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Identification and functional characterization of *de novo* *FOXP1* variants provides novel insights into the etiology of neurodevelopmental disorder

Elliot Sollis[1], Sarah A. Graham[1], Arianna Vino[1], Henning Froehlich[2], Maaike Vreeburg[3], Danai Dimitropoulou[1], Christian Gilissen[4], Rolph Pfundt[4], Gudrun A. Rappold[2,5], Han G. Brunner[3,4], Pelagia Deriziotis[1,†,*] and Simon E. Fisher[1,6,†,*]

[1]Language and Genetics Department, Max Planck Institute for Psycholinguistics, Nijmegen 6525 XD, The Netherlands, [2]Department of Human Molecular Genetics, Heidelberg University, Heidelberg 69120, Germany, [3]Department of Clinical Genetics and School for Oncology & Developmental Biology (GROW), Maastricht UMC, Maastricht 6202 AZ, The Netherlands, [4]Department of Human Genetics, Radboud Institute for Molecular Life Sciences, Nijmegen 6500 HB, The Netherlands, [5]Interdisciplinary Center of Neurosciences (IZN), Heidelberg University, Heidelberg 69120, Germany and [6]Donders Institute for Brain, Cognition and Behaviour, Nijmegen 6525 EN, The Netherlands

*To whom correspondence should be addressed. Tel: +31 243521923; Fax: +31 243521213; Email: pelagia.derizioti@mpi.nl (P.D.); Tel: +31 243521441; Fax: +31 243521213; Email: simon.fisher@mpi.nl (S.E.F.)

Abstract

De novo disruptions of the neural transcription factor *FOXP1* are a recently discovered, rare cause of sporadic intellectual disability (ID). We report three new cases of *FOXP1*-related disorder identified through clinical whole-exome sequencing. Detailed phenotypic assessment confirmed that global developmental delay, autistic features, speech/language deficits, hypotonia and mild dysmorphic features are core features of the disorder. We expand the phenotypic spectrum to include sensory integration disorder and hypertelorism. Notably, the etiological variants in these cases include two missense variants within the DNA-binding domain of FOXP1. Only one such variant has been reported previously. The third patient carries a stop-gain variant. We performed functional characterization of the three missense variants alongside our stop-gain and two previously described truncating/frameshift variants. All variants severely disrupted multiple aspects of protein function. Strikingly, the missense variants had similarly severe effects on protein function as the truncating/frameshift variants. Our findings indicate that a loss of transcriptional repression activity of FOXP1 underlies the neurodevelopmental phenotype in FOXP1-related disorder. Interestingly, the three novel variants retained the ability to interact with wild-type FOXP1, suggesting these variants could exert a dominant-negative effect by interfering with the normal FOXP1 protein. These variants also retained the ability to interact with FOXP2, a paralogous transcription factor disrupted in rare cases of speech and language disorder. Thus, speech/language deficits in these individuals might be worsened through deleterious effects on FOXP2 function. Our findings highlight that *de novo* FOXP1 variants are a cause of sporadic ID and emphasize the importance of this transcription factor in neurodevelopment.

Introduction

FOXP1 (forkhead-box protein P1; OMIM 605515) is a member of the forkhead-box family of transcription factors that is crucial for embryonic development (1). Heterozygous disruptions of the *FOXP1* gene result in global developmental delay and intellectual disability (ID) (OMIM 613670). The first cases of *FOXP1*-related disorder were individuals carrying *de novo* deletions of *FOXP1* and neighboring genes (2). Since then, additional cases have been found with either truncating/frameshift variants or deletions encompassing only *FOXP1*, confirming that disruption of one copy of this gene results in disorder (3–9). All reported etiological *FOXP1* variants to date have occurred *de novo*, consistent with the widespread observation of *de novo* protein-disrupting variants in cases of severe sporadic neurodevelopmental disorder (10).

Comparison of the phenotypes of patients with *FOXP1* disruptions has led to the delineation of a syndrome in which mild-to-moderate ID is frequently accompanied by features of autism spectrum disorder (ASD) (4–8). Some individuals present with additional behavioral problems such as obsessions and compulsions, aggression and hyperactivity (4,7,8). Macrocephaly and abnormal facial features have been reported in certain cases (6,7). Probands carrying *FOXP1* variants also show speech and language impairments, which range from moderate to severe and affect expressive language to a greater degree than receptive language (3–7). The speech deficits in these patients may be in part related to orofacial motor dysfunction, which has been noted in several cases (5,6).

The presence of speech and language impairments in this emerging *FOXP1*-related disorder is of particular interest because *FOXP1* is the closest paralogous gene to *FOXP2* (OMIM 605317). The *FOXP2* gene is disrupted in a rare form of speech and language disorder that is characterized by developmental verbal dyspraxia [also known as childhood apraxia of speech (CAS)] as well as deficits in expressive and receptive language affecting spoken and written domains (OMIM 602081) (11). FOXP1 and FOXP2 heterodimerize via a leucine zipper domain (12) and are co-expressed in several brain regions, with the potential to co-regulate downstream targets, including those involved in language, such as *CNTNAP2* (8,12,13).

In mouse and human fetal brain, *FOXP1* expression is observed in regions including the striatum, cerebral cortex (layers 3–5), hippocampus and thalamus (13,14). Mice with global *Foxp1* deletion die around embryonic day E14.5 (1). Mice with selective deletion of *Foxp1* in the brain are viable but display gross malformations of the striatum that develop post-natally, along with electrophysiological abnormalities in the hippocampus (15). These animals also exhibit learning and memory deficits and reduced social interests (15).

Here, we report the identification of three novel *de novo FOXP1* variants in individuals with ID through clinical whole-exome sequencing, including two missense variants in the FOX DNA-binding domain. We present detailed phenotypic information on the affected probands, extending the phenotype of *FOXP1*-related disorder. We report functional characterization of the three novel variants reported here, alongside three previously detected *de novo* etiological variants (4,8,16). Our functional investigations include the first analyses of missense *FOXP1* variants. We demonstrate deleterious effects on subcellular localization, transcriptional repression and protein–protein interactions for all six etiological variants, shedding light on the pathological mechanisms underlying *FOXP1*-related disorder.

Results

Clinical description of patients

Patient 1 is an 11-year-old boy from the USA born to healthy non-consanguineous parents (Table 1). At the age of 6 months, the patient was noted to have hypotonia and, at the age of 8 months, he was diagnosed with global developmental delay and macrocephaly. At the age of 12 months, he was noted by MRI to have mild, diffuse periventricular leukomalacia. He had delays in all motor milestones; he walked at 21 months and has fine motor problems. In addition, he presented with speech delays and has speech impairment, including articulation deficits. He has been diagnosed with mild ID; an IQ test revealed a wide range in his IQ score (between 50 and 80 in the different tasks). He displays a number of autistic features such as stereotypic behavior, obsessive-compulsive tendencies, ADHD and sensory processing disorder but does not fulfill criteria for classical autism. Anxiety-related behaviors are well-managed by Prozac. During childhood, he presented with recurrent infections of the skin and ear. Other findings were a large forehead and short stature. Biochemical tests (plasma amino acids and urine organic acids) were normal. Genetic investigations with normal results included chromosome analysis, chromosome microarray, fragile X syndrome screening and *PTEN* sequencing.

