

## ARTICLE

# A *de novo* *FOXP1* variant in a patient with autism, intellectual disability and severe speech and language impairment

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**FOXP1 (forkhead box protein P1) is a transcription factor involved in the development of several tissues, including the brain. An emerging phenotype of patients with protein-disrupting *FOXP1* variants includes global developmental delay, intellectual disability and mild to severe speech/language deficits. We report on a female child with a history of severe hypotonia, autism spectrum disorder and mild intellectual disability with severe speech/language impairment. Clinical exome sequencing identified a heterozygous *de novo* *FOXP1* variant c.1267\_1268delGT (p.V423Hfs\*37). Functional analyses using cellular models show that the variant disrupts multiple aspects of *FOXP1* activity, including subcellular localization and transcriptional repression properties. Our findings highlight the importance of performing functional characterization to help uncover the biological significance of variants identified by genomics approaches, thereby providing insight into pathways underlying complex neurodevelopmental disorders. Moreover, our data support the hypothesis that *de novo* variants represent significant causal factors in severe sporadic disorders and extend the phenotype seen in individuals with *FOXP1* haploinsufficiency.**

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## INTRODUCTION

*FOXP1* (forkhead box protein P1; OMIM 605515) belongs to the FOX gene family of transcription factor proteins, defined by the presence of a characteristic DNA-binding domain known as the forkhead box (FOX).<sup>1</sup> The mouse ortholog, *Foxp1*, is required for normal development of the heart, lung and esophagus<sup>2,3</sup> and acts as an accessory factor to Hox transcription factors in regulating the projection and connection of motor neurons to target muscles.<sup>4,5</sup> The importance of *Foxp1* in neurodevelopment is highlighted by studies in mice with a brain-specific *Foxp1* deletion.<sup>6</sup> These mutant mice show impaired neuronal development and present with autistic-like behaviors. In recent years, *FOXP1* variants have been reported in a number of patients with sporadic autism spectrum disorders (ASD), intellectual disability (ID), global developmental delay and moderate to severe speech delay, where expressive language is most severely affected (OMIM 613670).<sup>7,8</sup> The presence of speech and language impairments as characteristic of *FOXP1*-deficiency syndrome is interesting because the most similar gene to *FOXP1* is *FOXP2*, which is disrupted in a rare form of speech and language disorder (OMIM: gene 605317, disorder 602081).

*FOXP1* variants identified in patients with ASD and/or ID include whole gene deletions,<sup>7,9–12</sup> translocations,<sup>13</sup> nonsense variants,<sup>14</sup> missense variants<sup>15</sup> and frameshift variants.<sup>16</sup> The identification of whole gene deletions suggests that the mechanism of pathogenicity is haploinsufficiency. The severity of the phenotype indicates that these mutations are not inherited and in all cases where parental DNA has been tested the variants have been found to occur *de novo*. Using next-

generation sequencing, in this study we identified and characterized a *de novo* *FOXP1* variant in a female child with a history of severe hypotonia, ASD and mild ID with severe speech/language deficits.

## MATERIALS AND METHODS

### Clinical exome sequencing

Clinical exome sequencing of the proband and her unaffected parents was performed in the UCLA Clinical Genomics Center (see Lee *et al.*<sup>17</sup> for a detailed description of the UCLA clinical exome sequencing pipeline). There was only one high-quality nonsynonymous *de novo* coding variant identified in the patient, affecting the *FOXP1* gene. This sequence variant had a quality score  $\geq 500$ , no reads supporting the alternate allele in the parents and had never been observed in the general population (based on 1k Genomes Project, dbSNP135, NHLBI ESP). There were no clinically significant inherited homozygous, hemizygous, homoplasmic or compound heterozygous variants identified in the patient.

### Cell culture and transfection

Epstein–Barr virus-transformed live lymphoblast cell lines were obtained from the father, mother, proband and one unaffected sibling. Cultures were grown in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). HEK293 cells were grown in DMEM and SHSY5Y in DMEM-F12 (Invitrogen). Media were supplemented with 10% fetal bovine serum (Invitrogen). Transfections were performed using GeneJuice according to the manufacturer's instructions (Merck-Millipore, Amsterdam, The Netherlands).

