

## De Novo Mutations in *FOXP1* in Cases with Intellectual Disability, Autism, and Language Impairment

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Heterozygous mutations in *FOXP2*, which encodes a forkhead transcription factor, have been shown to cause developmental verbal dyspraxia and language impairment. *FOXP2* and its closest homolog, *FOXP1*, are coexpressed in brain regions that are important for language and cooperatively regulate developmental processes, raising the possibility that *FOXP1* may also be involved in developmental conditions that are associated with language impairment. In order to explore this possibility, we searched for mutations in *FOXP1* in patients with intellectual disability (ID; mental retardation) and/or autism spectrum disorders (ASD). We first performed array-based genomic hybridization on sporadic nonsyndromic ID (NSID) (n = 30) or ASD (n = 80) cases. We identified a de novo intragenic deletion encompassing exons 4–14 of *FOXP1* in a patient with NSID and autistic features. In addition, sequencing of all coding exons of *FOXP1* in sporadic NSID (n = 110) or ASD (n = 135) cases, as well as in 570 controls, revealed the presence of a de novo nonsense mutation (c.1573C>T [p.R525X]) in the conserved forkhead DNA-binding domain in a patient with NSID and autism. Luciferase reporter assays showed that the p.R525X alteration disrupts the activity of the protein. Formal assessments revealed that both patients with de novo mutations in *FOXP1* also show severe language impairment, mood lability with physical aggressiveness, and specific obsessions and compulsions. In conclusion, both *FOXP1* and *FOXP2* are associated with language impairment, but decrease of the former has a more global impact on brain development than that of the latter.

Developmental language disorders represent a heterogeneous group of conditions that are frequent but poorly understood. Although these disorders show high heritability, very little is known about the causative genes. *FOXP2* (MIM 605317) represents an important entry point into the molecular basis of developmental language disorders.<sup>1,2</sup> Patients heterozygous for mutations in *FOXP2* show verbal dyspraxia (MIM 602081), which is characterized by impaired coordinated mouth movements that are required for speech, as well as variable impairment of expressive and receptive language, without consistent nonverbal cognitive deficits. Recent studies suggest that *FOXP2* is also required for vocal learning in birds and that it might have played a role during the evolution of human language.<sup>3–5</sup>

*FOXP2* belongs to a subgroup of the FOX family of winged-helix/forkhead transcription factors, which also includes *FOXP1* (MIM 605515), *FOXP3* (MIM 300292), and *FOXP4* (MIM 608924).<sup>6</sup> Several observations suggest that *FOXP2* and its closest relative, *FOXP1*, may regulate common processes. For instance, *FOXP1* and *FOXP2* are expressed in distinct but also in overlapping regions of the developing bird, mouse, and human brain, including areas

associated with the production and processing of vocalization and language.<sup>7–10</sup> FOXP proteins act as transcriptional repressors by forming homo- or heterodimers.<sup>9,11,12</sup> Interestingly, *FOXP1* and *FOXP2* can physically interact in vitro, can repress the transcription of common targets in vivo by occupying the same binding sites, and cooperatively regulate lung and esophageal development in mice.<sup>11,13</sup>

Because *FOXP1* and *FOXP2* cooperate to control developmental processes, it was hypothesized that mutations in *FOXP1* could likewise be associated with language impairment.<sup>10,14</sup> However, sequencing of *FOXP1* in a cohort of individuals with developmental verbal dyspraxia did not reveal any pathogenic mutations.<sup>14</sup> Another possibility is that *FOXP1* disruption causes other developmental conditions that are associated with language impairment, such as intellectual disability (ID; mental retardation) and autism spectrum disorders (ASD [MIM 209850]).<sup>15</sup> In order to explore this possibility, we performed mutation analyses of *FOXP1* in patients with ID and/or ASD. Because *FOXP2* heterozygous mutations are sufficient to disrupt language development, we decided, likewise, to focus our attention on heterozygous

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mutations, which in the case of ID are more likely to arise de novo than to be transmitted. By analogy to the *FOXP2* paradigm, we also restricted our analysis to patients without specific morphological abnormalities (referred to herein as having the nonsyndromic form of ID and ASD).