Patient 2 is a 7-year-old Dutch boy born to healthy non-consanguineous parents (Table 1). His family history includes a maternal uncle diagnosed with a pervasive developmental disorder. The patient presented with global developmental delay, ID and speech and language problems. Delays in cognitive development (total IQ = 53 at the age of 5 years) and mild delays in motor development (walked at the age of 17 months) were noted. Currently, he can speak in sentences, but with poor pronunciation/articulation. The child neurologist described an apraxia of the tongue. When playing, he constantly talks and makes noises. He has also been diagnosed with a sensory integration disorder. In addition, his behavior can be demanding and impulsive; he has ADHD and received medication (Ritalin). He shows many features of pervasive developmental disorder such as stereotypic behavior, obsessive-compulsive tendencies and a great need for structure in daily life but does not fulfill criteria for classical autism. He has mild dysmorphic features including hypertelorism, small down slanted eyes, a short nose and mild retrognathia. Height, weight and head circumference measurements are normal for his age. Other findings include strabismus and enuresis. Investigations with normal results included screens for fragile X and Angelman syndromes, SNP array testing, *MECP2* and *TCF4* sequencing and metabolic tests. A screen for deletions/duplications of the poly-alanine stretch in exon 2 of *ARX* was negative.

Patient 3 is a 15-year-old Dutch girl born to healthy non-consanguineous parents (Table 1). The pregnancy was complicated by gestational diabetes. She was born at 38 weeks via C-section and had a birth weight of 4160 g. During her first months of life, she developed nystagmus and was prescribed glasses because of hypermetropia (+5 diopter). The patient showed developmental delays; she walked at the age of 21 months and has been diagnosed with severe ID. At 3 years, her BSID-II was equivalent to 18 months and at 5 years her development was equivalent to ~2.3 years (SON-R; non-verbal intelligence test). Her language skills at 12 years were equivalent to 3 years. Currently, she mostly lacks speech and can say a few words with poor articulation. In addition, the patient has behavioral problems: autistic features, anxiety, aggression, obsessive-compulsive behavior and screaming. Mild dysmorphic features include hypertelorism, prominent

Table 1. Phenotypic description of patients with *de novo* missense, nonsense and frameshift *FOXP1* variants

Patient	This study Patient 1	This study Patient 2	This study Patient 3	Hamdan *et al.* (4) Patient B	O'Roak *et al.* (8)	Srivastava *et al.* (16) Patient 41	Lozano *et al.* (7)
Variant (NP_116071.2)	p.R465G	p.R514C	p.Y439*	p.R525*	p.A339Sfs*4	p.W534R	p.V423Hfs*37
Age	11 years	7 years	15 years	9 years 11 months	9 years 5 months	2 years	14 years
Sex	Male	Male	Female	Male	Male	ND	Female
ID	Mild	Mild-to-moderate	Severe	Moderate	Severe	+	Moderate
ASD	Autistic features present	PDD-NOS	PDD-NOS	+	+	ND	+
ADHD	+	+	−	ND	ND	ND	+
Speech/language impairment or delay	+	+	+	+	+	ND	+
Regression	ND	−	−	ND	+	ND	ND
Anxiety	+	ND	+	ND	ND	ND	ND
Obsessions/compulsions	+	+	+	+	ND	ND	+
Aggression	ND	ND	+	+	ND	ND	+
Seizures	ND	ND	ND	ND	+	ND	ND
Motor delays	+	+	+	+	ND	ND	+
Hypotonia	+	+	+	ND	ND	+	+
Macrocephaly	+	−	−	ND	ND	+	+
Other findings	Sensory integration disorder, mild leukomalacia	Sensory integration disorder, hypertelorism, strabismus, enuresis	Hypertelorism, nystagmus	Jejunal and ileal atresia			

ND = no data; PDD-NOS = pervasive developmental disorder not otherwise specified.
NB. Whole-gene deletions are not included. See Le Fevre *et al.* (6) for a summary of these cases.

forehead and a broad tip of the nose. Menarche was at the age of 12 years. Her height and head circumference are normal (−1SD and +1SD, respectively in growth charts for Dutch children). Investigations with normal findings included karyotyping, fragile X screening, fluorescence *in situ* hybridization, SNP arrays and metabolic tests.

Clinical whole-exome sequencing

Clinical whole-exome sequencing revealed *de novo* heterozygous *FOXP1* variants in all three patients (Fig. 1, Table 2). Patient 1 carries a missense *FOXP1* variant p.R465G, which affects the first residue of the FOX DNA-binding domain. Patient 2 carries a missense *FOXP1* variant p.R514C within the DNA-recognition helix of the FOX DNA-binding domain. Patient 3 carries a stop-gain *FOXP1* variant p.Y439*, which truncates the protein between the leucine zipper dimerization domain and FOX DNA-binding domain.

Functional characterization of etiological *FOXP1* variants

Prior observations of *de novo* *FOXP1* deletions in sporadic ID strongly suggest that haploinsufficiency of this gene is the main pathogenic mechanism in *FOXP1*-related disorder (3–8). In addition, *de novo* truncating and frameshift *FOXP1* variants have been reported previously in individuals with ID and most likely result in haploinsufficiency via nonsense mediated decay (NMD) of the variant transcript. Indeed, this has been demonstrated for the p.A339Sfs*4 and the p.V423Hfs*37 variants (7,8) (in the case of the p.Y439* and p.R525* variants, no patient material was available to confirm NMD of the altered transcript).

We hypothesized that the *de novo* missense variants in *FOXP1* in Patients 1 and 2 would also result in a loss of protein function.

Only one other missense variant in *FOXP1* (p.W534R) has been reported to date and, to our knowledge, the effect on function has not been investigated (Table 2) (16). Therefore, we sought to characterize the effects of all three currently known missense variants in FOXP1 on protein function (Fig. 1C). For comparison, we performed parallel characterization on three etiological truncating and frameshift variants, including the p.Y439* variant found in Patient 3 and two previously reported variants (p.R525* and p.A339Sfs*4) (Fig. 1C, Table 2) (4,8). The p.R525* variant truncates the protein within the FOX domain and abolishes transcriptional repression activity (4). The p.A339Sfs*4 variant truncates the protein between the zinc finger and leucine zipper domains and results in mislocalization of the protein within the cell (8). We recently reported an additional etiological frameshift variant in *FOXP1* (p.V423Hfs*37) (Fig. 1C; Table 2) (7). Because functional analyses for this variant have been reported elsewhere (7), we did not assess the biological consequences of the p.V423Hfs*37 variant here.

