 3B and C). In addition, SOX5 and ZMYM2 interacted with a naturally-occurring alternative FOXP2 isoform (isoform III) that lacks the N-terminal 92 amino acids of other isoforms (Supplementary Material, Fig. S1), narrowing down the critical region to residues 93 to 258. The N-terminal region of FOXP2 also mediates interaction with the transcription factor TBR1, and with members of the PIAS family of proteins which are involved in FOXP2 post-translational modification, which suggests that this region may coordinate multiple protein–protein interactions (37,43). The region includes two polyglutamine tracts of unknown function that are expanded in FOXP2 relative to other FOXP family members. Shortening of these tracts to bring them into line with the sequence of FOXP1 does not affect interaction with TBR1 or PIAS proteins in cellular assays (37,43). Tract shortening also did not affect interaction with SOX5 and ZMYM2 in the current study, as expected given that these proteins also interact with FOXP1 (Supplementary Material, Fig. S2). Thus, the binding sites for these proteins may lie in the glutamine-rich regions flanking the polyglutamine tracts.

For the paralogous transcription factors SATB1 and SATB2, only C-terminally truncated FOXP2 forms containing an intact forkhead DNA-binding domain retained a full ability to interact with SATB1/2, suggesting that the interaction requires an intact forkhead domain together with additional elements from the N-terminal region of FOXP2 (Fig. 3D). Interestingly, a previous mass spectrometry study reported SATB1/2 in affinity purifications of FOXP1 and FOXP3 as well as of FOX proteins from several other subfamilies (49). Given that SATBs interact with diverse FOX proteins that have substantial structural divergence, it is highly likely that the forkhead DNA-binding domain itself plays an important role in mediating the interaction.

The second pair of paralogous transcription factors in our screen, NR2F1 and NR2F2, displayed normal interaction with a FOXP2 form truncated beyond residue 422, but reduced interaction with more severely truncated forms, suggesting a role for the

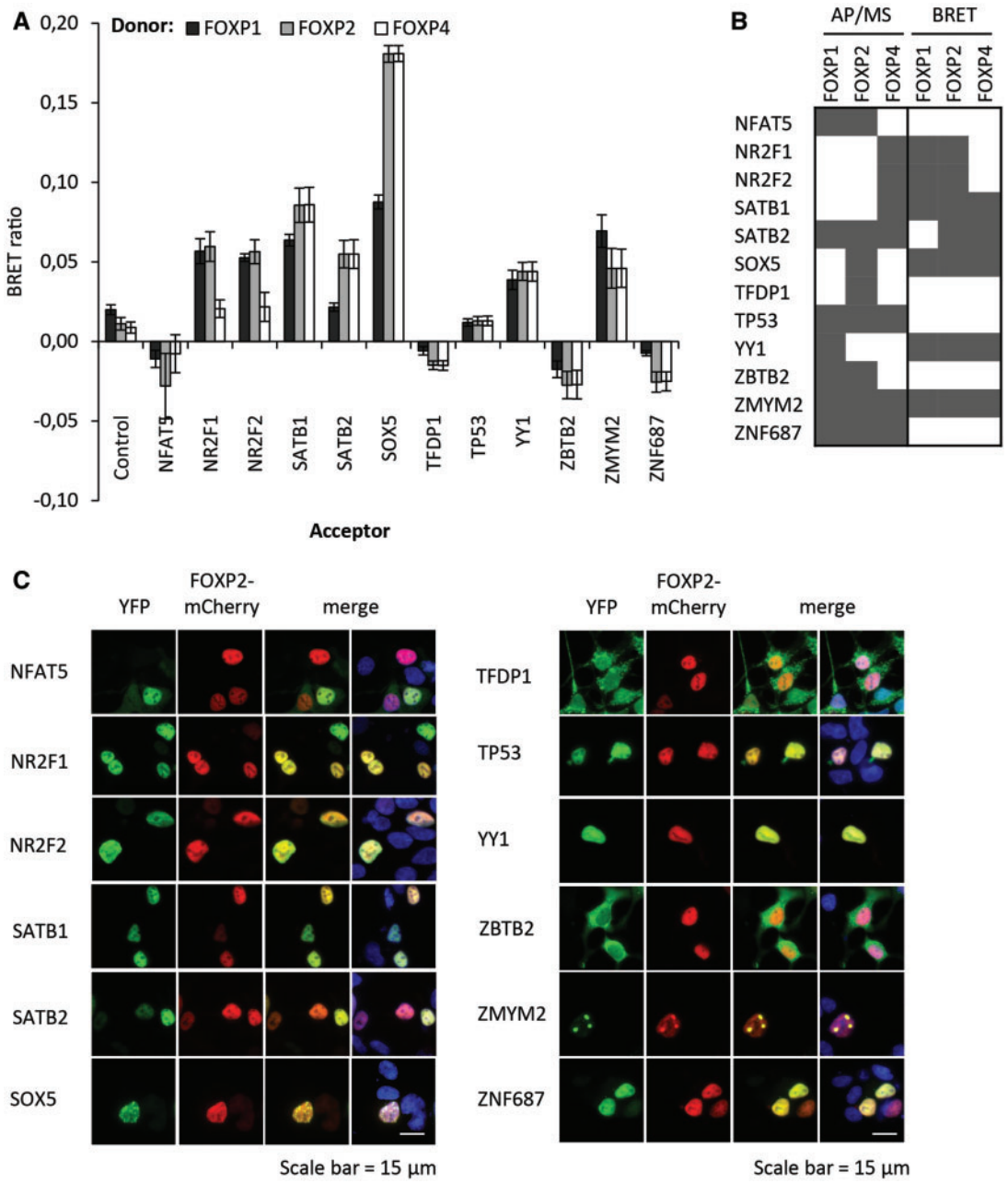

Figure 2. Validation of identified FOXP-interacting proteins. (**A**) Bioluminescence resonance energy transfer (BRET) assay for interaction of FOXP1, FOXP2 and FOXP4 variants with the putative FOXP-interacting transcription factors. HEK293 cells were transfected with *Renilla* luciferase-FOXP1, -FOXP2 or -FOXP4 (donor) and YFP (acceptor) fusion proteins of the putative FOXP-interacting transcription factors. The control acceptor protein is a nuclear-targeted YFP. Values are mean corrected BRET ratios ± S.E.M ($n = 3$). (**B**) Heatmap summarizing the results of the affinity purification followed by mass spectrometry (AP/MS) and the BRET assays. Grey shading indicates interaction. (**C**) Fluorescence micrographs of HEK293 cells transfected with YFP-fusions of the putative FOXP-interacting transcription factors, together with FOXP2 fused to mCherry. Nuclei were stained with Hoechst 33342 (Scale bar = 15 µm).