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### RNA extraction and cDNA synthesis

Total RNA was extracted from immortalized lymphoblasts using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and first-strand cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, USA).

### Nonsense-mediated decay

Nonsense-mediated decay (NMD) was assessed as previously described.<sup>16</sup> FOXP1 cDNA was PCR-amplified using forward 5'-GTCTACAGAACCCAAAGCCGC-3' and reverse 5'-GGTCTGCGCAATATCTGCTG-3' primers, followed by Sanger sequencing.

### Quantitative RT-PCR

Quantitative RT-PCR was performed and analyzed as previously described,<sup>18</sup> using primers specific for the amplification of FOXP1 c.1267\_1268delGT (forward 5'-CGTCACCCAAGGCCCTCTC-3'; reverse 5'-TTGCGTCGGAAGTAAGCAAAC-3'), followed by Sanger sequencing.

### DNA expression constructs and site-directed mutagenesis

Wildtype (WT) FOXP1 and FOXP2 were PCR amplified and cloned into pCR2.1-TOPO (Invitrogen).<sup>19</sup> The p.V423Hfs\*37 FOXP1 variant was generated using the pCR2.1-TOPO.FOXP1 as template with the QuikChange II Site-Directed mutagenesis kit (Agilent Technologies, Wilmington, DE, USA) and forward 5'-TGGTGGTTGTGATGAGAGGGCCTTGGG-3' and reverse 5'-CCCAAGGCCCTCTCATCACACCACCA-3' primers. FOXP cDNAs were subcloned using BamHI/XbaI restriction sites into pcDNA4HisMax, a modified pmCherry-C1 vector (Clontech, Saint-Germain-en-Laye, France), as well as pLuc and pYFP expression vectors.<sup>20</sup> All constructs were verified by Sanger sequencing.

### SDS-PAGE and western blotting

HEK293 cells were transfected with equimolar concentrations of FOXP1 expression plasmids and cultured for 48 h. Cell lysis, SDS-PAGE and western blotting were performed as previously described.<sup>19</sup> Membranes were probed with Clontech mouse anti-EGFP (for pYFP constructs) overnight at 4 °C, followed by incubation with HRP-conjugated goat anti-mouse IgG for 45 min at room temperature (Bio-Rad, Veenendaal, The Netherlands). Blots were stripped and re-probed with Sigma anti- $\beta$ -actin antibody to confirm equal protein loading.

### Fluorescence microscopy

HEK293 and SHSY5Y cells were fixed on coverslips as previously described.<sup>19</sup> YFP and mCherry fusion proteins were visualized by direct fluorescence, and nuclei were visualized with Hoechst 33342 (Invitrogen). Fluorescence images were obtained using an Axiovert A-1 fluorescence microscope with ZEN Image Software (Zeiss, Oberkochen, Germany).

### Luciferase reporter assays

Luciferase assays were carried out as previously described.<sup>19</sup>

### Bioluminescence resonance energy transfer (BRET) assays

BRET assays were performed as previously described.<sup>19,20</sup>

### Statistical significance

The statistical significance of the luciferase reporter assays was analyzed using a one-way analysis of variance and Tukey's *post hoc* test.

## RESULTS

### Clinical phenotype

The patient is a 14-year-old female, first-born child of nonconsanguineous parents. Her mother was 30 and her father was 32 years old at the time of her birth. She was born via C-section for oligohydramnios and preeclampsia and was noted to have a two-vessel umbilical cord and severe hypotonia at birth. During her first year she had severe

gross motor delays; she started to crawl at 12 months and walk at 20 months. The patient also had speech delays, with first words at 20 months. At 3 years of age, she was diagnosed with expressive and receptive communication deficits.

At present, only 50% of the patient's speech is coherent and she is not able to sustain a conversation. In particular, her speech lacks structure, has limited content and expansion and she repeats simple phrases (echolalia). Her preferred method of communication is via messaging through an electronic device. The patient performed poorly on language-skill evaluations: she scored 50 on the comprehensive assessment of spoken language (<0.1 percentile), 70 on the receptive one-word picture vocabulary test (2.0 percentile), 64 on the expressive one-word picture vocabulary test (1.0 percentile) and 78 on the Arizona articulation proficiency scale (3rd edition) (7.0 percentile). She has inconsistent production of 'j' and 'th', final consonant deletion with 'd', 't' and 'g', substitutes 'sh' for 'ch' and has difficulties with the vocalic 'r'. She continues to meet criteria for speech and language impairment as a discrepancy exists between her oral language skills in the areas of expressive and receptive language and her chronological age.