Effects of FOXP1 variants on protein expression

Wild-type (WT) FOXP1 and the six etiological FOXP1 variants were expressed as fusions with YFP and produced proteins at the expected molecular weights (Fig. 2A). Western blotting suggested that the p.A339Sfs*4 variant was expressed at a substantially higher level than WT FOXP1. We therefore quantified expression levels of all variants in live cells based on fluorescence intensity, which confirmed the increased expression level of the p.A339Sfs*4 variant (Fig. 2B). The dramatically increased expression of the p.A339Sfs*4 variant, but not of the p.Y439* variant, indicates that the region between residues 339 and 439 may play a role in the regulation of FOXP1 protein levels. This region
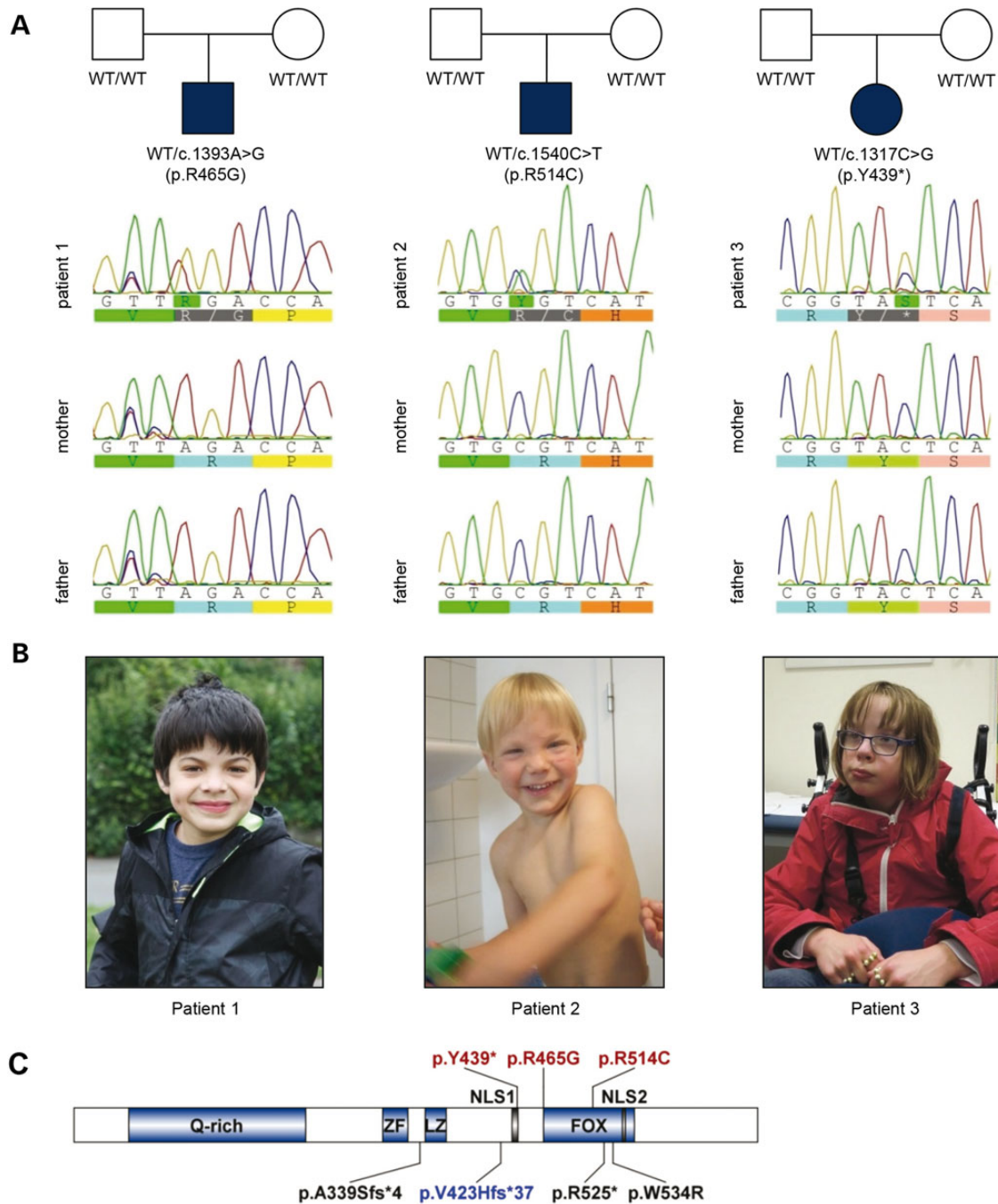
Figure 1. Identification of *de novo* *FOXP1* variants in three patients with neurodevelopmental disorder. (**A**) Pedigrees of the three patients with Sanger traces of genomic DNA from the probands and their unaffected parents (FOXP1 transcript accession number = NM_032682.5; FOXP1 protein accession number = NP_116071.2). (**B**) Photographs of Patients 1, 2 and 3 at 11, 3 and 12 years of age, respectively. (**C**) Schematic representation of the FOXP1 protein indicating *de novo* changes found in individuals with neurodevelopmental disorder. The three variants identified in this study are shown in red. The p.V423Hfs*37 variant characterized previously (7) is shown in blue, and three additional previously reported variants are shown in black. The major domains in FOXP1 are indicated: a glutamine-rich region (Q-rich), zinc finger (ZF), leucine zipper (LZ) and forkhead-box (FOX) domains, and two nuclear localization signals (NLS1 and NLS2).

contains the leucine zipper dimerization motif as well as a serine/threonine-rich region that may be subject to post-translational modification.

Subcellular localization of FOXP1 variants

Direct fluorescence imaging of YFP-tagged FOXP1 variants showed that the WT protein localized to the nucleus and was excluded from nucleoli (Fig. 3), as reported previously (7). Strikingly, all six FOXP1 variants showed aberrant protein localization, suggesting impaired function (Fig. 3). The p.A339Sfs*4 variant displayed a diffuse distribution in the nucleus and cytoplasm, consistent with previous observations (8). This variant lacks both nuclear localization signals (NLSs) (Fig. 1C) but is small enough to passively diffuse into the nucleus. The p.Y439* variant formed large cytoplasmic aggregates and was entirely absent

Table 2. *De novo FOXP1 variants in patients with sporadic neurodevelopmental disorder*

Proband	Genomic (hg19 coordinates)	Transcript NM_032682.5	Effect on protein	Protein NP_116071.2	PolyPhen2
This study; Patient 1	Chr3:71026829	c.1393A>G	Missense	p.R465G	Probably damaging
This study; Patient 2	Chr3:71021818	c.1540C>T	Missense	p.R514C	Probably damaging
This study; Patient 3	Chr3:71027010	c.1317C>G	Nonsense	p.Y439*	N/A
Hamdan *et al.* (4); Patient B	Chr3:71021785	c.1573C>T	Nonsense	p.R525*	N/A
O'Roak *et al.* (8)	Chr3:71050170_71050171	c.1017_1018insT	Frameshift	p.A339Sfs*4	N/A
Srivastava *et al.* (16); Patient 41	Chr3:71021758	c.1600T>C	Missense	p.W534R	Probably damaging
[a]Lozano *et al.* (7)	Chr3:71027059_71027060	c.1267_1268del	Frameshift	p.V423Hfs*37	N/A