region encompassing residues 330–422 (Fig. 3E). Accordingly, normal interaction was observed with a C-terminal fragment of FOXP2 beginning at residue 330, whereas a fragment beginning at residue 423 showed little or no binding (Fig. 3E). Residues 330–422 include a leucine zipper domain which mediates dimerization, and a zinc finger domain with unknown function. FOXP2 dimerization does not appear to be essential for interaction with NR2F1/2, because we found that a synthetic dimerization-deficient FOXP2 variant retained the ability to interact with NR2F1 (Supplementary Material, Fig. S2D) (26). Interestingly, the zinc finger domain in FOXP2 contains a short amino acid sequence (residues 360–367) with high similarity to the NR2 binding motif F/YSXXLXXL/Y, which has been shown to mediate the interaction of NR2F1/2 with other transcription factors and co-repressors (50) (Fig. 3F). This motif is conserved in FOXP1 but not FOXP4, which could explain why NR2F1/2 interacted with FOXP1 and FOXP2, but not FOXP4, in our BRET assays (Fig. 2A and B) – the presence of NR2F1/2 in FOXP4 complexes analysed by mass spectrometry may then be due to an indirect interaction mediated by FOXP1/2.

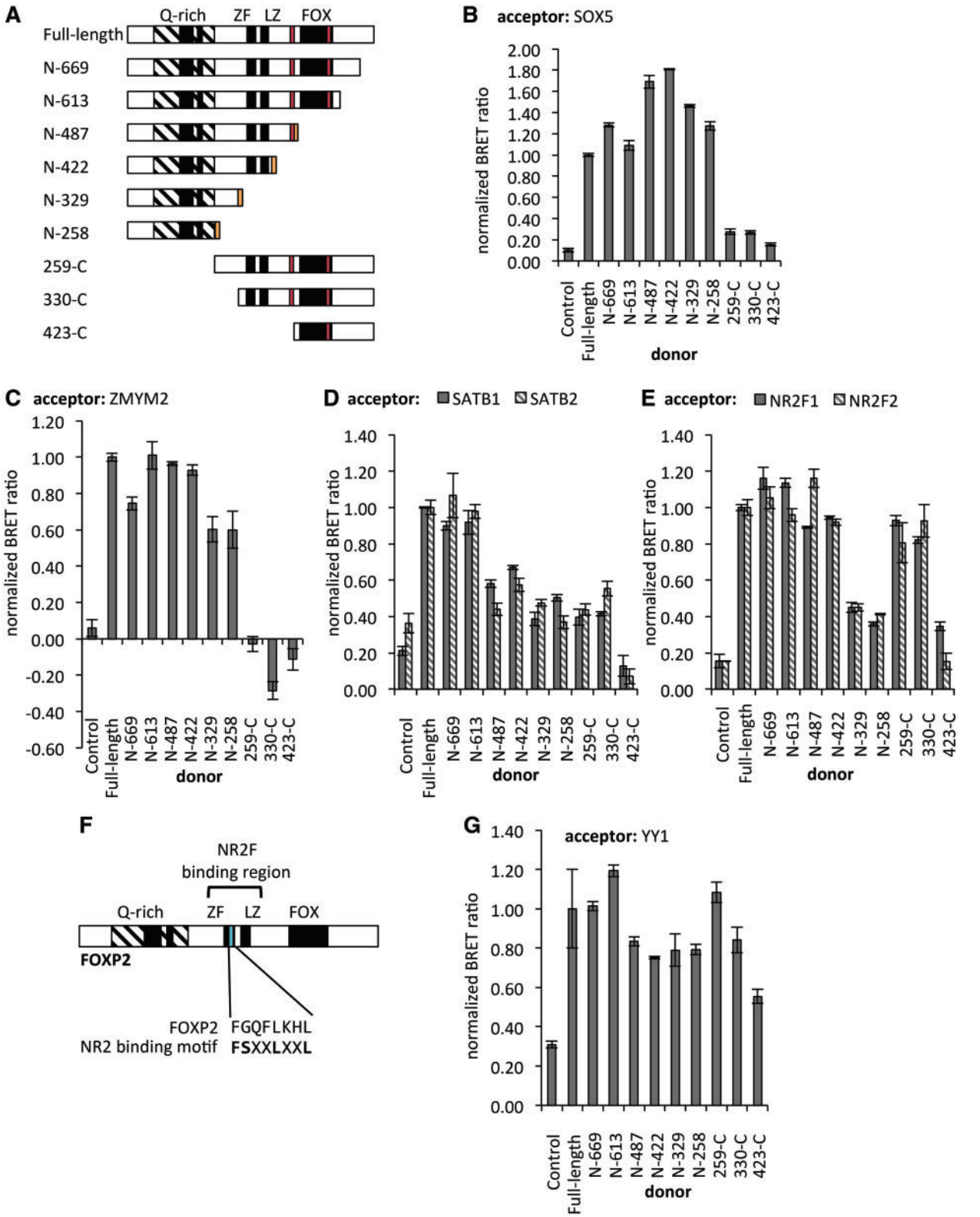

Figure 3. Mapping of the regions in FOXP2 that mediate protein–protein interactions (A) Schematic representation of synthetic truncated forms of FOXP2 isoform I (NM_014491.3, NP_055306.1). Known domains are labeled: glutamine-rich (Q-rich) region (hatched shading) including long and short polyglutamine tracts, zinc finger (ZF), leucine zipper (LZ), and forkhead domain (FOX). Nuclear localization signals are indicated with red bars. A synthetic nine-residue nuclear targeting sequence (orange bars) was appended to the C-terminus of variants that lack one or both of the endogenous nuclear localization signals. (B–E, G) BRET assay for interaction of synthetic truncated FOXP2 variants with FOXP-interacting transcription factors. HEK293 cells were transfected with truncated FOXP2 variants fused to Renilla luciferase (donor) and SOX5 (B), ZMYM2 (C), SATB1 (D), SATB2 (D), NR2F1 (E), NR2F2 (E) or YY1 (G) fused to YFP (acceptor). The control donor protein is a nuclear-targeted luciferase. Values are mean corrected BRET ratios normalized to full-length FOXP2 ± S.E.M. ($n = 3$). (F) Schematic representation of FOXP2 showing the putative NRF2 binding motif (light blue bar). The sequence alignment of the region shows the putative NRF2 binding motif in FOXP2 paralogues FOXP1 and FOXP4. Critical residues of the NRF2 binding motif are shown in bold.