She is in eighth grade of special education and her cognitive evaluation this year showed a full-scale IQ of 54 (0.1 percentile), verbal comprehension index score of 59 (0.3 percentile), working memory index of 50 (<0.1 percentile) and processing speed index of 70 (2.0 percentile). She has obsessive behavior traits, restricted interests, repetitive and perseverative behaviors, likes routine and received the diagnosis of ASD. Furthermore, she has episodes of aggression and was diagnosed with attention deficit hyperactivity disorder at 7 years of age.

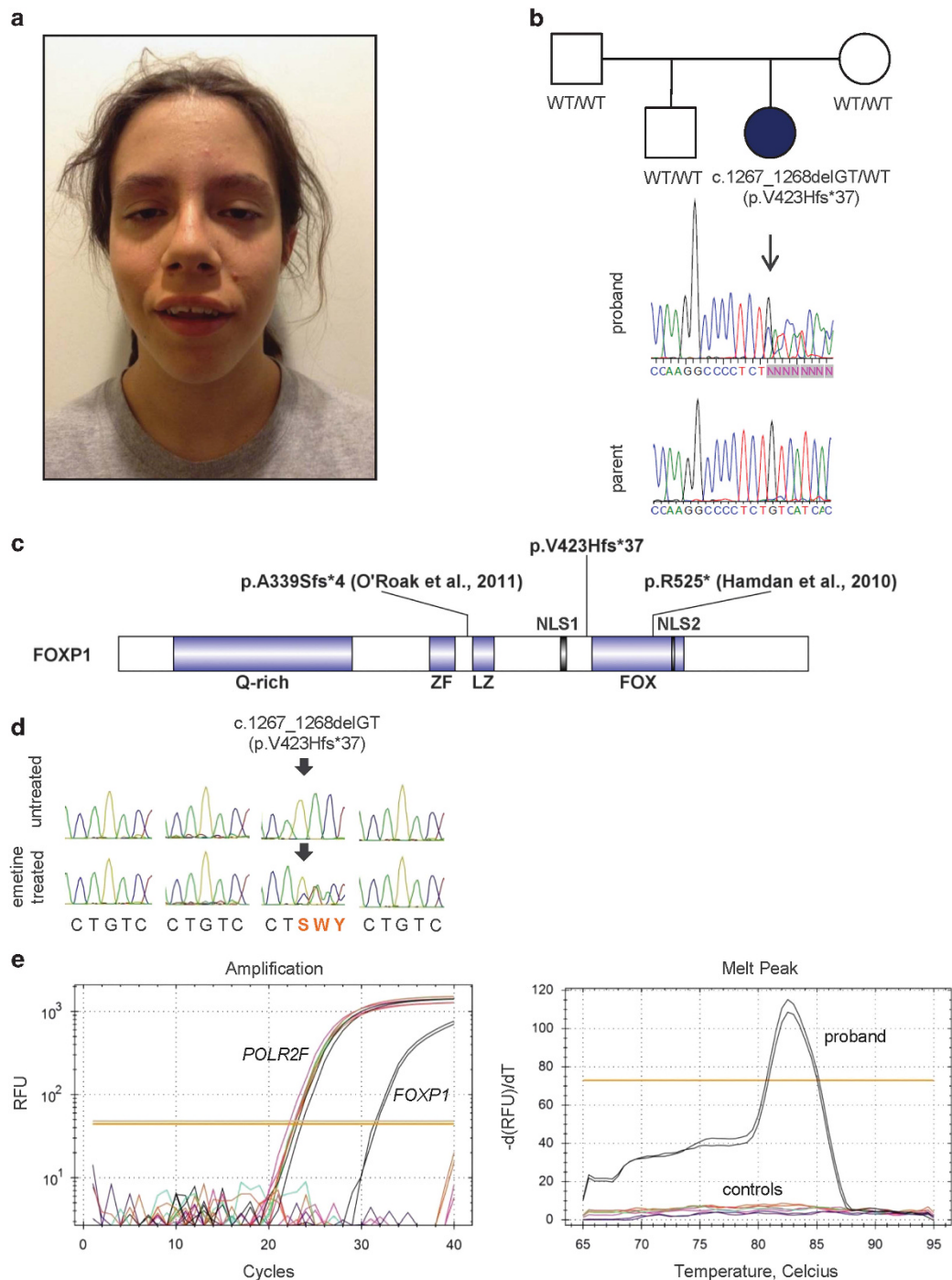
The patient is macrocephalic (+4 SD) and has underdeveloped ear lobes (Figure 1a). She also has mild ptosis, high palate, wide-spaced teeth and a hypotonic face. Her gait is abnormal with mild right foot dragging and a wide base. Her height is at 58th percentile (159 cm) and her weight is at 75th percentile (53.2 kg). Her adaptive physical education assessment indicated that she has difficulty with balance and overall low fitness scores. The rest of the physical examination was normal.