We studied sporadic cases with nonsyndromic ID (NSID), ASD, or both NSID and ASD, most of which were of French Canadian origin. We chose to study sporadic cases to increase the likelihood of identifying de novo mutations. The NSID cases were selected with the use of previously described criteria.<sup>16</sup> The sporadic ASD patients were diagnosed according to DSM-IV criteria and were selected on the basis of a positive Autism Diagnostic Interview-Revised (ADI-R) and/or Autism Diagnostic Observation Schedule-Generic (ADOS-G). We used 570 healthy individuals as controls, including 285 French Canadians and 285 European individuals who were evaluated and found not to have cognitive dysfunction, neuropsychiatric symptoms, or family history of neuropsychiatric problems, as detailed elsewhere.<sup>17</sup> Blood samples were collected from all members of these cohorts and from their parents, with informed consent and after approval of the study by institutional ethics committees. Genomic DNA was extracted from blood samples with the Puregene kit (Gentra). Paternity and maternity of each NSID and ASD patient proband trio were confirmed with the use of six informative microsatellite markers.

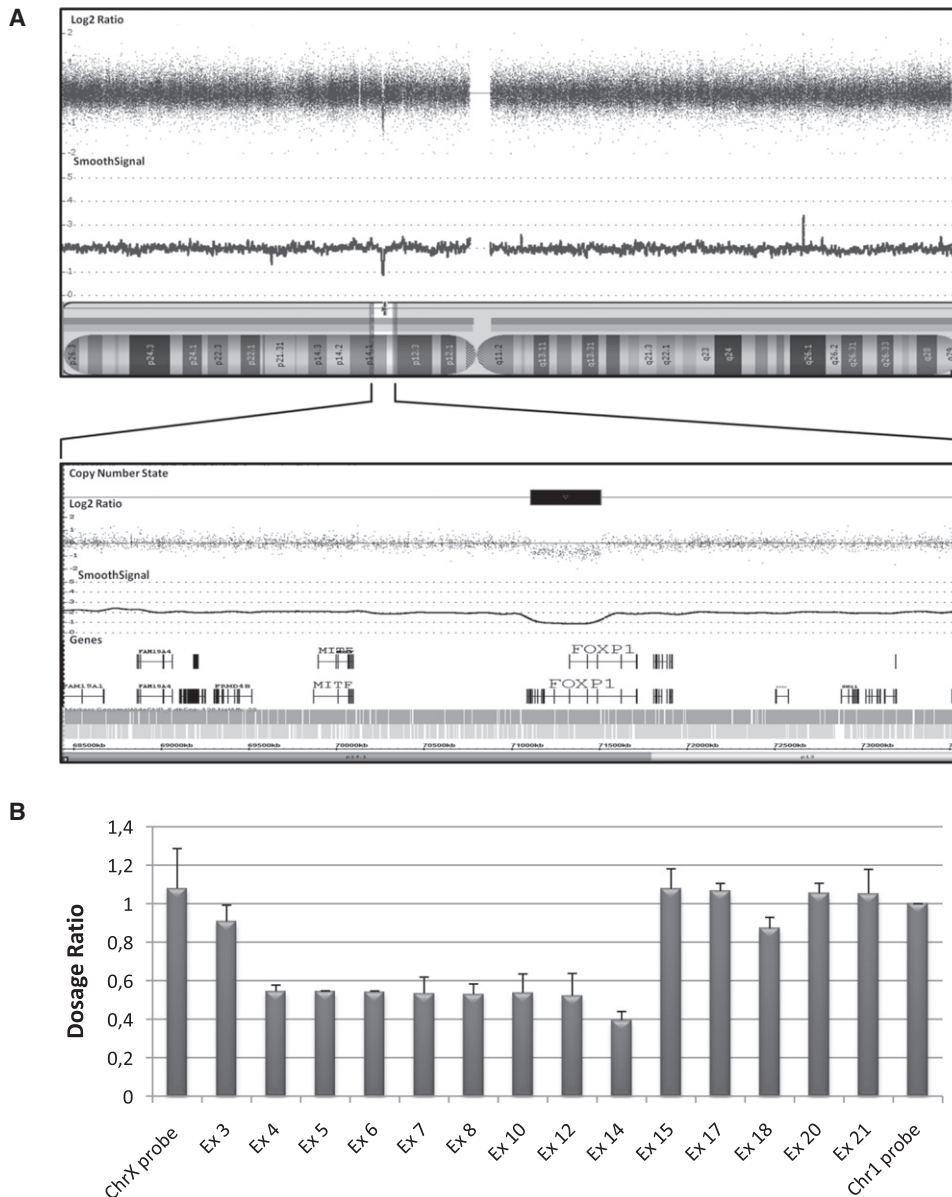
We initially determined whether *FOXP1* is affected by copy number changes in 80 individuals from the sporadic ASD cohort (including 27 with documented ID), using Genome-Wide Human Affymetrix 5.0 SNP arrays, and in 30 individuals from the sporadic NSID cohort, using Affymetrix 6.0 SNP arrays. Both parents of each case were also studied with these arrays. We identified a de novo intragenic *FOXP1* deletion in an NSID female patient (R0031608) (patient A). This ~390 kb deletion (genomic region on chromosome 3:71114875–71504640; hg18) encompasses exons 4–14 of the longest isoform of *FOXP1* (*FOXP1a*; Refseq no. NM\_032682.4), including sequence corresponding to its translation initiation site as well as to leucine zipper and zinc finger domains that are important for *FOXP1* dimerization and regulation of transcriptional activity (Figure 1A).<sup>11,13</sup> Multiplex ligation-dependent probe amplification (MLPA) analysis of *FOXP1* in patient A confirmed the loss of these exons (Figure 1B). No deletions encompassing *FOXP1* exons were identified in the other tested patients or their parents, nor have any been reported in the Database of Genomic Variants, a large public repository of structural variations in population controls.<sup>18</sup>