Finally, in the case of YY1, all of the truncated FOXP2 variants tested retained some degree of interaction, suggesting that the interaction between FOXP2 and YY1 may involve multiple binding sites (Fig. 3G).

Disorder-related FOXP variants disrupt interactions with transcription factors

We next investigated etiological variants of FOXP proteins related to neurodevelopmental disorders, assessing their effects on the transcription factor interactions that we identified. Heterozygous disruptions of the *FOXP2* gene cause a rare form of speech and language disorder (22). The disorder was first reported in a three-generation family, in which all of the affected members carry the FOXP2 variant p.R553H, which is unable to bind to its typical DNA target sequence (15,51). Several other variants in FOXP2 have since been reported in individuals with neurodevelopmental disorders; functional studies indicate that some of these are etiological variants whereas others are benign rare variants (Fig. 4A) (16–21). Etiological variants had been shown to severely disrupt core molecular aspects of FOXP2 protein function, such as transcriptional activity and subcellular localization (43). Using the BRET assay, we examined the effects of seven rare variants on the interaction of FOXP2 with the transcription factors identified in our interactor screen (Fig. 4B–H). Four of these seven rare variants had been shown to be benign in prior cellular assays (43). All four of these benign rare variants displayed normal interaction with the novel interaction partners, whereas almost all interactions involving one of the three pathogenic FOXP2 variants were either abolished or disrupted (Fig. 4B–I).

Heterozygous mutations of the *FOXP1* gene result in a neurodevelopmental syndrome characterized by mild to moderate ID with features of ASD and speech and language impairments (13,14). We examined the effects of seven etiological variants of FOXP1 on interactions with the six FOXP1-interacting transcription factors identified in our screen. The etiological FOXP1 variants comprised four truncated and three missense variants. The truncated variants did not interact with any of the transcription factors tested in our assays, whereas two of the three missense variants retained some degree of interaction with one or more interaction partner (Fig. 5B–H). The FOXP1 missense variants p.R465G and p.R514C retained the ability to interact with SOX5 (Fig. 5B). Accordingly, co-expression of these variants with SOX5 led to mislocalization of SOX5 into the nuclear aggregates formed by the abnormal FOXP1 proteins, an effect not observed when SOX5 was co-expressed with other FOXP1 variants (Fig. 5I). Thus, most etiological FOXP1 and FOXP2 variants represent a loss of function with respect to interaction with other neural transcription factors. However the FOXP1 p.R465G and p.R514C variants may exert dominant-negative effects *in vivo* by interfering with the function of interacting transcription factors such as SOX5. Such differences in the behaviour of different protein variants have the potential to contribute to phenotypic variation observed between individuals with FOXP1-related disorder (14).

FOXP2 is co-expressed with interacting transcription factors in specific neuronal subpopulations

We sought to identify the cell types in which the newly identified interactions between FOXP2 and transcription factors may be physiologically relevant. Of the seven FOXP-interacting transcription factors validated using the BRET assay, ZMYM2 and YY1 are ubiquitously expressed, while SOX5, SATB1, SATB2, NR2F1 and NR2F2 are all neural transcription factors that are expressed in subsets of neurons within the cerebral cortex (52–55). We therefore examined the co-localization of Foxp2 with the interacting neural transcription factors in mouse brain by immunostaining (Fig. 6). Brains from mice at postnatal day 3 were used because by this point in development the cortical layers have formed and layer-specific Foxp2 expression can readily be detected (35). Foxp2 was observed in the deep layers of the cortex, as well as in the striatum, as previously reported (Fig. 6) (35). Each of the five neural transcription factors showed a different pattern of expression in the cortex. Sox5 exhibited extensive co-expression with Foxp2 in deep layer cortical neurons (Fig. 6). Satb2 expression was found throughout all the six layers of the cortex and its expression coincided with Foxp2 in a subpopulation of neurons of layer V (Fig. 6). Satb1 was detected in layers IV to VI, including a subpopulation of Foxp2-expressing cells (Fig. 6). Nr2f1 was expressed in all the layers of the cortex, including the vast majority of Foxp2-positive cells in layers V and VI (Fig. 6). In contrast, and despite being expressed in a few scattered cells in the cortex layers I, IV and V, Nr2f2 was not co-expressed with Foxp2 in any cortical neurons (Fig. 6). However, Nr2f2 was found to be extensively co-expressed with Foxp2 in the Purkinje cells of the cerebellum, another key neuronal subtype with high Foxp2 expression (Fig. 7). These data are consistent with the prevailing view that expression of different combinations of transcription factors is essential to determining and maintaining cell identity and function.

Discussion

In this study, through mass spectrometry-based proteomics followed by targeted analysis using BRET, we identified and characterized the interactions between FOXP family proteins and seven neurally-expressed transcription factors. We demonstrated co-expression of the interactors with Foxp2 in functionally-relevant neuronal subtypes in the early postnatal mouse brain, confirming that the reported interactions are of potential physiological significance *in vivo*. We found that different interaction partners have distinct binding sites within the FOXP2 protein, and that disorder-associated variants in FOXP1 and FOXP2 generally cause broad disruption of protein–protein interactions, but in some cases may lead to a dominant-negative effect in which abnormal FOXP protein may interfere with the functions of a normal interactor.