Investigations with normal results included genetic testing for muscular dystrophy, mitochondrial disorders, fragile X syndrome and SNP microarray. Electroencephalography and nerve conduction studies were normal. Magnetic resonance imaging, performed at 12 years of age, revealed multiple nonenhancing subcortical and deep white matter abnormalities and an incidental finding of venous angioma in the left frontal lobe.

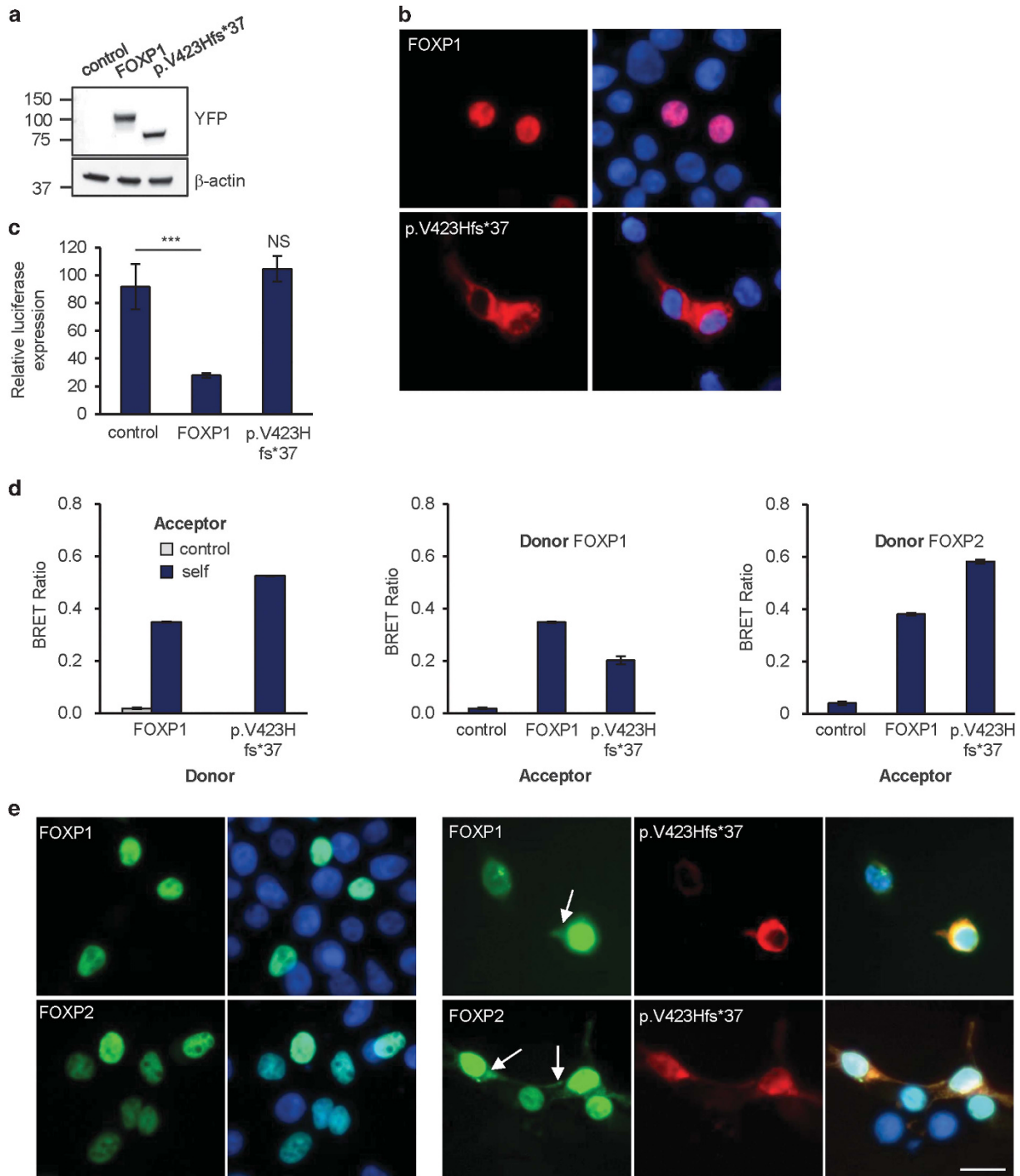
### De novo FOXP1 sequence variant identified by exome sequencing

