

Molecular networks implicated in speech-related disorders: FOXP2 regulates the SRPX2/uPAR complex

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It is a challenge to identify the molecular networks contributing to the neural basis of human speech. Mutations in transcription factor FOXP2 cause difficulties mastering fluent speech (developmental verbal dyspraxia, DVD), whereas mutations of sushi-repeat protein SRPX2 lead to epilepsy of the rolandic (sylvian) speech areas, with DVD or with bilateral perisylvian polymicrogyria. Pathophysiological mechanisms driven by SRPX2 involve modified interaction with the plasminogen activator receptor (uPAR). Independent chromatin-immunoprecipitation microarray screening has identified the uPAR gene promoter as a potential target site bound by FOXP2. Here, we directly tested for the existence of a transcriptional regulatory network between human FOXP2 and the SRPX2/uPAR complex. *In silico* searches followed by gel retardation assays identified specific efficient FOXP2-binding sites in each of the promoter regions of SRPX2 and uPAR. In FOXP2-transfected cells, significant decreases were observed in the amounts of both SRPX2 (43.6%) and uPAR (38.6%) native transcripts. Luciferase reporter assays demonstrated that FOXP2 expression yielded a marked inhibition of SRPX2 (80.2%) and uPAR (77.5%) promoter activity. A mutant FOXP2 that causes DVD (p.R553H) failed to bind to SRPX2 and uPAR target sites and showed impaired down-regulation of SRPX2 and uPAR promoter activity. In a patient with polymicrogyria of the left rolandic operculum, a novel FOXP2 mutation (p.M406T) was found in the leucine-zipper (dimerization) domain. p.M406T partially impaired the FOXP2 regulation of SRPX2 promoter activity, whereas that of the uPAR promoter remained unchanged. Together with recently described FOXP2-CNTNAP2 and SRPX2/uPAR links, the FOXP2-SRPX2/uPAR network provides exciting insights into molecular pathways underlying speech-related disorders.

INTRODUCTION

The development and functioning of the brain depend on very precise and complicated molecular networks that must be regulated both in time and in space. Animal models and

human genetic analyses have identified a large number of proteins participating in crucial brain processes, but little is known about how these individual proteins organize in regulatory and interacting networks. Deciphering the neurogenetic pathways that are associated with speech-related circuits of the

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brain, while being of prime scientific importance, represents an even more challenging problem given that speech is a unique human trait. Nevertheless, in recent years, progress has been made by studying families where impairment of speech-associated processes is inherited in a monogenic fashion. It has thus been possible to pinpoint genes that constitute key entry points into the molecular networks underlying such processes (1,2). In particular, rare loss-of-function mutations in the *FOXP2* gene (OMIM 605317) can cause a severe speech and language disorder (OMIM 602081) (3–5). *FOXP2*-related disorder is characterized by difficulties in mastering sequences of oral and facial movements, impairing speech articulation (developmental verbal dyspraxia, DVD), accompanied by additional problems with expressive and receptive language. *FOXP2* belongs to a family of transcription factors that have crucial functions in a wide range of physiological processes and that are characterized by a forkhead-box (FOX) DNA-binding domain (6). In addition to the generation and characterization of various animal models (7–12), subsequent studies have focussed on the identification of *FOXP2*-targeted promoter regions (13,14). At least some putative *FOXP2*-regulated genes are likely to represent important players in speech-associated pathways. Indeed, a recent study found that *FOXP2* directly regulates the *CNTNAP2* gene (OMIM 604569) which encodes a contactin-associated protein of the neurexin superfamily and showed that polymorphisms in this target gene are associated with specific language impairment (15).

Human genetic analyses have also implicated mutations of the sushi-repeat protein *SRPX2* (OMIM 300642) in a range of speech-related disorders. These include epileptic (rolandic/sylvian epilepsy), functional (DVD) and developmental (bilateral perisylvian polymicrogyria) disorders of the speech areas of the cortex (OMIM 300643, 300388) (16). Several proteins that physically interact with *SRPX2* have then been identified, suggesting the involvement of the extracellular matrix proteolysis machinery in the pathology and physiology of speech-associated circuits. Most notably, *SRPX2* was found to directly interact with *uPAR* (OMIM 173391), the plasminogen activator receptor of the urokinase type (also known as *PLAUR*), and the *SRPX2/uPAR* ligand–receptor interaction showed quantitative modification caused by a pathogenic mutation of *SRPX2* (17).

Interestingly, the *uPAR* promoter region was identified among the top 100 potential *FOXP2* targets in a high-throughput chromatin-immunoprecipitation microarray screen (ChIP-chip) using human neuronal models (14). Given that *FOXP2* and *SRPX2* mutations cause related disorders of speech processing and associated brain areas, this observation raised the exciting possibility of a functional link between the *FOXP2* transcription factor on the one hand and the *SRPX2/uPAR* complex on the other hand. In the present study, we show that human *FOXP2* down-regulates both the *SRPX2* and the *uPAR* genes and that this transcriptional regulation is lost when *FOXP2* bears a pathogenic p.R553H mutation that is known to cause DVD. Moreover, we report the discovery of a new *FOXP2* coding mutation in a patient with polymicrogyria of the left rolandic operculum and demonstrate that it partially impairs the proper *FOXP2* regulation of *SRPX2* promoter activity. Our study thus

identifies a novel genetic and regulatory network that is altered in disorders affecting speech processing and functioning/development of speech-related brain areas.