We next sequenced all the coding exons and intron-exon boundaries of the longest *FOXP1* isoform (*FOXP1a*; 16 coding exons) in 110 cases with NSID, 84 cases with ASD, and 51 cases with both NSID and ASD, as well as in 570 controls. We identified a de novo nonsense mutation, c.1573C>T (p.R525X), in a male patient with NSID and autism (R0024121) (patient B). This alteration abolishes the last 152 amino acids of *FOXP1*, including part of the

forkhead DNA-binding domain (FHD) and a conserved nuclear localization signal (NLS) (Figure 2A).<sup>19,20</sup> The FHDs of other *FOXP* proteins share more than 90% sequence identity with that of *FOXP1* (Figure 2B).<sup>21</sup> Alteration of the corresponding arginine residue in *FOXP3* (c.1189C>T [p.R397W]), as well as several other residues in the *FOXP3* FHD, has been reported to cause a lethal X-linked neonatal autoimmune disease known as IPEX (MIM 304790).<sup>22,23</sup> Furthermore, high-resolution analysis of the crystal structure of the human *FOXP2* FHD revealed that the residue homologous to p.R525, as well as residues downstream of it, plays an important role in DNA binding.<sup>21</sup> Indeed, pathogenic alterations in *FOXP2* that abolish the FHD (p.R328X) or that are located in proximity to the residue homologous to p.R525 (p.R553H) have been shown to cause verbal dyspraxia (Figure 2B).<sup>24,25</sup>

Sequencing also revealed a heterozygous missense mutation, c.643C>A (p.P215A), in both an NSID patient (n = 1/110) and a healthy individual (n = 1/570). In the case of the NSID patient, the variant was transmitted from an unaffected parent, further supporting the notion that it is not pathogenic. This variant was also previously reported in an individual with developmental verbal dyspraxia and in a control sample.<sup>14</sup> Three additional heterozygous missense variants (c.1333G>A [p.V445M], n = 1/570; c.1838C>A [p.T613N], n = 1/570; c.1709A>G [p.N570S], n = 2/570) were detected in *FOXP1*, but these did not affect any known functional domains and were present only in our cohort of controls, indicating that they are not pathogenic. No other amino-acid-altering variants were identified in any of the screened individuals, including the 570 healthy controls.

In order to test the effect of the p.R525X mutation on *FOXP1* activity, we took advantage of the well-documented ability of *FOXP1* to repress transcription from the SV40 promoter, which can be easily monitored with the use of a standard luciferase assay.<sup>9,20</sup> The p.R525X mutation was introduced into the full-length coding sequence of the *FOXP1* longest isoform (*FOXP1a*; obtained from Kazusa DNA Research Institute, Japan) and subcloned into the mammalian expression vector pcDNA4HisMax (Invitrogen). Human embryonic kidney (HEK) 293 cells were then cotransfected with the use of Fugene 6 (Roche) in 24-well plates, with 400 ng of pcDNA4HisMax without an insert or containing either the wild-type (WT) *FOXP1* or the *FOXP1-R525X* cDNA, along with 50 ng of pGL3-promoter construct (Promega) (in which the SV40 promoter drives a firefly luciferase reporter). In order to account for variations in transfection efficiency and variation in cell number, cells were also cotransfected with 50 ng of a *Renilla* luciferase construct (pRL-TK; Promega) driven by the HSV-thymidine kinase promoter, which is not affected by *FOXP1*. Cells were lysed 48 hr after transfection and quantified for firefly and *Renilla* luciferase activities with the use of the Dual Luciferase Reporter Assay System (Promega). As shown in Figure 2D, WT *FOXP1* was found to significantly inhibit the firefly luciferase signal (~50%