In sporadic cases of ASD, gene-discovery approaches have recently focused on identifying *de novo* variants by employing whole-exome sequencing in parent-child trios. This strategy has been successful in uncovering a substantial fraction of population risk and identifying variation conferring relatively larger biological effects.<sup>21</sup> Therefore, to identify putative variants that may affect protein function, we performed clinical exome sequencing<sup>17</sup> on DNA from the proband and her unaffected parents. Using this approach we identified a heterozygous *de novo* two-base deletion in FOXP1 present in the proband that was subsequently validated and confirmed as *de novo* by Sanger sequencing (Figure 1b). The FOXP1 variant has been submitted to the NCBI ClinVar database (<http://www.ncbi.nlm.nih.gov/clinvar/>; ClinVar accession number SCV000189225).

This variant, c.1267\_1268delGT (numbered from the first coding nucleotide in NM\_032682.5), is located in exon 15 of FOXP1



**Figure 1** Identification of a *de novo* *FOXP1* variant in a patient with hypotonia, autism, mild ID and speech/language deficits. **(a)** Photograph of the patient at 14 years of age showing macrocephaly, mild ptosis, wide-spaced teeth and a hypotonic face. **(b)** Family pedigree showing Sanger traces of genomic DNA from the patient and one unaffected parent. The affected proband (shaded symbol) carries a heterozygous *de novo* two-base deletion (-GT relative to mRNA; c.1267\_1268delGT) that introduces a frameshift and results in a premature stop codon. Site of deletion is indicated by black arrow. **(c)** Schematic representation of the *FOXP1* protein indicating the change found in the patient. The *FOXP1* protein has a glutamine-rich (Q-rich) region and zinc-finger (ZF), leucine zipper (LZ) and FOX DNA-binding domains, as well as two nuclear localization signals (NLS1 and NLS2). The change in *FOXP1* is predicted to yield a C-terminally truncated protein (p.V423Hfs\*37) that lacks the FOX DNA-binding domain and NLS. Other truncated *FOXP1* variants previously reported in ASD (p.A339Sfs\*4) and ID (p.R525\*) are also shown. **(d)** Sanger traces of *FOXP1* cDNA from immortalized lymphoblasts derived from the affected proband and controls (father, mother and unaffected sibling). Two sets of cultures were grown in parallel, one of which was treated with 100  $\mu$ g/ml emetine for 7 h to inhibit NMD. In untreated cells, there is no evidence of the variant transcript, suggesting that the majority of these transcripts are degraded by NMD. Sanger traces from left to right: father, mother, proband, unaffected sibling. Arrows indicate site of deletion. **(e)** Quantitative RT-PCR amplification of variant *FOXP1* transcripts in cDNA derived from the proband and controls (father, mother and unaffected sibling) (left panel). Melting curve analysis was performed to assess the specificity of the amplification (right panel). Primers were designed to specifically detect the variant allele and amplification of c.1267\_1268delGT was only seen in proband-derived cDNA (black lines). Results represent two technical replicates per condition. *POLR2F* was used as a control. RFU, raw fluorescence unit.