RESULTS

Bioinformatic detection of FOX, FOXP and FOXP2 consensus-binding sites in *SRPX2* and *uPAR* promoters

FOX transcription factors bind to consensus DNA sites in the *cis* regulatory regions of their target genes. Consensus-binding sites with increased levels of specificity have been defined for transcription factors of the general *FOX* family (*FOX* sites: TRTTKRY, where R = A or G, K = T or G and Y = T or C), for members of the *FOXP* subfamily (*FOXP* sites: TATTTT) and for *FOXP2* itself (*FOXP2* sites: AATTTG and ATTTGT). The *FOXP2* consensus sequence ATTTGT is contained within the *FOXP* sites and the *FOXP* sites also conform to the more general *FOX* sites (18). The promoter regions of the human *SRPX2* and of the human *uPAR* genes were screened *in silico* for the presence of *FOX*, *FOXP* and *FOXP2* sites, including approximately 1.5 kb of DNA sequence 5' to each corresponding and canonical transcription start site (TSS) (*SRPX2*: GenBank accession no. NM_014467; *uPAR*: GenBank accession no. NM_002659). *In silico* and 5'-RACE (rapid amplification of cDNA ends) polymerase chain reaction (PCR) experiments confirmed that the *SRPX2* and *uPAR* brain transcripts detected so far did not display any alternative and more distal 5' TSS (data not shown). Several consensus sequences of various types (*FOX*, *FOXP*, *FOXP2*) were identified in the promoter regions of both genes (Table 1, Supplementary Material, Fig. S1). Seven sites [*SRP1*–*SRP7*, from 3' to 5' of the (+) strand] were identified for *SRPX2*: *SRP1*, *SRP2*, *SRP5* and *SRP7* were of the *FOX* type and *SRP3*, *SRP4* and *SRP6* fitted the consensus for *FOXP2*. Six sites (*UP1*–*UP6*) were detected for *uPAR*: *UP1*–*UP3* were of the *FOX* type and *UP4*–*UP6* were of the *FOXP* type. Some of these sites (*SRP1*, *UP2*, *UP5* and *UP6*) were composed of more than one consensus sequence. The presence of such consensus-binding sites *in silico* was thus consistent with a possible regulation of *SRPX2* and of *uPAR* by transcription factors of the *FOX* family. Notably, comparative searches of those consensus-binding sites (*SRP1*–7 and *UP1*–6, respectively) in the *SrpX2* and *UpAR* promoter sequences from chimpanzee and mouse revealed subtle to dramatic differences with their human promoter counterparts, with the most obvious evolutionary modification being the lack of any of the *UP1* to *UP6* consensus forkhead binding site in the mouse *UpAR* promoter region (Supplementary Material, Fig. S1).

FOXP2 expression leads to a significant decrease in native *SRPX2* and *uPAR* transcripts

The possible regulation of *SRPX2* and *uPAR* by *FOXP2* was then addressed by measuring by quantitative RT–PCR (qRT–PCR) the expression of these genes in the presence of exogenous human *FOXP2* protein encoded by the major transcript of *FOXP2* (isoform 1; GenBank accession no. NM_014491). When HEK293T cells were transfected with a

Table 1. *In silico* detection of FOX, FOXP and FOXP2 consensus-binding sites

	Sequence ^a	Consensus-binding site ^b			Position (nt) ^c
		FOX	FOXP	FOXP2	
<i>SRPX2</i>					
SRP1 ^d	TGTTTGTgagTGTTTAT	+	–	–	–110; –120
SRP2	GTAAACA	+	–	–	–305
SRP3	AATTG	–	–	+	–664
SRP4	TATTGT	+	+	+	–767
SRP5	TGTTGGC	+	–	–	–919
SRP6	TATTGT	+	+	+	–1260
SRP7	TGTTGAT	+	–	–	–1373
<i>uPAR</i>					
UP1	TATTAC	+	–	–	–131
UP2 ^e	ACAAACAAACAAACA	+	–	–	–693; –697; –701
UP3	ATCAATA	+	–	–	–746
UP4	ATAAATA	+	+	–	–832
UP5 ^e	TATTATTTATTAT	+	+	–	–1160; –1164; –1168
UP6 ^f	ATAAATAAATAAATAAATA	+	+	–	–1319; –1323; –1327; –1331

The promoter regions of the human *SRPX2* and *uPAR* genes were screened *in silico* for the presence of FOX, FOXP and FOXP2 consensus-binding sites (Supplementary Material, Fig. S1). nt, nucleotide.

^aCore binding sites are represented in capital letters.

^b(+) indicates the presence of a consensus-binding site and (–) indicates the absence of a consensus-binding site.

^cPosition relative to the canonical TSS.

^dTwo non-overlapping sites.

^eThree overlapping sites.

^fFour overlapping sites.

construct expressing recombinant FOXP2 protein, *SRPX2* mRNA levels were significantly reduced (43.6% decrease, $P < 0.05$, two-tailed unpaired *t*-test) across replicate experiments when compared with control cells transfected with the corresponding non-recombinant vector (Fig. 1). Similarly, the amounts of *uPAR* transcripts also showed significant and replicated decrease (38.6% decrease, $P < 0.01$, two-tailed unpaired *t*-test) in FOXP2-transfected cells (Fig. 1). This indicated the existence of a functional link between FOXP2 and both the *SRPX2* and the *uPAR* genes.