The interaction partners we identified have important, yet diverse, roles in neurodevelopment. Sox5 is expressed in deep cortical layers during embryonic and early postnatal stages and controls timing of the generation of distinct corticofugal neuron subtypes (52). Loss of Sox5 results in aberrant differentiation and abnormal migration of cortical projection neurons (52). Satb2 is a key regulator of cortical development and is expressed throughout all cortical layers (53,56). The neural functions of Satb1 have not been as extensively investigated as its roles in the immune system (57), but it is reported to regulate development of cortical interneurons and facilitate cortical neuron plasticity by modulating dendritic spine density (58,59). Nr2f1 regulates the differentiation of motor neurons, axonal projection and cortical arealization and, together with Nr2f2, plays a role in cell migration and regulation of neurogenesis (55,60–63). The interactions we demonstrated between these proteins and FOXP family members may therefore impact on a range of crucial processes in cortical development and maturation. Protein–protein interactions and their downstream consequences are naturally limited to the cell populations in which

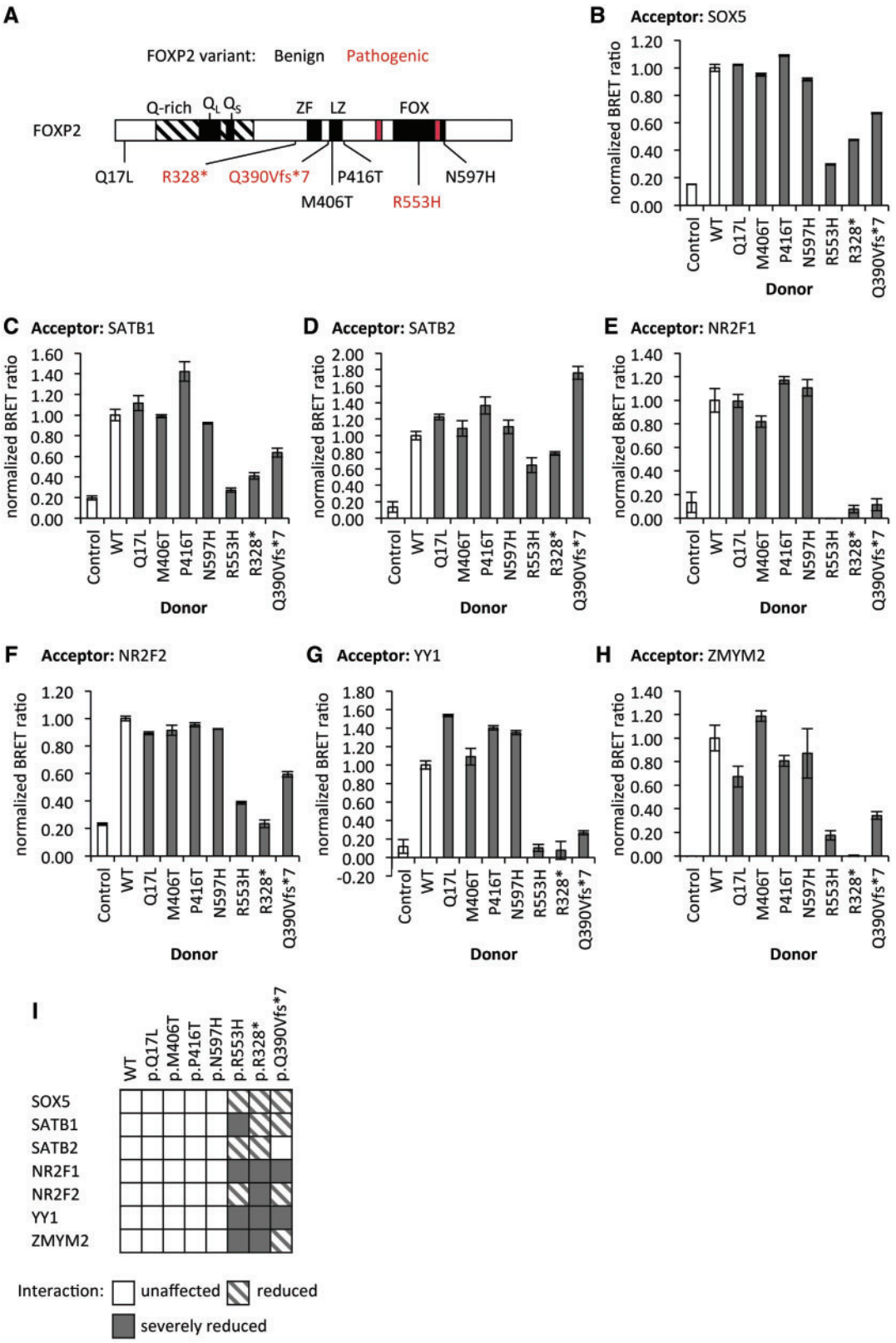

Figure 4. Etiological mutations in FOXP2 disrupt protein interactions (**A**) Schematic representation of the FOXP2 protein isoform I (NM_014491.3, NP_055306.1) showing rare variants found in individuals with neurodevelopmental disorders. Pathogenic variants are shown in red and benign variants in black. Known domains are labeled: glutamine-rich (Q-rich) region (hatched shading), long (Q_L) and short (Q_S) polyglutamine tracts, zinc finger (ZF), leucine zipper (LZ) and forkhead domain (FOX). Nuclear localization signals are indicated with red bars. (B–H) BRET assays for interaction of FOXP2 variants with FOXP-interacting proteins. HEK293 cells were transfected with Renilla luciferase-FOXP2 wild type (WT) or variants (donor) and YFP (acceptor) fusions of SOX5 (B), SATB1 (C), SATB2 (D), NR2F1 (E), NR2F2 (F), YY1 (G) or ZMYM2 (H). The control acceptor protein is a nuclear-targeted YFP. Values are mean corrected BRET ratios normalized to wild-type FOXP2 ± S.E.M. ($n = 3$). (**I**) Heatmap summarizing the results of the BRET assays between FOXP2 variants and the FOXP-interacting transcription factors. White squares indicate an unaffected interaction; a hatched pattern, a reduced interaction; and grey shading, a severely reduced interaction.

the proteins are co-expressed. Our analysis of co-expression of Foxp2 and interacting transcription factors in early postnatal mouse cortex shows that the subpopulations of co-expressing cells vary from a minor fraction of cells to the majority of deep layer projection neurons. Foxp1 and Foxp4 show different patterns of expression in the developing cortex compared with Foxp2, leading to further combinations of protein–protein interactions with differing functional outcomes.