**Figure 2** Functional characterization of the *de novo* FOXP1 variant. (a) Immunoblot of FOXP1 proteins fused to YFP in transfected HEK293 cells demonstrates similar levels of expression between WT FOXP1 and the mutant protein. Control denotes cells transfected with empty pYFP vector. Equal protein loading was confirmed by stripping and reprobing with an anti-β-actin antibody. (b) Fluorescence imaging of HEK293 cells transfected with WT or mutant FOXP1 fused to mCherry (red). The p.V423Hfs\*37 variant displays aberrant localization to the cytoplasm. Nuclei were stained with Hoescht 33342 (blue). Scale bar=10 μm. Concordant results were seen in SHSY5Y cells, as shown in Supplementary Figure 3. (c) Luciferase reporter assays for transcriptional regulatory activity of FOXP1 variants in HEK293 cells. The FOXP1 p.V423Hfs\*37 variant failed to repress expression of the luciferase reporter, indicating loss of transcriptional repressor activity. Values are expressed relative to the control (\*\*\*) $P < 0.001$ ; NS, not significant). The mean ± SEM of three independent experiments performed in triplicate is shown. (d) BRET assays for interaction between the p.V423Hfs\*37 variant and WT FOXP proteins in live HEK293 cells. Bars represent the corrected mean BRET ratios ± SEM of one experiment performed in triplicate. (e) Fluorescence images of HEK293 cells transfected with WT and mutant FOXP1. In the absence of the p.V423Hfs\*37 variant (fused to mCherry; red), FOXP1 and FOXP2 are nuclear (fused to YFP; green) (left panel). When coexpressed with the p.V423Hfs\*37 variant, FOXP1 and FOXP2 mislocalize to the cytoplasm (right panel). Nuclei were stained with Hoescht 33342 (blue). Scale bar = 10 μm. Concordant results were seen in SHSY5Y cells, as shown in Supplementary Figure 3.



(numbered from NG\_028243.1). The change is predicted to introduce a frameshift into the encoded protein, resulting in premature termination and loss of the DNA-binding domain and nuclear localization signals (NLSs) (p.V423Hfs\*37 according to HGVS recommendations) (Figure 1c). Two potentially causative heterozygous *de novo* FOXP1 truncating variants have been reported previously in cases of ASD (c.1014\_1015insA (p.A339Sfs\*4)) and ID (c.1573C>T (p.R525\*)) with speech and language deficits<sup>14,16</sup> (see Figure 1c for a comparison between the three truncated protein variants of FOXP1).

Because the variant introduces a stop codon before the final exon boundary in FOXP1, the mutant transcript may be subject to NMD. Therefore, we assessed the expression of FOXP1 transcripts in proband-derived lymphoblasts. Under normal cell culture conditions, it was found that only the WT allele was expressed (Figure 1d). Following inhibition of NMD, expression of both the variant and WT alleles could be detected, indicating that variant transcripts are generally degraded by NMD (Figure 1d). To investigate whether any of the variant transcripts escape this process, we performed quantitative RT-PCR with primers specific for the variant FOXP1 allele and found that c.1267\_1268delGT FOXP1 is expressed in low levels in proband-derived lymphoblasts (Figure 1e and Supplementary Figure 1).

#### Functional characterization of the FOXP1 sequence variant in human cells

To examine the effects of variant transcripts escaping NMD, we performed functional characterization of the protein encoded by the mutated transcript. WT and variant forms of FOXP1 were expressed as YFP- or mCherry-fusion proteins in HEK293 cells. The p.V423Hfs\*37 protein variant was expressed at a similar level to the WT protein as assessed by western blotting (Figure 2a). In contrast to FOXP1 that localized to the nucleus, the p.V423Hfs\*37 variant fused to mCherry localized to the cytoplasm, consistent with loss of both nuclear localization signals (Figure 2b). Of note, when the p.V423Hfs\*37 variant was fused to YFP, it formed aggregates in the cytoplasm (Supplementary Figure 2). Similar results were obtained when examining the subcellular localization of the FOXP1 variant in transfected human neuroblastoma SHSY5Y cells (Supplementary Figure 3). The lack of the FOX DNA-binding domain suggests that the FOXP1 variant is unlikely to retain DNA-binding capacity. Luciferase reporter assays demonstrated that the p.V423Hfs\*37 variant is unable to repress transcription, consistent with loss of DNA-binding capacity (Figure 2c).