FOXP2 represses *SRPX2* and *uPAR* promoters

We next tested whether expression of FOXP2 could modify the activities of the *SRPX2* and of the *uPAR* promoters by using a luciferase reporter assay. The promoter regions of each gene as defined above were subcloned into the appropriate vector (pGL3) 5' to the coding sequence of the firefly luciferase reporter gene. Either construct (pGL3-*SRPX2*, pGL3-*uPAR*) was cotransfected with a FOXP2-containing vector (pcDNA4/HisMax-FOXP2) or with an empty (pcDNA4/HisMax) vector and with a β -galactosidase expression vector (pHSV-LacZ) for the normalization of transfection efficiencies. Significant and replicated repression of the *SRPX2* promoter by FOXP2 protein was detected in pcDNA4/HisMax-FOXP2 transfected cells when compared with pcDNA4/HisMax-transfected cells (80.2% decrease in luciferase expression, $P < 0.0005$, two-tailed unpaired *t*-test) (Fig. 2A). As a positive control, FOXP2 was also found to repress the SV40 promoter (data not shown), as already described (18). Using the same experimental procedure, dramatic and reproducible repression (77.5% decrease in luciferase expression, $P < 0.0005$, two-tailed unpaired *t*-test) of the

uPAR promoter by FOXP2 was shown (Fig. 2B). Together with the qRT-PCR data, the reporter assays shown here demonstrated the ability of the human FOXP2 protein to repress the activities of *SRPX2* and *uPAR* promoters.

FOXP2 directly binds to consensus sites in the *SRPX2* and *uPAR* promoters

Repression of the *SRPX2* and *uPAR* promoter activities and transcript expressions by FOXP2, and the presence of FOX, FOXP and FOXP2 consensus-binding sites within the promoter regions of *SRPX2* and of *uPAR*, strongly suggested that FOXP2 could directly bind to these promoter regions. We tested this by carrying out electrophoretic mobility shift assays (EMSA) for the 13 consensus-binding sites (SRP1-7 and UP1-6) that had been detected *in silico* in the *SRPX2* and *uPAR* promoter sequences (Supplementary Material, Fig. S1 and Table S1). Nuclear extracts were prepared from HEK293T cells transfected with the FOXP2 expression construct (pcDNA4/HisMax-FOXP2). These extracts were able to bind to a positive control consensus FOXP2 site as previously shown (18) and to DNA probes corresponding to the UP2 and UP6 sites of the *uPAR* promoter region, as well as to the SRP1 and SRP2 sites of the *SRPX2* promoter region (Fig. 3, Supplementary Material, Fig. S2). In contrast, no binding was observed when using nuclear extracts prepared from HEK293T cells transfected with an empty construct (pcDNA4/HisMax; line pcDNA4 in each panel of Fig. 3). The gel shifts that were observed with SRP1, SRP2, UP2 and UP6 probes were due to binding by FOXP2 protein, since addition of an N-terminal FOXP2 antibody caused larger complexes or 'supershifts' to occur (line FOXP2Ab in each panel of Fig. 3). By comparison, no supershift was

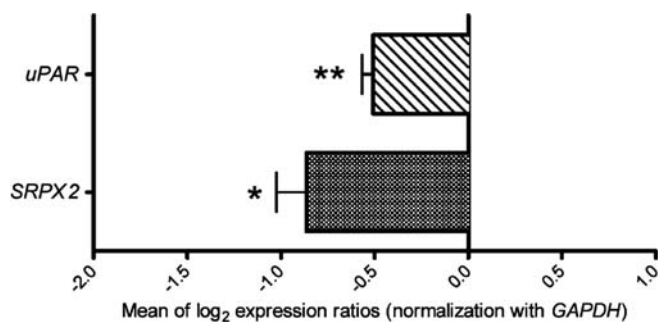


Figure 1. qRT-PCR experiments. Wild-type FOXP2 protein down-regulates the native *SRPX2* and *uPAR* genes. qRT-PCR experiments were performed from HEK293T cells transfected with pcDNA4/HisMax-FOXP2 construct. Expression changes are given as the mean of log₂ expression ratios of cells transfected with pcDNA4/HisMax-FOXP2 compared with cells transfected with non-recombinant pcDNA4/HisMax vector and are normalized for equal expression of the *GAPDH* internal control. Values represent the mean of comparisons of five independent cDNA syntheses. *P*-values were calculated using two-tailed unpaired *t*-test. **P* < 0.05 and ***P* < 0.01.

obtained when a non-specific IgG antibody was used. Binding to either of the SRP1, SRP2, UP2 and UP6 probes was specific, as demonstrated by competitive impairment with each corresponding unlabeled probe (lines UPwt2, UPwt6, SRwt1, SRwt2 in Fig. 3). Moreover, mutant (Supplementary Material, Table S1) and unlabeled forms of SRP1, SRP2, UP2 and UP6 probes (lines UPmut2, UPmut6, SRmut1, SRmut2 in Fig. 3) failed to compete with the FOXP2-binding abilities of their labeled and wild-type counterparts, as also shown for an irrelevant NFK unlabeled probe. Altogether, these data demonstrated efficient specific binding of FOXP2 to consensus sites situated within the promoter regions of both the *SRPX2* and *uPAR* genes.