**Figure 1. De Novo *FOXP1* Deletion at 3p14.1 in Patient A**

(A) The Affymetrix Genome-Wide Human SNP 6.0 Array result for patient A is shown above the ideogram of chromosome 3 (Netaffx version 28). Each dot represents a SNP or a copy number marker, with normal copy number having a log2 ratio of ~0 and deleted regions less than -1. The CNV analysis was performed according to the manufacturer's procedure with the use of the Affymetrix reference library, GenomeWideSNP\_6.hapmap270.na30r1.a5, with a regional GC correction. The de novo deletion (chr3: 71109689–71508061; hg18) is boxed, and the only gene affected with this deletion, *FOXP1*, is shown.

(B) Mapping of *FOXP1* deleted exons in patient A. Using MLPA analysis,<sup>35</sup> we mapped the deletion breakpoints between exon 4 and exon 14 of *FOXP1*. MLPA probes targeting 14 exons (Ex) of *FOXP1* were custom designed with the ProSeek software.<sup>36</sup> These 14 probes were compared to two control probes on chromosome X and chromosome 1. MLPA was performed on 50 ng of genomic DNA. Probe amplification products were run on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The data were analyzed with the GeneMapper software version 4.0 (Applied Biosystems). The dosage ratio (DR) was calculated as follows: DR = (peak height *FOXP1* / peak height chromosome 1 probe) in the patient carrying the deletion / (peak height *FOXP1* / peak height chromosome 1 probe) in her unaffected mother, who has no copy number variation in *FOXP1*. The DR has a theoretical value of ≤ 0.7 for a deletion, between 0.7 and 1.3 for a normal situation, and ≥ 1.3 for a duplication. Values represent mean of samples ± standard deviation of samples run in triplicate. Data show that exons 4–14 are deleted in patient A.

reduction;  $p < 0.001$ ), as compared with an empty vector, whereas *FOXP1*-R525X failed to induce any reduction in luciferase levels, indicating that the p.R525X alteration impaired the *FOXP1* ability to repress the SV40 promoter. Collectively, these results, along with the position of the

p.R525X alteration in the FHD, strongly suggest that the de novo p.R525X alteration disrupts *FOXP1* function. The mechanism(s) underlying this loss of *FOXP1* activity could involve altered nuclear localization, as suggested by the disruption of one of the NLS in the *FOXP1* mutant



protein, impaired DNA binding, as suggested from the crystal structure of the FOXP2 FHD, or a combination of both. Although mutant and WT proteins were detected at comparable levels in transfected HEK293 cells, we cannot also exclude the possibility that the p.R525X mutation induces nonsense-mediated decay of *FOXP1* mRNA in the patient's cells (Figure 2E). Additional work is needed to address these mechanistic questions.

The phenotypes of both patients with de novo mutations in *FOXP1* are summarized in Table 1. Patients A and B were born from nonconsanguineous French Canadian parents after uneventful pregnancies and deliveries. Patient B was operated on for atresia of the midjejunum and midileum gut during the neonatal period. Their initial development was characterized by global delay, with severe language impairment. They started to walk between the ages of 18 and 20 mo. Patient A was not clearly pronouncing any word until 3 yrs of age and started to associate words at 4 yrs of age, whereas patient B said his first words at the age of 6 yrs. The patients exhibited no deficits of oromotor coordination that could explain this delay in learning to talk.

Patients A and B were assessed with the ADI-R and the ADOS-G at 6 yrs 8 mo and at 2 yrs 7 mo, respectively. Despite some autistic features in reciprocal social interaction (social avoidance with peers and decreased inhibition with adults) and restricted interests and repetitive behaviors (delayed echolalia and stereotyped language, self-injury, perceptual fixations), patient A would not have been considered as being autistic because of subthreshold scores in the communication area. In contrast, patient B showed scores above the diagnostic threshold for autism in all areas.