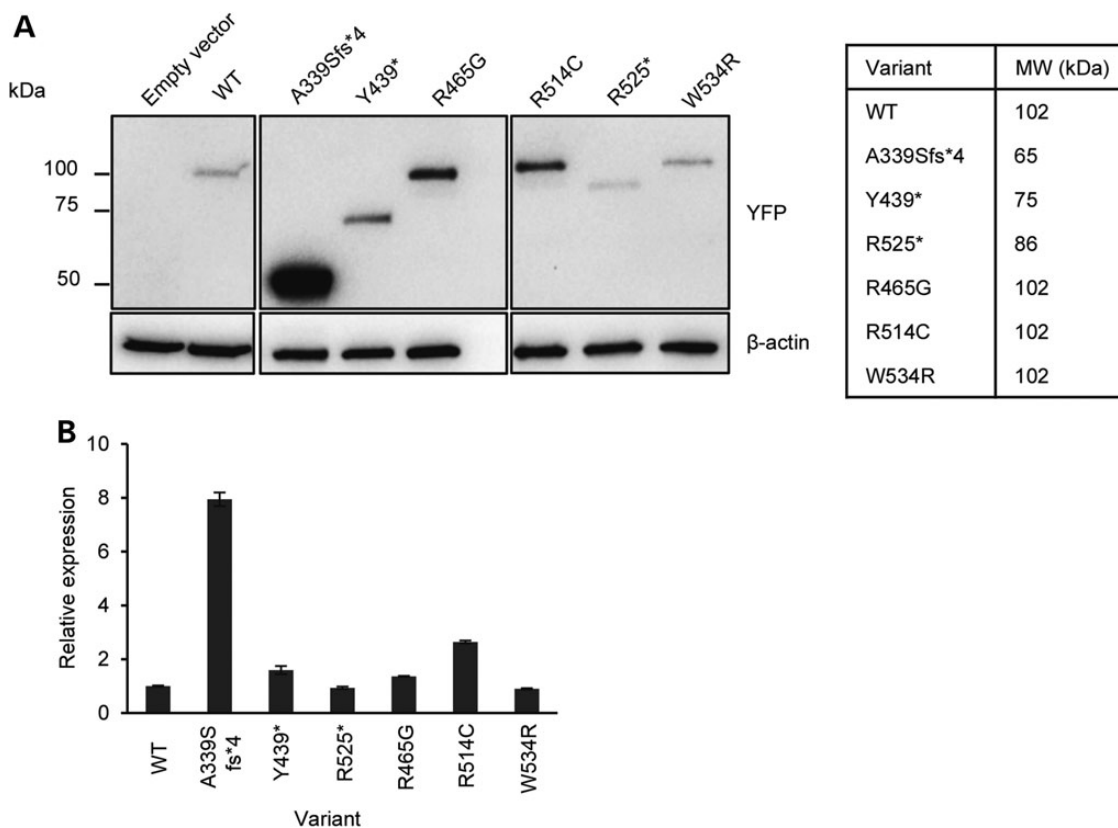N/A, not available; [a]Functional characterization described in Lozano *et al.* (7).

Figure 2. Effects of *FOXP1* variants on protein expression. (**A**) Immunoblot of whole-cell lysates of cells expressing YFP-tagged FOXP1 variants probed with anti-EGFP antibody. Blot was stripped and re-probed with anti-β-actin antibody to confirm equal loading. The predicted molecular weights of the YFP-FOXP1 fusion proteins are indicated on the right hand side. (**B**) Relative expression of FOXP1 protein variants in live cells as assessed by YFP fluorescence (average of three experiments ± S.D).

from cell nuclei, similar to the recently reported p.V423Hfs*37 variant, which is truncated at a similar position in the protein (7). The p.R525* variant was also excluded from the nucleus, despite retaining one of the two NLSs, and formed large aggregates, suggesting misfolding of the aberrant protein.

Notably, all three missense variants displayed dramatically disturbed localization patterns, despite having intact NLSs (Figs. 1C and 3). The p.R465G and p.R514C variants formed cytoplasmic and nuclear aggregates, whereas the p.W534R variant was observed exclusively in cytoplasmic aggregates.

The more severe effects of the p.W534R variant on protein localization may be due to dramatic destabilization of the FOX domain, as suggested by molecular modeling (p.W534R ΔΔG +5.35 kcal/mol; p.R465G ΔΔG +0.83 kcal/mol; p.R514C ΔΔG +0.74 kcal/mol).

Transcriptional repression by *FOXP1* variants

To assess the effect of variants on the ability of FOXP1 to regulate transcription, we performed luciferase reporter assays using the
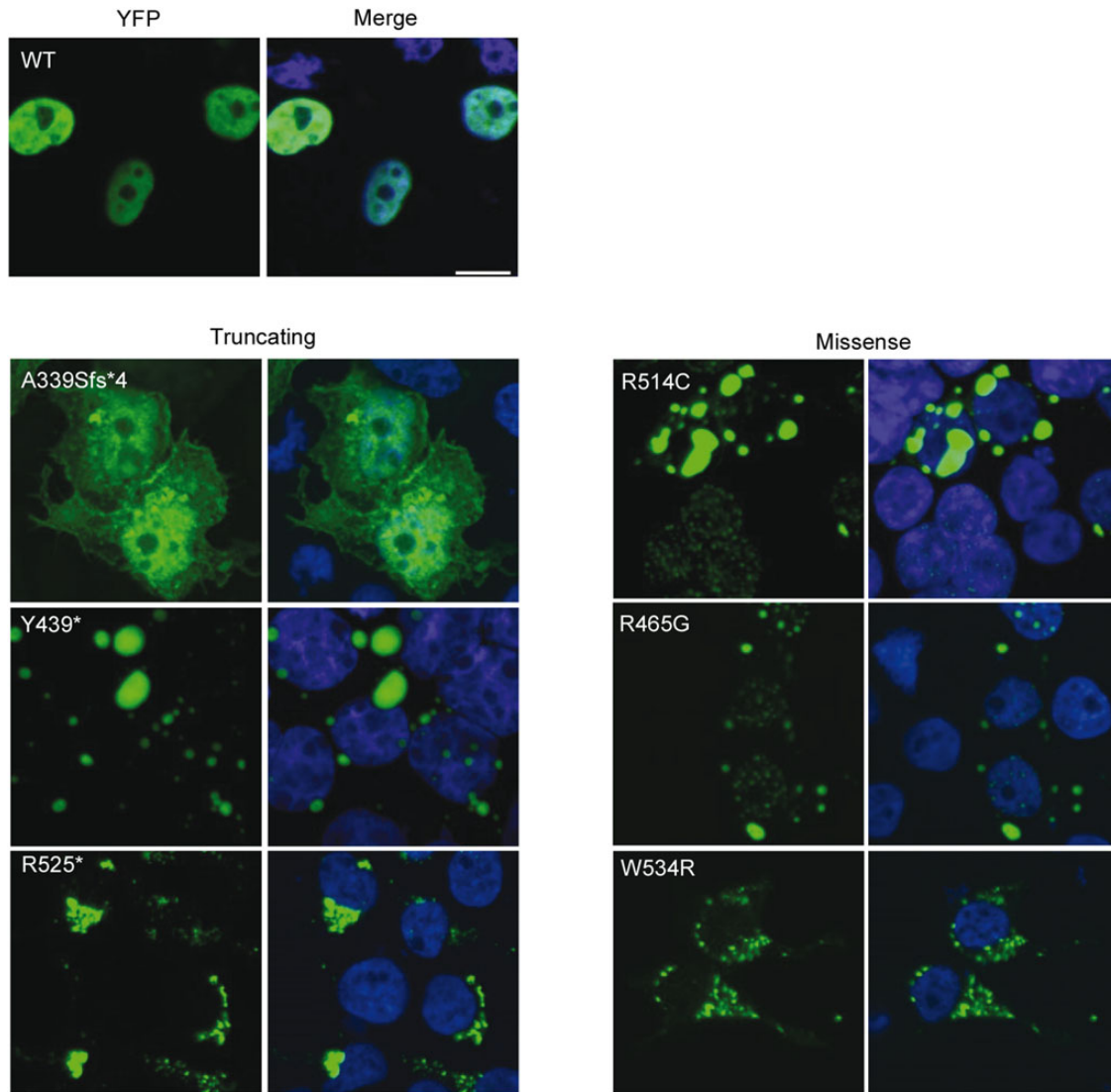
Figure 3. *FOXP1* variants severely disrupt cellular localization. Fluorescence imaging of cells expressing YFP-tagged FOXP1 variants (green). Nuclei were stained with Hoechst 33342 (blue). Scale bar, 10 μm.
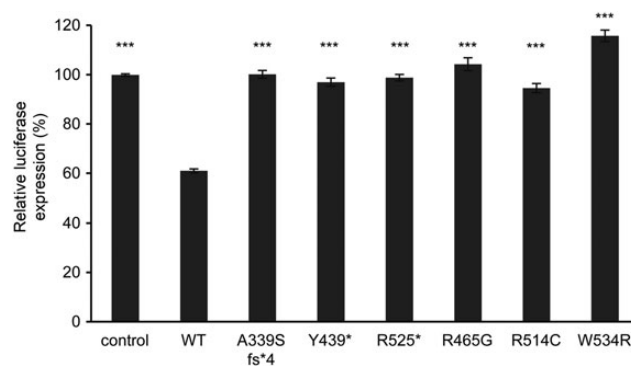