In addition to roles in cortical development, our findings highlight potential interactions in the cerebellum. We found co-expression of Nr2f2 and Foxp2 in Purkinje cells of the cerebellum; expression of the individual proteins in Purkinje cells has been reported previously in separate studies (34,35,54). Nr2f2 regulates cerebellar growth and patterning (54), while Foxp2 has been implicated in dendritic outgrowth and arborization of Purkinje cells (64) and mice lacking functional Foxp2 have small cerebellums (65). Foxp4, which is similarly expressed in Purkinje cells, may also be involved in the maintenance of dendritic arborization (66).

FOXP1, FOXP2 and FOXP4 have all been implicated in human genetic disorders with neurodevelopmental phenotypes. Several of the FOXP-interacting transcription factors identified in this study are known to cause neurodevelopmental disorders through haploinsufficiency. SOX5 haploinsufficiency results in ID and language impairment (OMIM #616803) (67,68). *De novo* mutations in SATB2 cause syndromic ID, usually with absent or near absent speech, and often cleft palate or dental anomalies (OMIM #612313) (69–71). Mutations in NR2F1 cause Bosch-Boonstra-Schaaf optic atrophy syndrome, a dominant disorder characterized by optic atrophy and ID, frequently accompanied by hypotonia, seizures, ASD and oromotor dysfunction (OMIM #615722) (72,73). YY1 haploinsufficiency leads to a syndrome that includes ID often accompanied by motor problems and various congenital malformations (OMIM #617557) (74,75). ZMYM2 *de novo* mutations have been found in large-scale exome sequencing studies of cases of ASD (76). However, since ZMYM2 is ubiquitously expressed, further cases of disruptions are needed in order to confidently establish its involvement in neurodevelopmental disorders. Of note, following completion of experiments for this study, recurrent *de novo* mutations in ZNF148, another transcription factor identified in our mass spectrometry screen, have been identified in individuals with developmental delay and ID (OMIM #617260) (77). The differing levels of ID and speech/language impairment associated with disruption of FOXP proteins and their interacting transcription factors suggests that reduced levels of these transcription factors lead to differing, but potentially related, disturbances in downstream gene expression and cortical development.

Most of the identified FOXP-interacting transcription factors control cortical development in an interconnected fashion, cooperating with each other but also forming positive and negative loops of regulation between them (78). For instance, to maintain the specific neuronal identities in deeper layers of the cortex, SOX5 directly represses Fezf2, a key node of the cortical transcriptional network, in layer VI and the subplate (79), which at the same time is repressed by TBR1 (80), an ASD-related transcription factor also known to interact with FOXP2 and FOXP1 (37). In contrast, in layer V, SATB2 promotes the expression of SOX5 and Fezf2, while Fezf2 represses SATB2 (81). Also, NR2F1, SATB2 and SOX5 control the development of cortical projection neurons at least in part by repressing the same target gene, encoding the transcription factor BCL11B (52,63,82,83). Strikingly, NR2F1 and NR2F2 physically interact with transcription factors BCL11B and BCL11A (50), which also plays roles in cortical development by repressing TBR1 (84). BCL11A haploinsufficiency has recently been identified as a cause of sporadic ID (85). Therefore, a number of genetically-distinct neurodevelopmental disorders may have shared roots in this complex regulatory network of transcription factors that orchestrates cortical development, and our results contribute to extend the characterization of this network. It will be interesting in the future to further determine the specific roles the FOXPs play in this network and, in general, better understand the transcriptional architecture underlying cortical development.

Clearly the proteins investigated here are only a subset of the biologically-important interactors of FOXP proteins. Our mass spectrometry screens provide the largest list of candidate FOXP-interacting proteins reported to date, and represent a resource for further investigation of protein networks surrounding these transcription factors. We elected to focus on transcription factors with potential roles in neurodevelopment, and a logical next step would be to consider proteins mediating transcription factor activity, such as chromatin modifying and remodelling complexes. For example, the candidate FOXP-interacting proteins include the chromatin remodeler SETD2, haploinsufficiency of which leads to Luscan-Lumish syndrome (OMIM #616831), a neurodevelopmental disorder characterized by ID and macrocephaly accompanied by speech delay (86,87). In addition, FOXP proteins have roles in multiple tissues and candidate interactors in our screen may be of relevance to the function of FOXPs in non-neuronal contexts. For example, FOXP1, FOXP2 and FOXP4 are all expressed in the embryonic heart, and mice lacking Foxp1 or Foxp4 die before birth due to heart defects (8,35,88,89). Heterozygous mutations in our confirmed FOXP-interaction transcription factor NR2F2 cause a variety of congenital heart defects (90), and a critical role in heart development has also been described for our confirmed interactor YY1 (91). Therefore, the FOXPs may cooperate with NR2F2 and YY1 to regulate downstream target genes important for cardiac development.

Our strategy employed affinity purification coupled to mass spectrometry to screen for potential FOXP-interacting transcription factors, followed by validation of selected candidate interactors using a BRET assay. These two methods have different strengths in relation to identifying protein interaction partners. The affinity-purification method gives a proteome-wide picture of protein interactions, but may miss weak or transient interactions because it is performed on cell lysates, and may suffer from false positives due to non-specific and indirect interactions. In contrast, BRET is a targeted method which monitors protein interactions in living cells, can detect transient interactions, is less vulnerable to false positives, but may give false negatives due to the constraints on energy transfer (42). We show here that this two-stage approach is effective in identifying high-confidence protein interaction partners with potential biological importance.

In conclusion, our findings situate the FOXPs within a broader molecular network that orchestrates cortical development, and thus provide novel clues as to how these proteins work to build a functioning brain, and why their deficiency leads to neurodevelopmental disorders.