Pathogenic mutation of FOXP2 disrupts its functional links with *SRPX2* and *uPAR*

Rare mutations in transcription factor FOXP2 lead to DVD (3–5); hence, targets downstream to FOXP2 are obvious candidates for being involved in pathophysiological mechanisms. Based on the data presented above, *SRPX2* and *uPAR* may be considered particularly strong candidates because of the demonstrated relationship between the *SRPX2/uPAR* complex and disorders of the speech cortex, including DVD itself (13,14). This question was addressed by studying the most well-characterized aetiological mutation of FOXP2 (p.R553H), assessing its functional effects on the *SRPX2* and *uPAR* genes. p.R553H is a pathogenic substitution in the DNA-binding domain of the FOXP2 protein, found in all 15 affected members of an extensively studied multigenerational family segregating speech and language disorder (the KE family) (3).

qRT-PCR experiments showed that, in contrast to wild-type FOXP2, p.R553H mutant FOXP2 showed a significant and complete loss of repression of native *SRPX2* and *uPAR* expression (*P* < 0.001 and *P* < 0.01, respectively, two-tailed unpaired *t*-test) (Fig. 4A). Luciferase assays yielded consistent data; the p.R553H mutation led to significant losses of repression of *SRPX2* and *uPAR* promoters when compared with

wild-type FOXP2 (*P* < 0.0001 for each, two-tailed unpaired *t*-test) (Fig. 4B). There even was increased activity (*P* < 0.0001, two-tailed unpaired *t*-test) of the *SRPX2* promoter in the presence of mutant p.R553H FOXP2 (Fig. 4B), when compared with cells transfected with a pcDNA4/HisMax empty vector. Overall, these experiments demonstrated a strong reduction in the capacity of mutant p.R553H FOXP2 protein to down-regulate *SRPX2* and *uPAR*.

Loss of down-regulation of *SRPX2* and of *uPAR* by mutant FOXP2 could be partly due to disrupted nuclear localization (18). Indeed, alteration of FOXP2 nuclear targeting was confirmed in the present study by immunocytochemistry with Xpress epitope antibody after transfection of HEK293T cells with tagged constructs allowing expression of wild-type or p.R553H FOXP2 (Fig. 5). While increased cytoplasmic localization was clearly detected when compared with wild-type FOXP2, a proportion of mutant p.R553H FOXP2 protein still showed nuclear localization, consistent with previously published data (18). FOXP2 DNA-binding capabilities can be affected by the p.R553H substitution (18) and this question was thus more specifically addressed with respect to the *SRPX2* (SRP1 and SRP2) and *uPAR* (UP2 and UP6) binding sites using the same EMSA procedure as described above. Mutant p.R553H FOXP2 not only displayed reduced binding to a previously defined consensus site used as a control (18), but also showed unambiguous impairment (of variable degree) in its ability to bind to the consensus SRP1, SRP2, UP2 and UP6 target sites in the promoter regions of *SRPX2* and of *uPAR* (Fig. 3).

A novel FOXP2 missense mutation in a patient with left opercular polymicrogyria partially impairs regulation of *SRPX2*

The regulatory link between FOXP2 and *SRPX2* prompted us to screen for *FOXP2* mutations in a series of 32 patients presenting with disorders of the speech cortex that can be caused by *SRPX2* mutations [rolandic epilepsy (RE) with speech impairment, perisylvian polymicrogyria] or with other disorders of the same clinical spectrum [continuous spike-and-waves during sleep (CSWS) and Landau-Kleffner syndromes] (19). One heterozygous missense mutation (c.T1591C) of *FOXP2* was found in a girl of Turkish origin presenting with focal epilepsy with CSWS and cognitive and language deficits. Magnetic resonance imaging (MRI) showed polymicrogyria of the left rolandic operculum (Fig. 5A and B). Her father also carried the mutation (Fig. 5B) but did not have any neurological problem and had normal MRI (data not shown), as verified by three independent and experienced radiologists and neuropaediatricians. The proband's sister and brother also had the mutation (data not shown). Their clinical phenotype could not be fully assessed and no MRI was performed, but no obvious neurological abnormality was noted. No compound heterozygous mutation was found in the patient and her mother and their karyotypes were normal; comparative genomic hybridization analysis of the patient's DNA using Agilent 60K oligonucleotides array did not detect any abnormality in the FOXP2 genomic area (data not shown). The mutation replaced a highly conserved methionine residue with a threonine