Figure 4. *FOXP1* variants abolish transcriptional repression. Luciferase reporter assays using the SV40 promoter. The mean ± S.E.M. of three independent experiments is shown. Values are expressed relative to the control. All variants were significantly different to WT FOXP1 (***$P < 0.001$).

SV40 promoter (Fig. 4) (4,7,17). As reported previously, WT FOXP1 repressed luciferase activity ($P < 0.001$). All six *FOXP1* variants showed significant ($P < 0.001$) loss of repressive activity, similar to the p.V423Hfs*37 variant reported previously (7), suggesting that they would not be able to regulate transcription of target genes.

Protein interactions of FOXP1 variants

To examine the effects of the FOXP1 variants on self-association, and on interaction with WT FOXP1 and FOXP2 proteins, we employed the bioluminescence resonance energy transfer (BRET) assay, which monitors protein–protein interactions in live cells (7,18,19).

The p.Y439* variant retained the ability to interact with WT FOXP1 and FOXP2 and to self-associate (Figs. 5A and 6A, Supplementary Material, Fig. S1), similar to the recently reported p.V423Hfs*37 variant (7). Strikingly, co-transfection of the p.Y439* variant with WT FOXP1 or FOXP2 resulted in translocation of the WT proteins into cytoplasmic aggregates (Figs. 5B and 6B). The other two truncating variants, p.A339Sfs*4 and p.R525*, showed a complete loss of interaction with WT FOXP1 and FOXP2 and were also unable to self-associate (Figs. 5A and 6A, Supplementary Material, Fig. S1). Consistent with this loss of interaction, the localization of WT FOXP1 and FOXP2 was not perturbed by co-transfection with these variants (Figs. 5B and 6B). In the case of the p.A339Sfs*4 variant, the loss of interaction is expected owing to the lack of the leucine zipper motif. However,

the p.R525* variant retains the leucine zipper and could therefore be expected to interact with itself and WT FOXP proteins. Furthermore, the loss of interaction with WT proteins is unlikely to be due to mislocalization of the variant because the variant also fails to self-associate (Figs. 5 and 6, Supplementary Material, Fig. S1). The absence of interactions may be due to misfolding of this variant.

Of the three missense variants, the p.R465G and p.R514C variants retained the ability to interact with WT FOXP1 and FOXP2 and to self-associate (Figs. 5A and 6A, Supplementary Material, Fig. S1). Accordingly, when co-expressed with WT FOXP1 or FOXP2 proteins, the p.R465G and p.R514C variants led to mislocalization of the WT proteins in nuclear aggregates (Figs. 5B and 6B). These variants may therefore sequester WT protein and exert a dominant-negative effect in patient cells. In contrast, the p.W534R variant showed loss of interaction with WT FOXP1 and FOXP2 and a reduced ability to self-associate (Figs. 5A and 6A, Supplementary Material, Fig. S1). Furthermore, co-expression of p.W534R with FOXP1 or FOXP2 did not affect localization of the WT proteins (Figs. 5B and 6B). The loss of interactions resulting from the p.W534R variant may be due to protein misfolding.
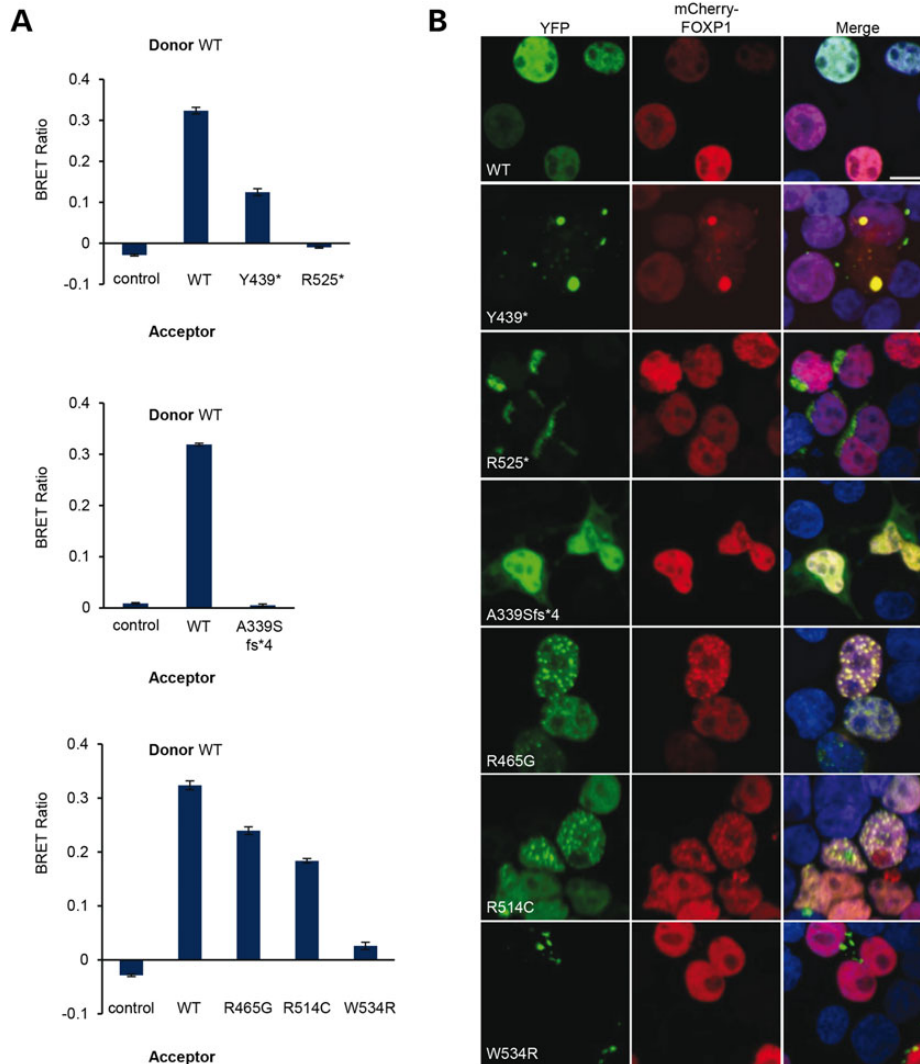

Figure 5. FOXP1 variants disrupt interactions with WT FOXP1. (**A**) BRET assays for interaction between WT FOXP1 and FOXP1 variants. Bars represent the corrected mean BRET ratios ± S.D. of one experiment performed in triplicate. (**B**) Fluorescence imaging of cells co-transfected with FOXP1 variants and WT FOXP1. FOXP1 variants fused to YFP are shown in green (left panel) and WT FOXP1 fused to mCherry is shown in red (middle panel). Nuclei were visualized using Hoechst 33342 (blue). Scale bar, 10 μm.