Materials and Methods

DNA constructs

The cloning of human FOXP2 (NM_014491), FOXP1 (NM_032682), FOXP4 (NM_001012426), has been described previously (20). The coding sequences of TFDP1 (NM_007111), NR2F1 (NM_005654),

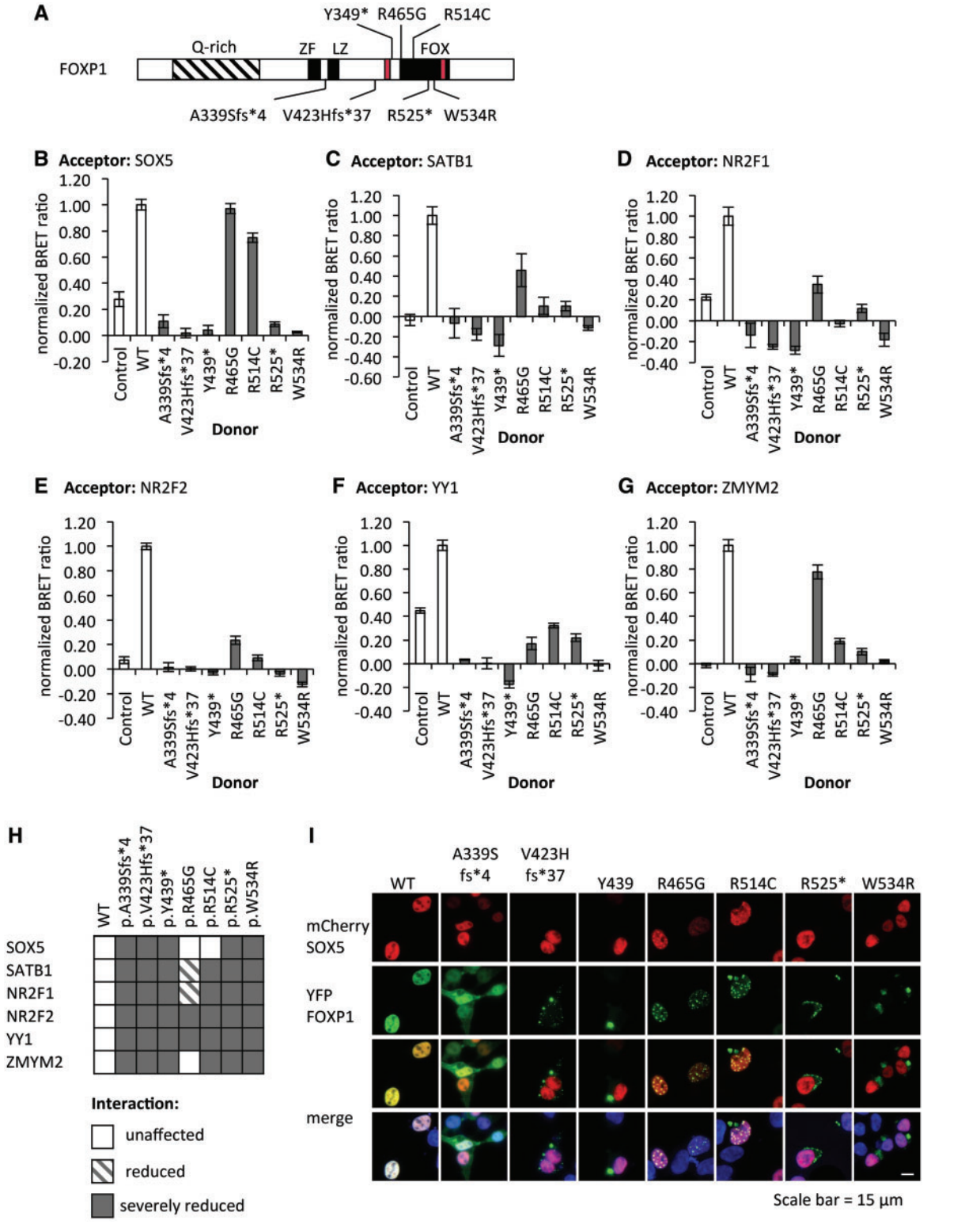

Figure 5. Etiological mutations in FOXP1 disrupt protein interactions. (**A**) Schematic representation of the FOXP1 protein (NM_032682, NP_116071) showing rare variants found in individuals with neurodevelopmental disorders. Known domains are labeled: glutamine-rich (Q-rich) region (hatched shading), zinc finger (ZF), leucine zipper (LZ) and forkhead domain (FOX). Nuclear localization signals are indicated with red bars. (**B–G**) BRET assays for interaction of FOXP1 variants with the FOXP-interacting proteins. HEK293 cells were transfected with Renilla luciferase-FOXP1 wild type (WT) or variants (donor) and YFP (acceptor) fusions of SOX5 (B), SATB1 (C), NR2F1 (D), NR2F2 (E), YY1 (F) or ZMYM2 (G). The control acceptor protein is a nuclear-targeted YFP. Values are mean corrected BRET ratios normalized to wild-type FOXP1 ± S.E.M. ($n = 3$). (**H**) Heatmap summarizing the results of the BRET assays between FOXP2 variants and the FOXP-interacting transcription factors. White squares indicate an unaffected interaction; a hatched pattern, a reduced interaction; and grey shading, a severely reduced interaction. (**I**) Fluorescence micrographs of HEK293 cells transfected with YFP-fusions of FOXP1 wild-type or etiological variants, together with SOX5 fused to mCherry. Nuclei were stained with Hoechst 33342. (Scale bar = 15 µm).