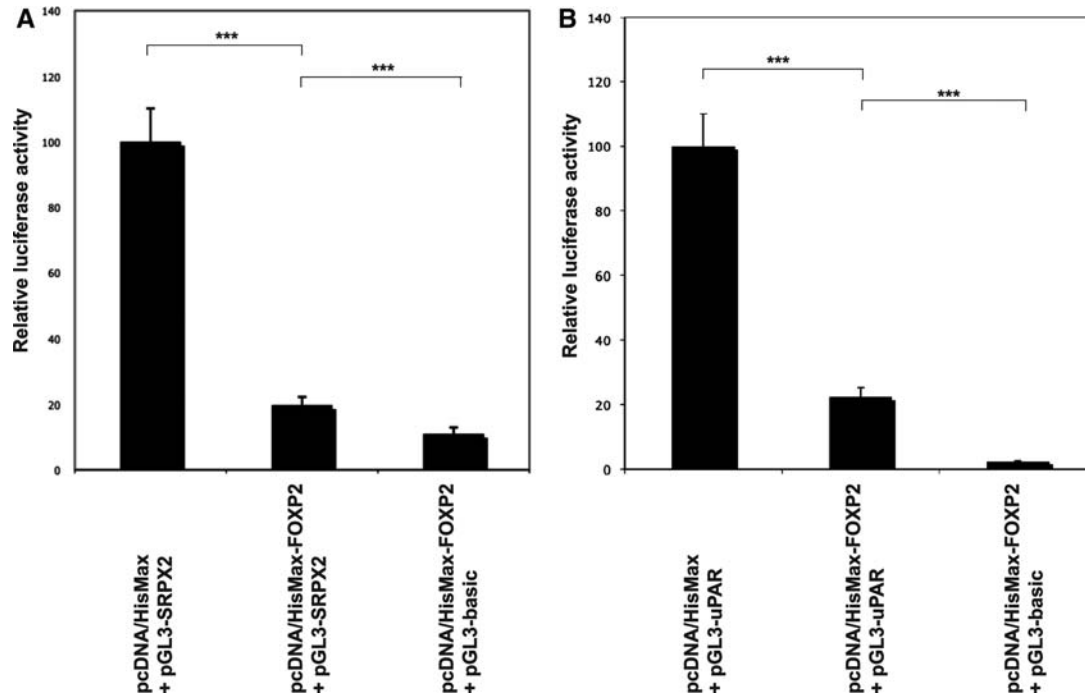


Figure 2. Luciferase reporter assays. Wild-type FOXP2 protein down-regulates the activities of the *SRPX2* (A) and of the *uPAR* (B) promoters. Either of the *SRPX2* (pGL3-SRPX2) and *uPAR* (pGL3-uPAR) promoter constructs was co-transfected into HEK293T cells with FOXP2-containing (pcDNA4/HisMax-FOXP2) or with empty (pcDNA4/HisMax) vector and with a β -galactosidase expression vector (pHSV-LacZ) for the normalization of transfection efficiencies. In parallel, an empty (promoterless) vector (pGL3-basic) was transfected with pcDNA4/HisMax-FOXP2 and with pHSV-LacZ. Transcriptional activities were determined by quantifying the luciferase activity of cellular extracts prepared 48 h after transfection. Data show the mean \pm SD relative activity from three experiments done in triplicate. Statistical significances were determined by two-tailed unpaired *t*-test. ****P* < 0.0005.

(p.M406T) within the leucine-zipper domain of FOXP2 and was neither found in the SNP database (<http://www.ncbi.nlm.nih.gov/snp> at NCBI) nor in 222 unrelated control individuals (33 of Turkish origin + 189 Caucasians). In analyses of HEK293T cells transfected with tagged mutant constructs, p.M406T led to partial alteration in the nuclear localization of FOXP2 (Fig. 5C). Luciferase reporter assays were then conducted and showed that the p.M406T mutation led to significant loss of repression of the *SRPX2* promoter, when compared with wild-type FOXP2 (*P* < 0.0001, two-tailed unpaired *t*-test) (Fig. 5D). In contrast to p.R553H (Fig. 4B), p.M406T FOXP2 partially retained the ability to repress *SRPX2* promoter activity and had no effect on the repression of *uPAR* promoter activity (Fig. 5D).

DISCUSSION

The present study characterized a novel molecular regulatory network associated with the disorders of cortical speech areas and of speech processing. In particular, we demonstrated the direct down-regulation of *SRPX2*, a gene implicated in X-linked RE associated with DVD (RESDX syndrome) or with bilateral perisylvian polymicrogyria (BPP) (16), by the FOXP2 transcription factor, mutated in people with autosomal dominant DVD (3–5). Similarly, FOXP2 also down-regulated the *uPAR* gene, which encodes a receptor of SRPX2, which in turn displays a modified interaction with the mutant SRPX2 protein (17). Hence, our study shows that FOXP2 co-regulates the expression of the ligand

SRPX2 and of its receptor, uPAR. That there is connectivity between FOXP2 targets had already been suggested (14); as far as we are aware, this is the first clear demonstration of regulated targets with a direct interaction between them. Our findings are also in good agreement with the usual down-regulating effects of FOXP2 on its direct targets as identified by CHIP-chip experiments (13,14); furthermore, whereas the *SRPX2* promoter region was not present on the microarrays used in these previous studies, *uPAR* was detected among the 100 most significant FOXP2 targets in human neuronal models (14).

Wild-type FOXP2 was able to bind to several consensus sites in the promoter regions of *SRPX2* (SRP1 and SRP2) and *uPAR* (UP2 and UP6). None of these four sites corresponded to the canonical and specific FOXP2 consensus sequences; instead, SRP1, SRP2 and UP2 were of the FOX type and UP6 of the FOXP type. The binding of FOXP2 to less stringent FOX or FOXP sites has already been observed (14). Among the four FOXP2 binding sites studied here, three (SRP1, UP2 and UP6) consisted of more than one fork-head consensus sequence; this situation would be consistent with the binding of FOXP2 through homo- or heterodimerization, as already proposed (20), and may also favor cross-binding to FOX and FOXP consensus sites. Both the regulations of *SRPX2* and of *uPAR* were abolished by a p.R553H FOXP2 mutation that is known to cause DVD (3); the expression of p.R553H FOXP2 even led to significant increases in the activity of the *SRPX2* promoter when compared with the control situation. This latter result is in line