Figure 6. FOXP1 variants disrupt interactions with FOXP2. (**A**) BRET assays for interaction between WT FOXP2 and FOXP1 variants. Bars represent the corrected mean BRET ratios ± S.D. of one experiment performed in triplicate. (**B**) Fluorescence imaging of cells co-transfected with WT FOXP2 and FOXP1 variants. FOXP1 variants fused to YFP are shown in green (left panel), and WT FOXP2 fused to mCherry is shown in red (middle panel). Nuclei were visualized using Hoechst 33342 (blue). Scale bar, 10 μm.
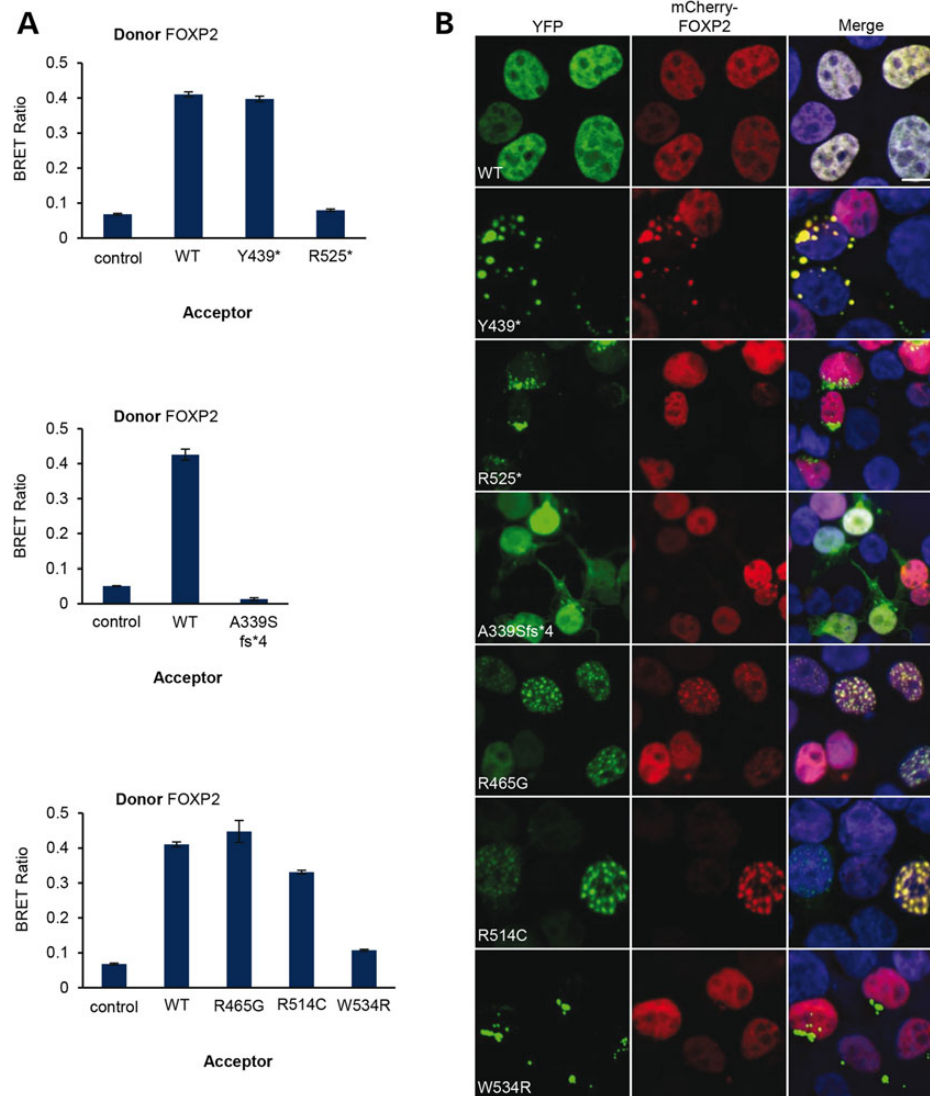
FOXP1 as a risk factor in ID and CAS

Given the striking effects of the *de novo* variants identified in individuals with ID on FOXP1 protein function, we also assessed the effects of five additional *FOXP1* variants observed in probands with ID or CAS (Table 3, Supplementary Material, Fig. S2A). Three of these variants—p.Q76dup, p.P215A, p.N570S—are known to have been inherited from unaffected parents, and the p.P215A and p.N570S variants have subsequently been found to occur at low frequency in the general population (Table 3) (4,5,20). These variants may represent risk factors for ID or cause a less severe form of *FOXP1*-related disorder that manifests primarily as a speech impairment. In this case, they might have effects on protein function that are milder in comparison to the effects of *de novo* variants. The other two variants—p.I107T and p.N597T—are of unknown inheritance status but have not been observed in the general population, such that it is possible that they may be causal *de novo* variants in the probands (5,21). The p.I107T variant was found in a proband with CAS and was attributed a potentially causal role on the basis of the relationship of FOXP1 to FOXP2 (21).

We found that all five variants were expressed and showed normal nuclear localization and transcriptional repression activity (Supplementary Material, Figs S2B, S2C, S3A, S3B). Furthermore, all variants were able to self-associate and to interact with WT FOXP1 and FOXP2 (Supplementary Material, Figs S3C, S4, S5). Therefore, our data do not support the contribution of these variants to the disorders in these patients. The lack of functional effects of the inherited variants suggests that the involvement of FOXP1 in neurodevelopmental disorder may be limited to highly penetrant *de novo* variants. Of particular note, the lack of effects of the p.I107T and p.N597T variants on protein function emphasizes the importance of performing functional characterization of missense variants observed in known disorder genes before attributing a causal role, especially when the inheritance status is unknown.

Discussion

Here, we report three novel *de novo* FOXP1 variants, including two missense changes, detected by clinical whole-exome sequencing

Table 3. Variants of unknown significance in *FOXP1*

Genomic (hg19 coordinates)	dbSNP	Minor allele frequency[a]	Transcript NM_032682.5	Effect on protein	Protein NP_116071.2	PolyPhen2	Phenotypes	Inheritance
Chr3:71161740_71161741	N/A	0/13006	c.226_228dup	Inframe insertion	p.Q76dup	N/A	ID (5)	Inherited
Chr3:71096114	rs146606219	24/13006	c.643C>G	Missense	p.P215A	Probably damaging	ID (4,5), CAS (20), controls (4,5,20)	Inherited
Chr3:71019900	rs140161845	23/13006	c.1709A>G	Missense	p.N570S	Benign	ID (5), controls(4)	Inherited
Chr3:71015140	N/A	0/13006	c.1790A>C	Missense	p.N597T	Benign	ID (5)	Unknown
Chr3:71102887	N/A	0/13006	c.320T>C	Missense	p.I107T	Possibly damaging	CAS (21)	Unknown

N/A, not available; [a]from NHLBI Exome Sequencing Project.