Patients A and B were evaluated with a series of cognitive and behavioral assessment tools at 15 and 9 yrs of age, respectively. Assessment with the Leiter International Performance Scale-R (Brief IQ), an estimate of nonverbal intellectual functioning, revealed ID in the mild to moderate range. Results from the Vineland Adaptive Behavior Scale were consistent with the presence of ID in both patients, showing severe deficits in adaptive behaviors across the range of subscales (communication, daily living skills, socialization). Formal testing of patient A via the Clinical Evaluation for Language Fundamentals confirmed the presence of severe language impairment. Specifically, expressive language was severely limited, with oral communication characterized by single words or very short, simple sentences. Expressive vocabulary, equivalent to about 4.5 yrs, was slightly better than other expressive skills, such as morphology and repetition of increasingly complex sentences (both below 4 yrs) but was nonetheless mostly limited to relatively simple, frequent words. In several tests, she gave responses to

expressive language items by miming the required words, indicating good comprehension of the question but an inability to produce the answer verbally. In support of this, receptive language abilities were somewhat more developed, as indicated by her understanding of a number of more complex relational and numerical concepts. Assessment of patient B with the Preschool Language Scale also confirmed severe language impairment. In terms of expressive communication, he scored at an age equivalence of 1 yr 11 mo. He had a range of about 100 words in his vocabulary and communicated with single words or short sentences (two to three words). In contrast, he was able to understand more complex relational concepts and performed at an age equivalence of 3 yrs 7 mo in auditory comprehension tasks.

Both patients also showed a particular behavioral profile. Clinically significant problems were noted on the Vineland Maladaptive Behavior scale for patient A and were characterized by both internalizing (e.g., social withdrawal, anxiety) and externalizing (e.g., impulsivity, mood lability, temper tantrums, sulking, disobedience, physical aggression) behaviors. Parent responses on the Aberrant Behavior Checklist also indicated the presence of behavioral problems in both patients. In patient A, irritability was rated as the most significant problem, with all other problem scales (lethargy, stereotypy, hyperactivity, inappropriate speech) reaching clinically significant levels. Similarly, patient B had significantly elevated problems on the irritability, stereotypy, and hyperactivity subscales and clinically significant speech problems. A number of specific obsessions and compulsions (e.g., looking in mirrors, touching people's hair, nail biting, removing silicone in the house, hoarding objects) were described by the parents and confirmed via the Repetitive Behavior Scale. On this scale, ritualistic, restricted, and "sameness" behavior were most often reported for patient A (e.g., mealtime and bedtime rituals, rigid routines, listens to the same music continuously, strong attachment to specific objects). Stereotyped and self-injurious behavior (e.g., turning in circles, spinning objects, hitting and biting self) were also reported for patient B.

In summary, we report two patients with de novo deleterious intragenic mutations in *FOXP1*. These patients share strikingly similar phenotypes, including mild to moderate ID with severe language impairment, autism and /or autistic features, mood lability with physical aggressiveness, and specific obsessions and compulsions. The patients with *FOXP1* disruption described herein do not show verbal dyspraxia, but their phenotype nevertheless overlaps with that of patients with *FOXP2* disruption to the extent that they also show language impairment. Two other patients with de novo deletions encompassing *FOXP1* were recently described.<sup>26,27</sup> These patients, who

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(E) Immunoblot performed on total protein extracts from HEK293 cells transfected with pcDNA4-HisMax-based constructs expressing FOXP1 or FOXP1-R525X in frame with an N-terminal Xpress tag, as described in the text. Proteins were resolved on SDS-PAGE, and FOXP1 and FOXP1-R525X were detected with the use of a monoclonal antibody against the N-terminal Xpress tag (Invitrogen). The blot was stripped and probed with an anti-alpha-tubulin antibody (Abcam) as an internal loading control.