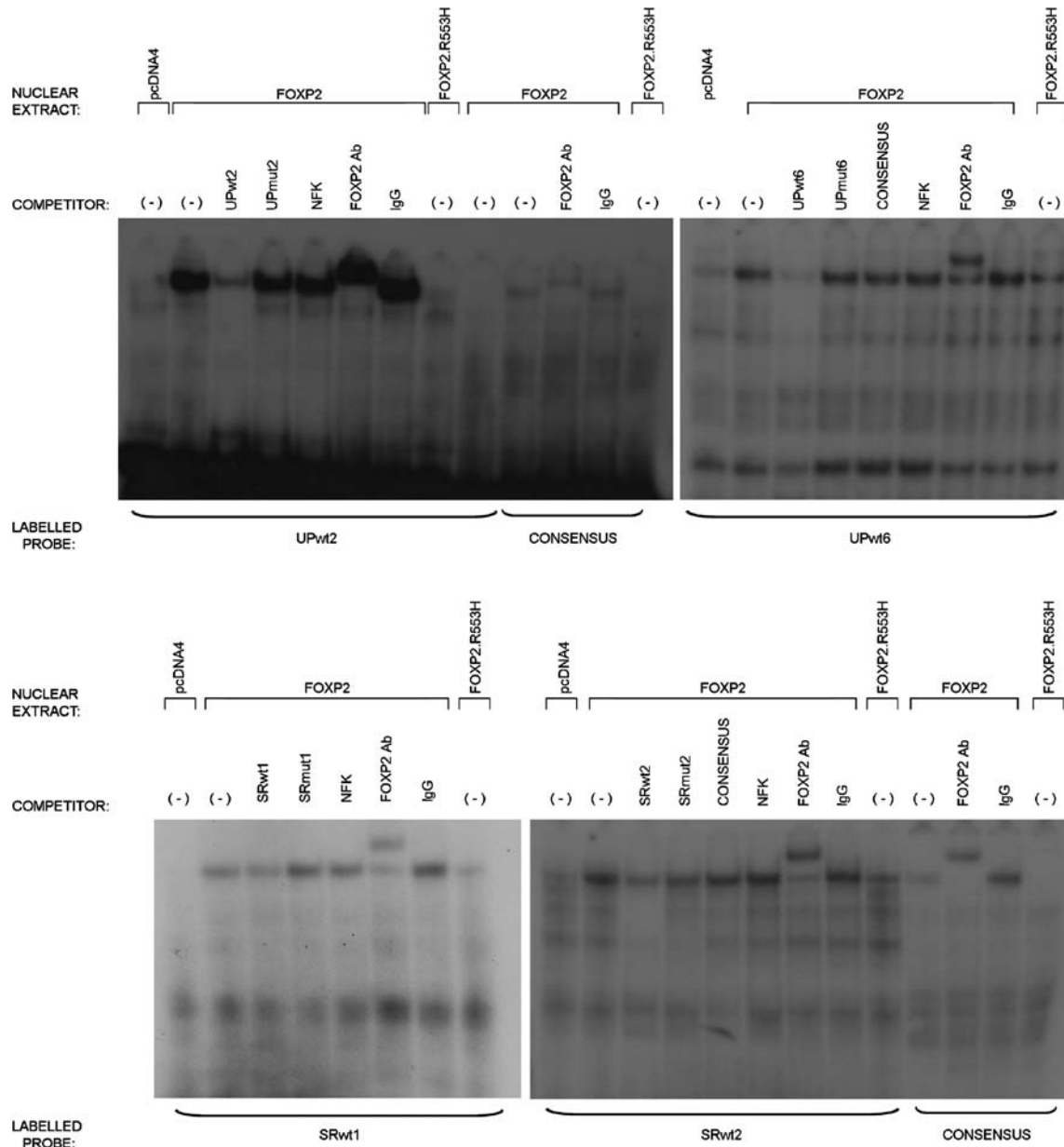


Figure 3. Electrophoretic mobility shift assays. FOXP2 directly binds sites identified within the *SRPX2* and *uPAR* promoters. EMSAs were used to determine whether direct interactions occurred between FOXP2 and the promoter regions of the *SRPX2* and *uPAR* genes. DNA probes were designed based on the presence of predicted FOX, FOXP and FOXP2-binding sites (Table 1, Supplementary Material, Table S1). Radiolabeled probes that had shown binding in earlier experiments (Supplementary Material, Fig. S2) were incubated with nuclear extracts taken from HEK293T cells transfected with either an empty vector (pcDNA4), wild-type FOXP2 or FOXP2.R553H. The binding of FOXP2 to a known consensus binding site (CONSENSUS) is shown as a positive control. Binding assays were also performed in the presence of competition from unlabeled wild-type (wt), mutant (mut) and irrelevant (NFK) probes. In each case, FOXP2 binding to the labeled wild-type probe was efficiently impaired via competition with unlabeled wild-type competitor probe, but not by mutant or irrelevant competitor probes, displaying the specificity of the interaction. Addition of an antibody directed to FOXP2 (FOXP2 Ab) caused a supershift to occur in each case, an effect not observed when an irrelevant control antibody (IgG) was added. This confirmed that the identity of the protein causing gel retardation is indeed FOXP2.

with the previous investigations of the p.R553H mutant form using the SV40 promoter as a reporter (18) and may be due to the interference of the mutant protein with the transactivation capabilities of endogenous FOXP proteins expressed in HEK293T cells. Consistent with the losses of regulation of *SRPX2* and of *uPAR*, mutant FOXP2 also showed partial alteration in nuclear localization and disrupted binding to the *SRPX2* and *uPAR* target sites. Altogether, these data argued

in favor of a pathogenic involvement of the FOXP2-*SRPX2*/*uPAR* regulatory link.

The data presented here demonstrated the existence of a novel regulatory and functional link, FOXP2-*SRPX2*/*uPAR*, displaying impairment in various disorders involving speech-related areas and networks. These findings are reminiscent of the recent functional link that was found between FOXP2 and the gene coding for the contactin associated