Table 4. Summary of functional characterization of etiological *FOXP1* variants

Variant	Expression levels	Cellular localization	Transcriptional repression	Interaction with FOXP1	Interaction with FOXP2
p.R465G	Similar to WT	Nuclear and cytoplasmic aggregates	No	Yes	Yes
p.R514C	Similar to WT	Nuclear and cytoplasmic aggregates	No	Yes	Yes
p.Y439*	Similar to WT	Cytoplasmic aggregates	No	Yes	Yes
p.A339Sfs*4	Greater than WT	Nuclear and cytoplasmic	No	No	No
p.R525*	Similar to WT	Cytoplasmic aggregates	No	No	No
p.W534R	Similar to WT	Cytoplasmic aggregates	No	No	No

in patients presenting with ID. We performed functional characterization of these variants, together with three previously reported *de novo* variants, and found that all six severely disrupted multiple aspects of protein function (Table 4). Notably, missense variants had similarly deleterious effects on protein activity as truncating and frameshift variants, confirming that these variants have a causal role in disorder in these patients. The loss of function demonstrated by molecular screens for missense, nonsense and frameshift variants reported here, coupled with the observation of whole-gene deletions of *FOXP1* in patients with ID (2,3,5), indicates that haploinsufficiency is the main pathogenic mechanism in individuals with *FOXP1*-related disorder. However, the retention of dimerization capacity of some of the variants suggests that the abnormal protein could exert a dominant-negative effect by preventing WT FOXP1 and FOXP2 from binding to DNA and modulating transcription.

The three patients in our study share phenotypic characteristics, including developmental delay, mild-to-severe ID, autistic features, speech/language impairment (including articulation and pronunciation deficits), hypotonia and obsessive-compulsive tendencies (Table 1). These traits are consistent with those previously described for individuals with whole-gene deletions or protein-disrupting *FOXP1* variants (3–8). We observed additional physical features such as hypertelorism, and neurological traits such as sensory integration disorder, which have not been previously been described, thereby extending the phenotype associated with *FOXP1*-related disorder. Interestingly, Patient 2 was noted to have apraxia of the tongue in addition to articulation and pronunciation problems. Deficits with learning and executing the fine motor sequences of the mouth, lips, tongue and larynx required for speech are seen in cases with disruptions of *FOXP2*, which is a paralog of *FOXP1* (11). Oro-motor dysfunction has also been previously reported in individuals with *FOXP1*

deletions in the absence of dyspraxic speech (5,6). Assessment of speech skills is warranted in cases of *FOXP1* disruption to ascertain if problems with orofacial praxis are a common phenotypic trait in *FOXP1*-related disorder.

The loss of transcriptional repression activity we observed for missense variants in our assays is consistent with the mechanism of DNA-binding by FOX transcription factors. The arginine at position 514, which is mutated in Patient 2, is conserved in 49/50 human FOX proteins (Supplementary Material, Fig. S6). No structure has been reported for FOXP1 bound to DNA, but the equivalent residue in FOXP2 (R553) is in the DNA-recognition helix and makes van der Waals contacts plus a water-mediated hydrogen bond to DNA bases and is therefore important for sequence-specific DNA binding (22). The tryptophan at position 534 in FOXP1, which is mutated in a patient previously described (16), is conserved in all 50 human FOX proteins (Supplementary Material, Fig. S6). The side chain of the equivalent residue in FOXP2 (W573) is in a β-strand in the FOX domain and makes a hydrogen bond to the DNA backbone (22). The arginine at position 465 in FOXP1, which is mutated in Patient 1, is conserved in all human FOXP subfamily proteins (Supplementary Material, Fig. S6). Furthermore, all human FOX proteins (with the exception of the FOXO subfamily) have a positively charged residue at this position (Supplementary Material, Fig. S6). The equivalent residue in FOXP2 (R504) lies at the N-terminus of the FOX domain and is important for DNA binding because it makes hydrogen bonds to the DNA backbone via its side chain (22).

Strikingly, missense variants affecting residues equivalent to FOXP1 p.R514 have been reported in five FOX-related monogenic disorders. A missense variant affecting the equivalent residue in FOXP2 (R553) is responsible for CAS and expressive and receptive language deficits in a multigenerational pedigree (KE family) (11). In cellular assays, this variant (p.R553H) has been shown to cause

mislocalization of the protein, which forms nuclear and cytoplasmic aggregates (17). The effects of the FOXP1 p.R514C variant identified here on protein localization are slightly more severe than the effects of the FOXP2 p.R553H variant, possibly as a consequence of the substitution with cysteine rather than histidine. The FOXP2 p.R553H variant also resulted in loss of DNA-binding and transcriptional repression activity (17), consistent with our observations on the FOXP1 p.R514C variant. Missense variants affecting residues homologous to FOXP1 p.R514 have also been reported in lymphedema-distichiasis syndrome (OMIM 153400) (FOXC2 p.R121H, heterozygous), Bamforth-Lazarus syndrome (OMIM 241850) (FOXE1 p.R102C, homozygous), alveolar capillary dysplasia with misalignment of pulmonary veins (OMIM 265380) (FOXF1 p.R97H, heterozygous) and blepharophimosis, ptosis and epicanthus inversus (OMIM 110100) (FOXL2 p.R103C, heterozygous) (23–26).

A variant affecting the residue homologous to FOXP1 W534 has been reported in FOXC1 (p.W152G) in a case of aniridia and congenital glaucoma (OMIM 601090) and had a severe impact on protein function (27). Variants affecting residues homologous to R465 have not been reported. However, while experiments were being performed for the current study, an additional missense variant in FOXP1 (p.P466L) was reported in a child with global developmental delay (28). The relevant residue is adjacent to the R465 residue affected by the missense variant in Patient 1. Missense variants affecting the amino acid equivalent to P466 have been reported in FOXF1 (p.P49S; p.P49Q) in alveolar capillary dysplasia with misalignment of pulmonary veins (OMIM 265380) (26). In sum, the FOXP1 variants identified in our patients mirror disease-causing variants across multiple FOX proteins, highlighting the key role of these amino acids in DNA binding by these transcription factors. This comparison of pathogenic variants in FOX family proteins underscores the value of considering paralogs and 3D structure together with functional studies in evaluating the etiological contribution of novel variants arising from next-generation sequencing.

The identification of FOXP1 disruptions in cases of ID revealed the critical role of this transcription factor in neurodevelopment. However, the precise functions of this gene in brain development remain unclear. In particular, neural target genes regulated by FOXP1 remain to be identified. *Foxp1* is expressed in the striatum, cerebral cortex (layers 3–5), hippocampus and thalamus (13). Investigations of the consequences of loss of this protein in the mouse brain were initially precluded because global deletion of both copies of *Foxp1* results in embryonic lethality owing to cardiac defects (1). Recently, mice have been engineered that lack *Foxp1* only in the brain (15). These animals display an imbalance of excitatory to inhibitory input in hippocampal neurons, but the most prominent effects are in the striatum. Following largely normal embryonic brain development, the mice develop enlargement of the ventral region of the striatum with reduction in the dorsal volume and increased ventricular volume during the first three post-natal weeks (15). Moreover, striatal neurons display altered morphology. In addition to morphological and electrophysiological abnormalities, mice lacking *Foxp1* in the brain have behavioral alterations with potential relevance for the neurological phenotype of human individuals carrying FOXP1 disruptions (15). Specifically, these animals display hyperactivity, increased repetitive behaviors, impaired short-term memory, reduced social interests and elevated anxiety.