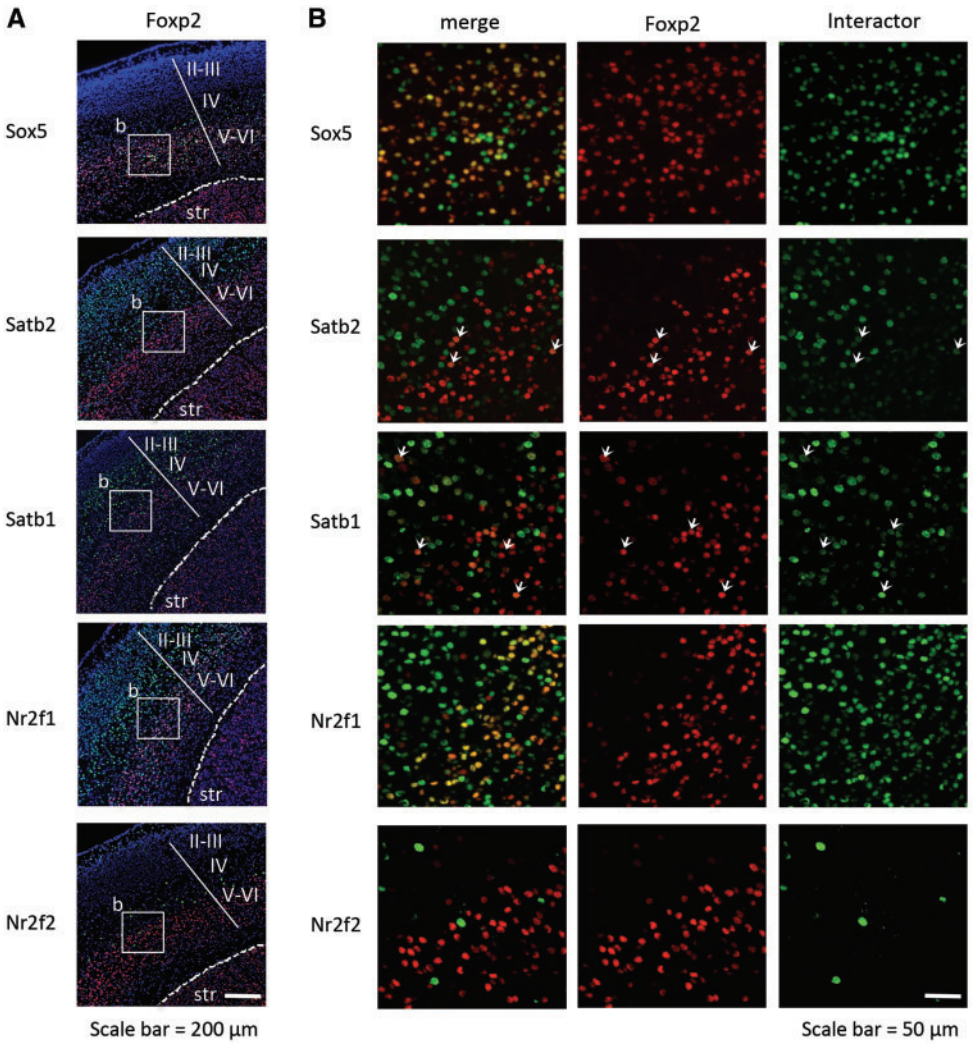

Figure 6. Foxp2 is co-expressed with Sox5, Satb1, Satb2 and Nr2f2 in the cerebral cortex. (**A**) Immunofluorescence experiments to assess endogenous co-expression of Sox5, Satb1, Satb2, Nr2f1 and Nr2f2 with Foxp2 in the cerebral cortex of P3 mice brains. A white line indicates the cerebral cortex, layer numbers are indicated. A dashed white line demarks the striatum (str) (scale bar = 200 μm) (**B**) Magnified views of the region indicated with a white square in (A). White arrows indicate cells that co-express both Foxp2 and the Foxp2-interacting transcription factor (Scale bar = 50 μm). For (A) and (B), Foxp2 protein expression is shown in red; Foxp2-interacting proteins, in green; and nuclei stained with DAPI, in blue.

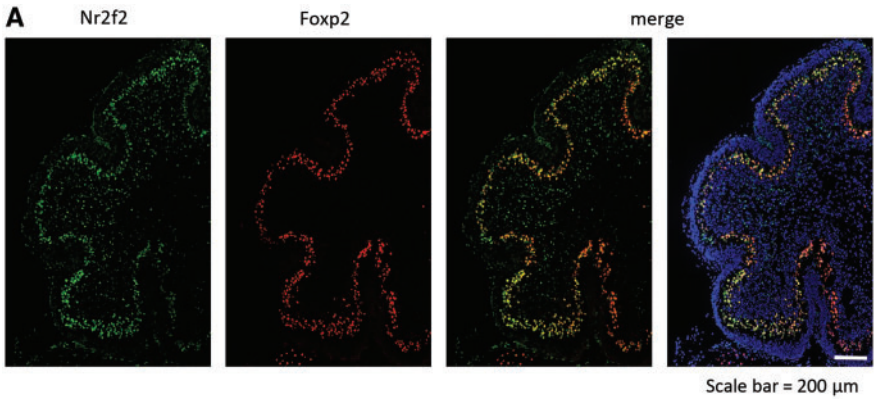

Figure 7. Foxp2 is co-expressed with Nr2f2 in the Purkinje cells in the cerebellum. Immunofluorescence experiments to assess endogenous co-expression of Nr2f2 with Foxp2 in the cerebellum of P3 mice brains. Foxp2 protein expression is shown in red; Nr2f2, in green; and nuclei stained with DAPI, in blue. (Scale bar = 200 μm).

NR2F2 (NM_021005), SATB1 (NM_002971), SATB2 (NM_001172509), ZBTB2 (NM_020861), TP53 (NM_000546), ZNF687 (NM_001304763), ZMYM2 (NM_003453), NFAT5 (NM_138714), YY1 (NM_003403) and SOX5 (NM_001330785) were amplified using the primers listed in Supplementary Material, Table S3 and cloned into pCR2.1-TOPO. Human fetal brain cDNA was used as template for PCR, except in the cases of NR2F2 and NFAT5, which were amplified from the plasmids pAAV-hNR2F2-RFP (Addgene, #22926) and pEGFP-NFAT5 (Addgene, #13627), respectively. The cloning of FOXP2 variants p.Q17L, p.M406T, p.P416T, p.R553H, p.N597H, p.R328*, P.Q390Vfs*7, of synthetic truncated forms of FOXP2, and of FOXP1 variants p.A339Sfs*4, p.V423Hfs*37, p.Y439*, p.R465G, p.R514C, p.R252*, and p.W534R has been described previously (14,20). For expression of FLAG-tagged protein for affinity purification, the coding regions of FOXP1, FOXP2 and FOXP4 were subcloned into the pEF1αFLAGbio expression vector, provided by Dr. J.W. Wang (92). For expression of fusion proteins with *Renilla* luciferase, YFP and mCherry tags, cDNAs were subcloned into the pLuc, pYFP, and pmCherry expression vectors, respectively, which have been described previously (20,42). To generate the nuclear-targeted *Renilla* luciferase and YFP control plasmids used in the BRET assay, a peptide including the nuclear localization signal of the SV40 large T antigen (CGYGPKKKRKVGGLDN) was appended into the C-terminus of the pLuc and pYFP plasmids by ligating synthetic double-stranded oligonucleotides between the BamHI and XbaI sites of the pLuc and pYFP vectors (42). Addition of this signal causes a significant redistribution of protein to the nucleus, as previously reported in (42). All constructs were verified by Sanger sequencing.