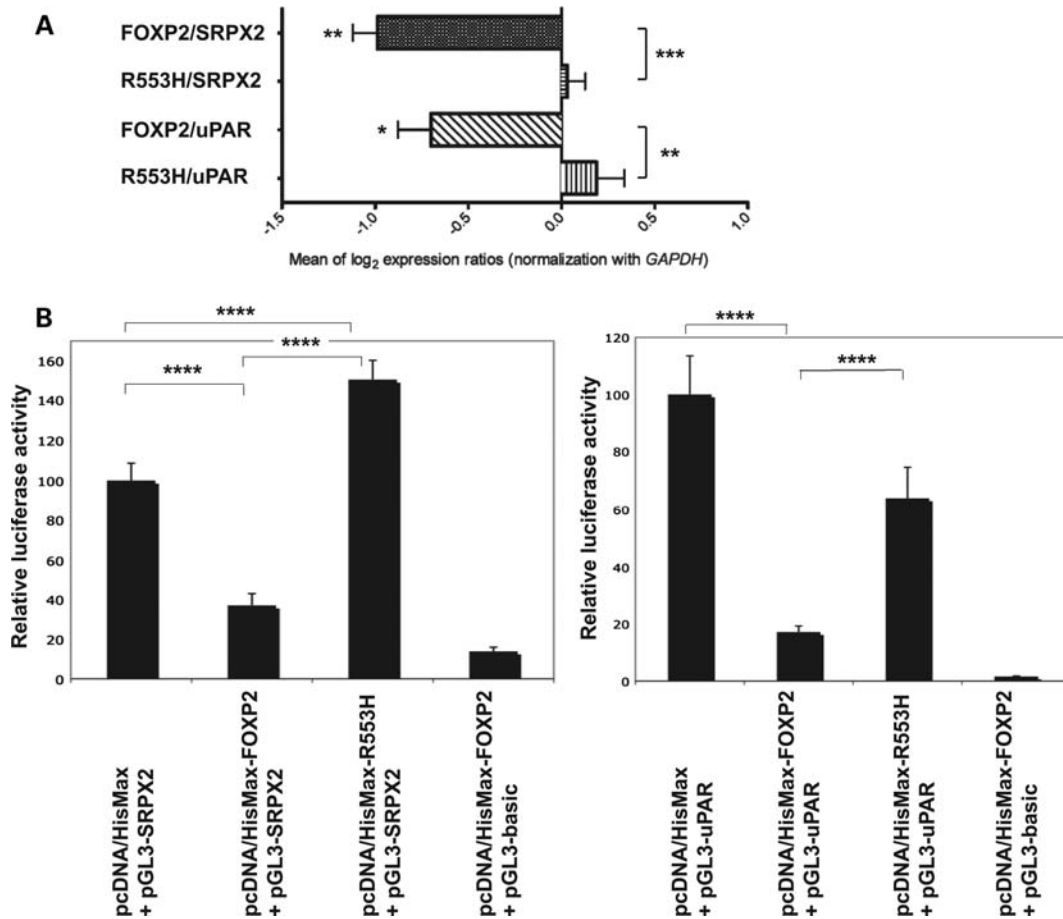


Figure 4. Regulation of, and binding on, the *SRPX2* and *uPAR* promoters is altered by a pathogenic p.R553H FOXP2 mutation. (A) qRT-PCR experiments showing loss of down-regulation of the native *SRPX2* and *uPAR* expressions by mutant FOXP2. Expression changes are given as the mean of log₂ expression ratios of HEK293T cells transfected with pcDNA4/HisMax-FOXP2 (wild-type FOXP2) or with pcDNA4/HisMax-R553H (mutant p.R553H FOXP2), when compared with HEK293T cells transfected with non-recombinant pcDNA4/HisMax vector, and are normalized for equal expression of the *GAPDH* internal control. Values represent the mean of comparisons of five independent cDNA syntheses. *P*-values were calculated using two-tailed unpaired *t*-test. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. (B) Luciferase reporter assays showing loss of down-regulation of the *SRPX2* and *uPAR* promoter activities by mutant p.R553H FOXP2. Either of the *SRPX2* (pGL3-SRPX2) and *uPAR* (pGL3-uPAR) promoter constructs was co-transfected into HEK293T cells with FOXP2-containing (pcDNA4/HisMax-FOXP2 or pcDNA4/HisMax-R553H) or with empty (pcDNA4/HisMax) vector and with a β-galactosidase expression vector (pHSV-LacZ) for the normalization of transfection efficiencies. In parallel, the empty (promoterless) vector (pGL3-basic) was transfected with pcDNA4/HisMax-FOXP2 and with pHSV-LacZ. Transcriptional activity was determined by quantifying the luciferase activity of cellular extracts prepared 48 h. after transfection. Data show the mean ± SD relative activity from three experiments done in triplicate. Statistical significances were determined by two-tailed unpaired *t*-test. *****P* < 0.0001.

protein-like 2, *CNTNAP2* (15). Whereas FOXP2 mutations cause DVD accompanied by expressive and receptive language deficits, *CNTNAP2* shows genetic association not only with specific language impairment (15) but also with autism (21–23) and with epilepsy and schizophrenia (24). Moreover, a homozygous *CNTNAP2* mutation in Amish children has been shown to cause focal epilepsy followed by language regression, behavioral problems and mental retardation (25). Hence, both the FOXP2-*CNTNAP2* and the FOXP2-*SRPX2/uPAR* regulatory pathways make direct and functional molecular links between various disorders of the brain that are diagnostically distinct, but are well known to share clinical, epidemiological and neurobiological features. Epilepsy, autism, DVD and specific language impairment may well rely on partially overlapping molecular networks. More precisely, there is long known and increasing evidence

for a connection between idiopathic focal epilepsies—RE particularly—on the one hand and cognitive, attention, speech and reading disabilities on the other hand (26–29). FOXP2 regulates the expression of both components of the SRPX2/*uPAR* complex involved in epileptic seizures of the rolandic area; interestingly, the centrotemporal electroencephalographic hallmark of RE has shown increased frequency in patients with DVD (30). To the best of our knowledge, no rolandic seizure has ever been reported in people with FOXP2 mutations. When compared with mutations in SRPX2, mutations in FOXP2 very likely disrupt the regulation of many more pathways that may have modulatory effects on the susceptibility to epileptic seizures.