FOXP proteins are able to form homodimers and to heterodimerize with other FOXP proteins through a leucine zipper domain.<sup>22</sup> Of note, FOXP1 and FOXP2 show partly overlapping neural expression patterns<sup>23,24</sup> such that FOXP1/FOXP2 heterodimers could regulate downstream targets, including those involved in language development, such as *CNTNAP2*.<sup>16,25</sup> Therefore, we investigated the effect of the *de novo* FOXP1 variant on the ability of the protein to homodimerize and to interact with WT FOXP1 and FOXP2 using the BRET assay. This method can monitor protein–protein interactions in live cells and has been successfully employed to study FOXP interactions.<sup>19,20</sup> The FOXP1 protein variant retains the leucine zipper domain required for homo- and heterodimerization with other FOXP proteins, and our results demonstrate that the p.V423Hfs\*37 variant can interact with itself, and with WT FOXP1 and FOXP2 in transfected cells (Figure 2d). Strikingly, coexpression of the p.V423Hfs\*37 variant with WT FOXP1 or FOXP2 resulted in colocalization of p.V423Hfs\*37 and WT proteins in the cytoplasm, indicating that the FOXP1 variant could interfere with the function of

the WT protein as a result of heterodimerization (Figure 2e and Supplementary Figure 3).

#### DISCUSSION

Here we report a patient with a *de novo* 2-bp deletion in FOXP1. This change leads to degradation of variant transcripts and encodes a nonfunctional protein, indicating that the patient phenotype likely results from FOXP1 haploinsufficiency. Furthermore, we demonstrate that a subset of variant FOXP1 transcripts escapes NMD and sequesters WT FOXP1 and FOXP2 outside the nucleus, suggesting that, if translated, the p.V423Hfs\*37 variant may also be exerting a dominant negative effect. In this case, the biological effects would be due to a combination of mechanisms, with the main pathological mechanism being haploinsufficiency. Future knockdown FOXP1 studies (eg, using shRNA constructs) should be informative in assessing the impacts of FOXP1 haploinsufficiency at the molecular level.

Our patient shares several phenotypic characteristics with other individuals who carry FOXP1 variants that disrupt protein function, including autism, ID, severe language deficits and macrocephaly.<sup>7</sup> Together, these observations indicate that FOXP1 haploinsufficiency results in a distinct syndrome that includes aspects of both ID and ASD, as well as other clinical features.<sup>7</sup> Targeted screens for FOXP1 variants are therefore warranted in probands with clinical features consistent with the syndrome.

Even though speech and language were affected in our patient, she did not have childhood apraxia of speech (difficulty with coordinating the complex sequences of orofacial muscle movements required for speech), and this is the most prominent feature in cases with pathogenic FOXP2 mutations.<sup>26,27</sup> In individuals with FOXP2 variants, speech articulation problems are accompanied by impairments in multiple aspects of expressive and receptive language.<sup>28,29</sup> In our patient, expressive language was more severely affected than receptive language, in agreement with phenotypes seen in other individuals with FOXP1 loss-of-function variants.<sup>7,11,14</sup> Thus far, FOXP1 variants have not been observed to cause language disorder in the absence of more global cognitive dysfunction.<sup>30</sup> The observed differences between the phenotypes of FOXP1 and FOXP2 haploinsufficiency may reflect differences in neural expression patterns<sup>23,24</sup> and/or regulation of shared and nonshared downstream targets.

Our investigation provides additional evidence that a significant proportion of severe sporadic neurodevelopmental disorders may result from *de novo* variants. Furthermore, this study highlights the value of experimental characterization of variants detected through next-generation DNA sequencing in understanding the molecular and cellular networks underlying major neurodevelopmental disorders.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)