**Table 1. Clinical Phenotype of the Patients with De Novo FOXP1 Mutations**

	Patient A (R0031608)	Patient B (R0024121)
FOXP1 de novo mutation	del (exons 4–14)	c.1573C>T (p.R525X)
Age (yrs: mo) / sex	15:11 / F	9:11 / M
ADI-R / ADOS-G	–/–	+/+
<b>Leiter International Performance Scale-R</b>		
BRIEF IQ: standard score (percentile)	58 (0.3)	48 (0.1)
<b>Pre-school Language Scale: Age Equivalent (Yrs: Mo)</b>		
Auditory comprehension	ND	3:7
Expressive communication	ND	1:11
Total language score	ND	2:7
<b>Clinical Evaluation of Language Fundamentals: Age Equivalent (Yrs:Mo)</b>		
<b>Expressive subtests</b>		
Morphology	< 4:0	ND
Recalling sentences	< 4:0	ND
Expressive vocabulary	< 4:5	ND
<b>Receptive subtests</b>		
Concepts and following directions	4:3	ND
Basic concepts	> 4:0	ND
Sentence structure	< 4:0	ND
<b>Vineland Adaptive Behavior Scales: Percentile Rank</b>		
Communication	1	< 1
Daily living	2	< 1
Socialization	< 1	1
Adaptive behavior composite	1	< 1
Internalizing behavioral abnormalities	clinically significant <sup>a</sup>	ND
Externalizing behavioral abnormalities	clinically significant <sup>a</sup>	ND
Maladaptive behavior index	clinically significant <sup>a</sup>	ND
<b>Aberrant Behavior Checklist: Raw Score (SD above Normal Mean)<sup>b</sup></b>		
Irritability subscale	26 (2.3)	31 (2.9)
Lethargy subscale	19 (1.7)	8 (0.2)
Stereotypy subscale	7 (1.6)	14 (2.9)
Hyperactivity subscale	24 (1.6)	42 (2.6)
Inappropriate speech subscale	6 (1.6)	6 (1.5)
<b>Repetitive Behavior Scale-Revised: Raw Scores<sup>c</sup></b>		
Stereotyped behavior	3	7
Self-injurious behavior	1	16

**Table 1. Continued**

	Patient A (R0031608)	Patient B (R0024121)
Compulsive behavior	3	6
Ritualistic behavior	6	6
Sameness behavior	6	11
Restricted behavior	4	10
<b>Other Tests</b>		
Fragile X testing	negative	negative
AGH	del FOXP1 (exons 4–14)	normal
CT scan	normal	ND

ND, not determined; AGH, array genomic hybridization. Affymetrix SNP arrays (5.0 or 6.0) were used for AGH.

<sup>a</sup> Clinically significant behavior problems on these scales correspond to the extreme top 2% of children of the same age.

<sup>b</sup> Clinically elevated: 1.5–1.9 SDs, significantly elevated:  $\geq 2.0$  SDs, where reported SDs are standard deviations from the normative ABC mean (community versus score).<sup>33</sup>

<sup>c</sup> All subscale scores correspond to significant case identification, except for the “self-injurious behavior” for patient A, as previously outlined.<sup>34</sup>

were less than 4 yrs of age when studied, showed developmental and speech delay, but formal cognitive or behavioral evaluation could not be performed. In one case, the deletion spans 750 kb and affects three other genes whereas in the other case, the deletion spans 1 Mb and extends over 500 kb on the 3' end of FOXP1. Even though this latter deletion does not affect any other known genes, the possibility that it encompasses elements that are important for the expression of genes located in its vicinity cannot be ruled out. Nevertheless, these cases reinforce our conclusion that FOXP1 haploinsufficiency affects cognitive development. Although an association between vitiligo (MIM 193200), an autoimmune form of skin depigmentation, and an intronic variant in FOXP1 was recently reported,<sup>28</sup> none of our patients displayed signs of such a condition.

Although FOXP1 and FOXP2 can cooperate to regulate transcription and are both associated with language impairment, FOXP1 disruption appears to have a more global impact on brain development than FOXP2 disruption. The difference between the phenotypic consequence of FOXP1 and FOXP2 haploinsufficiency might be explained at least in part by some difference in their expression patterns.<sup>7,9</sup> Alternatively, FOXP heterodimers and homodimers may have different biochemical properties. For instance, FOXP1-FOXP2 heterodimers may regulate targets that are involved in language impairment, whereas the respective FOXP homodimers may regulate additional targets that are associated with other developmental processes. Vernes et al. have found that FOXP2 directly regulates the expression of CNTNAP2, a gene that encodes a member of the neuroligin superfamily of transmembrane proteins implicated in neuronal recognition and cell adhesion.<sup>29</sup> CNTNAP2 has been associated with language

impairment and autism.<sup>29–32</sup> It is tempting to speculate that FOXP1 homodimers or FOXP1-FOXP2 heterodimers also regulate *CNTNAP2* expression and that *FOXP1* haploinsufficiency affects language development and possibly causes ID and autism by disrupting this regulatory interaction. FOXP1 and FOXP2 thus represent interesting entry points for dissecting the molecular mechanisms underlying neurodevelopmental disorders such as ID, autism, and language impairment.

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## Web Resources

The URLs for data presented herein are as follows:

Database of Genomic Variants, <http://projects.tcag.ca/variation/>  
Online Mendelian Inheritance of Man (OMIM), <http://www.ncbi.nlm.nih.gov/omim/>

UCSC Genome Browser, <http://www.genome.ucsc.edu/>

Uniprot, <http://www.uniprot.org/>

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