We have shown that several FOXP1 variants found in patients with ID and language impairment result in aberrant interactions with FOXP2. FOXP1 is co-expressed with FOXP2 in regions of the brain, including the striatum and a small subset of cortical neurons (13,29). Striatal dysfunction in mice lacking *Foxp1* in the

brain is particularly intriguing because of alterations affecting this region in mice with one functional copy of *Foxp2* (30,31). These animals show impairments in motor-skill learning which may relate to electrophysiological anomalies in the striatum, including impaired long-term depression in corticostriatal synapses (30,31). Future studies aimed at identifying downstream targets of the FOXP1 protein in the striatum, in the presence or absence of FOXP2 protein, will be crucial in furthering our understanding of ways that these transcription factors work together to regulate gene expression during neurodevelopment.

While FOXP1 disruptions reported to date in humans are all heterozygous, no abnormalities have yet been described in mice with heterozygous deletion of *Foxp1* either globally or in a brain-restricted manner (1,15). However, there has been no detailed analysis of brain anatomy or electrophysiology in mice with heterozygous *Foxp1* deletion. It is challenging to find appropriate phenotypes to assay in mouse models of human neurodevelopmental disorders because the surface phenotypes in the two species may differ while still reflecting a common neurobiological deficit. In the case of mice with heterozygous disruptions of *Foxp2* mirroring the genotype of humans with speech and language disorder, detailed characterization was required to uncover behavioral and electrophysiological deficits (32). Furthermore, these deficits relate to motor circuitry and motor-skill learning behavior and not to vocalization, which is not an appropriate proxy for human speech (32). In future, it will be of interest to conduct detailed characterization of mice with heterozygous *Foxp1* disruption to try to identify deficits, which might model the underlying molecular mechanism of neurodevelopmental disorder seen in patients.

In summary, we report three new patients presenting with sporadic ID and carrying FOXP1 disruptions. Our clinical assessment of these patients contributes to the delineation of the core phenotype of FOXP1-related disorder and has identified novel features, which may help identify future cases. Furthermore, functional characterization of the FOXP1 variants found in these patients has both confirmed the diagnosis of FOXP1-related disorder and shed light on the molecular mechanisms that underlie this condition. Future work should focus on elucidating the role of this transcription factor in brain development.

Materials and Methods

Exome sequencing

In the case of Patient 1, whole-exome sequencing was performed by GeneDx (MD, USA). In the case of Patients 2 and 3, routine diagnostic exome sequencing was carried out as previously described (33). All variants were validated by Sanger sequencing in the probands and parents and were found to be *de novo*. Informed oral consent was obtained for the use of the data and photographs according to relevant institutional and national guidelines and regulations.

Variants are described throughout this article according to the following reference sequences: FOXP1 transcript = NM_032682.5, FOXP1 protein = NP_116071.2. Variants have been submitted to the NCBI ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar, accession #SCV000246199, #SCV000246200, #SCV000246201).

Protein modeling of FOXP1 variants

The *de novo* FOXP1 variants (p.R465G, p.R514C and p.W534R) were modeled using the YASARA structural-simulation software (http://www.yasara.org/). The protein model for human FOXP2 bound

to NFAT and DNA (RCSB Protein Data Bank 2AS5) served as a structural template. The FoldX plugin for YASARA was used to calculate ΔΔG values.

Cell culture and transfection

HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum (both from Invitrogen). Transfections were performed using GeneJuice, according to manufacturer's instructions (Merck-Millipore).

DNA constructs

WT FOXP1 and FOXP2 were amplified by PCR and cloned into pCR2.1-TOPO (Invitrogen) as described (19). FOXP1 variant constructs were generated using the QuikChange II Site-Directed Mutagenesis Kit (Agilent) (primer sequences are listed in Supplementary Material, Table S1). The c.1017_1018insT (p.A339Sfs*4) variant construct was directly synthesized by GenScript USA, Inc., as described previously (8). FOXP cDNAs were subcloned using BamHI/XbaI restriction sites into pLuc (18), pYFP (18) and a modified pmCherry-C1 expression vector (Clontech). All constructs were verified by Sanger sequencing.

Western blotting

Cells were transfected with equimolar concentrations of FOXP1 expression plasmids and cultured for 24 h. Whole-cell lysates were extracted by treatment with lysis buffer (100 mm Tris pH 7.5, 150 mm NaCl, 10 mm EDTA, 0.2% Triton X-100, 1% PMSF, protease inhibitor cocktail; all from Sigma–Aldrich) for 10 min at 4°C, before centrifuging at 10 000×g for 30 min at 4°C to remove cell debris. Proteins were resolved on 4–15% Tris–Glycine gels and transferred onto polyvinylidene fluoride membranes (both Bio-Rad). Blots were probed with mouse anti-EGFP (for pYFP constructs; 1:8,000; Clontech) overnight at 4°C, followed by incubation with HRP-conjugated goat anti-mouse IgG for 45 min at room temperature (1:2000; Bio-Rad). Proteins were visualized using Novex ECL Chemiluminescent Substrate Reagent Kit (Invitrogen) and the ChemiDoc XRS+ System (Bio-Rad).

Protein expression analysis

Cells were transfected in clear-bottomed 96-well plates in triplicate with equimolar concentrations of FOXP1 expression plasmids, together with a modified pmCherry-C1 plasmid to normalize for transfection efficiency. YFP and mCherry fluorescence intensities were measured in live cells 42 h post-transfection in a TECAN M200PRO microplate reader at 37°C and 5% CO_2. For each well, the background-subtracted YFP intensity was divided by the background-subtracted mCherry intensity. Triplicate conditions were averaged.

Fluorescence microscopy

Cells were seeded onto coverslips coated with poly-L-lysine (Sigma–Aldrich) and were fixed 24 h post-transfection using 4% paraformaldehyde (Electron Microscopy Sciences) for 10 min at room temperature. YFP and mCherry fusion proteins were visualized by direct fluorescence. Nuclei were visualized with Hoechst 33342 (Invitrogen). Fluorescence images were obtained using an LSM510 confocal microscope with LSM Image Software (Zeiss).

Luciferase reporter assays

Cells were seeded in 24-well plates and transfected with 45 ng of firefly luciferase reporter construct (pGL3-prom; Promega), 5 ng of *Renilla* luciferase normalization control (pRL-TK; Promega) and 200 ng FOXP1 expression construct (WT or mutant in pYFP) or empty vector (pYFP; control). Firefly luciferase and *Renilla* luciferase activities were measured 48 h post-transfection in a TECAN F200PRO microplate reader using the Dual-Luciferase Reporter Assay system (Promega). The statistical significance of the luciferase reporter assays was analyzed using a one-way analysis of variance and a Tukey's *post hoc* test.

BRET assay

BRET assays were performed as previously described (18,19). In summary, cells were transfected with pairs of *Renilla* luciferase and YFP-fusion proteins in 96-well plates. *Renilla* luciferase and YFP fused to a C-terminal nuclear localization signal were used as control proteins. EnduRen luciferase substrate (Promega) was added to cells 36–48 h after transfection at a final concentration of 60 μm and incubated for 4 h. Emission measurements were taken with a TECAN F200PRO microplate reader using the Blue1 and Green1 filters and corrected BRET ratios were calculated as follows: $[\text{Green1}_{\text{(experimental condition)}}/\text{Blue1}_{\text{(experimental condition)}}] - [\text{Green1}_{\text{(control condition)}}/\text{Blue1}_{\text{(control condition)}}]$. YFP fluorescence was then measured separately, with excitation at 505 nm and emission at 545, to quantify expression of the YFP-fusion proteins. *Renilla* luciferase and YFP fused to a C-terminal nuclear localization signal were used as control proteins.

Supplementary Material

Supplementary Material is available at *HMG* online.

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