Cell culture and transfection

HEK293 cells were obtained from ECACC (cat. No. 85120602) and cultured in DMEM supplemented with 10% fetal bovine serum. Transient transfections were performed using GeneJuice (Merck-Millipore) according to the manufacturer's instructions. To generate stable cell lines expressing FLAG-tagged FOXP proteins, HEK293 cells were transfected with pEF1αFLAGbio containing the coding sequence of FOXP1, FOXP2, or FOXP4 using GeneJuice (Merck-Millipore) according to the manufacturer's instructions. Single clones were isolated following selection with culture medium containing 10 μM puromycin. Expression of the tagged proteins in selected clones was confirmed by western blotting of cell lysates using anti-FLAG antibody (Sigma). Selected stable cell lines were maintained in DMEM supplemented with 10% fetal bovine serum and 5 μM puromycin.

Affinity purification and mass spectrometry (AP/MS)

HEK293 cell lines stably expressing FLAG-tagged FOXP1, FOXP2 or FOXP4, or control cell lines, were expanded to twenty 15 cm dishes, washed with PBS, and harvested by scraping. The affinity-purification procedure has been described previously (93). Briefly, nuclear extracts were dialysed into buffer C-100 (20 mM HEPES pH 7.6, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 100 mM KCl, 20% glycerol) and then incubated with anti-FLAG M2 agarose beads (Sigma) and benzonase (Novagen) for 3 h at 4°C. Beads were washed five times for 5 min with buffer C-100 containing 0.02% NP-40 (C-100*) and bound proteins were eluted four times for 15 min at 4°C with buffer C-100* containing 0.2 mg/ml FLAG-tripeptide (Sigma). The purification of FOXP1, FOXP2 or FOXP4 was checked by western blot using anti-FLAG antibody. The elution fractions were pooled and proteins were TCA-precipitated, resolved by SDS-polyacrylamide gel electrophoresis, stained with Colloidal Blue Staining Kit (Invitrogen) and analysed by mass spectrometry as described previously (93–96).

Mass-spectrometry analyses were performed on three independent purifications from FOXP1- and FOXP2-expressing cell lines, and two independent purifications from FOXP4-expressing cell lines. Each purification was performed in parallel with stable cell lines transfected with empty control vector. To remove non-specific interactors, peptide lists were filtered using peptides found in the control conditions or in the CRAPome database, a collection of common contaminants in AP/MS data (97). Proteins with a Mascot score lower than 45 were also excluded.

Western blotting

Proteins from cell lysates were resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membranes using a TransBlot Turbo Blotting apparatus (Bio-Rad). Membranes were blocked in PBS containing 5% milk and 0.1% Tween-20 and incubated overnight at 4°C with primary antibody. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG for 45 min at room temperature. Proteins were visualized using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) and a ChemiDoc XRS+ imaging system (Bio-Rad).

BRET assay

BRET assays were performed as described previously (42). Briefly, HEK293 cells were transfected in 96-well plates with plasmids encoding YFP- and luciferase-fusion proteins. After 36–48 h, Enduren live cell luciferase substrate (Promega) was added at a final concentration of 60 μM. Cells were cultured for a further 4 h, and emission readings (integrated over 10 s) were taken using a TECAN F200PRO microplate reader using the Blue1 and Green1 filter sets. Expression levels of the YFP-fusion proteins were measured by taking fluorescent readings using the filter set and dichroic mirror suitable for green fluorescent protein (excitation 480 nm, emission 535 nm). The corrected BRET ratio was calculated with the following formula: $[Green1_{(experimental\ condition)}/Blue1_{(experimental\ condition)}]-[Green1_{(control\ condition)}/Blue1_{(control\ condition)}]$. The control conditions used luciferase or YFP fused to a C-terminal nuclear localization signal (42).

Fluorescence microscopy

HEK293 cells were seeded on coverslips coated with poly-L-lysine. Cells were transfected with plasmids encoding proteins of interest fused to YFP or mCherry. Cells were cultured for 30 h post-transfection, and then fixed with 4% paraformaldehyde. Nuclei were stained with Hoechst 33342. Fluorescence images were acquired using an Axiovert A-1 fluorescent microscope with ZEN Image software (Zeiss).

Immunofluorescence

Immunofluorescence experiments were performed on mouse brain sections at postnatal day 3 (P3). Brains were harvested and embedded in optimal cutting temperature (OCT) compound on dry ice. Sagittal sections were prepared at a thickness of 4 μm using a Leica CM1950 cryostat and then preserved at

−20°C. Tissue sections were fixed in ice-cold acetone for 10 min at −20°C, blocked using 10% donkey serum in PBS for 1 h at room temperature, and incubated overnight at 4°C with primary antibody diluted in 2% donkey serum. The following antibodies were used: goat anti-FOXP2 N-16 antibody (sc-21069, Santa Cruz), rabbit anti-FOXP2 antibody (ab16046, Abcam), rabbit anti-SOX5 (ab94396, Abcam), goat anti-SATB1 E-15 antibody (sc-5990, Santa Cruz), rabbit anti-SATB2 antibody (ab34735, Abcam), rabbit anti-NR2F1 (ab181137, Abcam) and rabbit anti-NR2F2 (ab42672, Abcam). After washing, tissue sections were incubated with fluorescently-labeled secondary antibodies diluted in 2% donkey serum: donkey anti-rabbit IgG Alexa488 (a21206, Invitrogen) and donkey anti-goat IgG Alexa594 (a11058, Invitrogen). Slides were mounted with VectaShield Antifade Mounting Medium with DAPI (Vector Labs) and then imaged using Axiovert A-1 fluorescent microscope with ZEN Image software (Zeiss).

Supplementary Material

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. The authors declare that they have no conflict of interests.

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