A novel p.M406T FOXP2 heterozygous mutation was found in a girl with developmental defects of the speech cortex, i.e. polymicrogyria of the left rolandic operculum, hence recalling

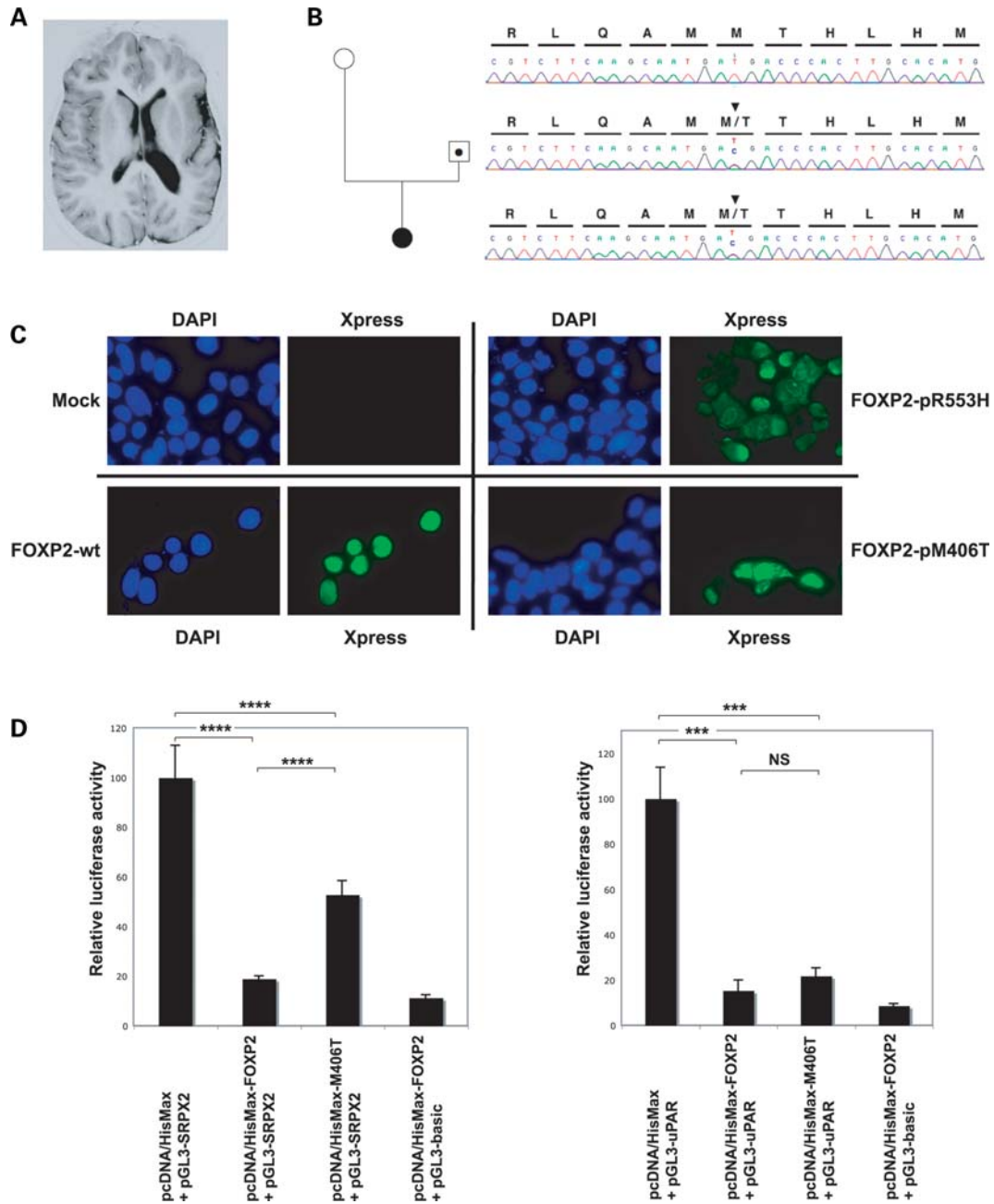


Figure 5. Novel p.M406T FOXP2 mutation in a patient with polymicrogyria of the left rolandic operculum partially impairs proper regulation of *SRPX2*. (A) MRI (axial inversion recovery section) of the patient with left opercular polymicrogyria. (B) c.T1591C mutation of *FOXP2*. The nucleotide sequences and translations are shown above the direct sequencing trace from PCR-amplified fragment. *Top*: Section of a wild-type sequence in the unaffected mother (white circle). *Middle*: Section of a sequence with the mutation in the carrier father (dotted square). *Bottom*: Section of a sequence with the mutation in the affected proband (full-blackened circle). Two more siblings were carriers, but did not show obvious neurological problems, although no MRI was performed (sequence traces not shown). (C) p.R553H and p.M406T mutant FOXP2 proteins display altered intracellular localizations. HEK293T cells were transfected with pcDNA4/HisMax (mock) vector (top, left), with pcDNA4/HisMax-FOXP2 (wt) vector (bottom, left), with pcDNA4/HisMax-R553H (p.R553H) vector (top, right) or with pcDNA4/HisMax-M406T (p.M406T) vector (bottom, right). Wild-type FOXP2 displayed unambiguous nuclear localization. Mutant p.R553H FOXP2 showed both nuclear and cytoplasmic localizations, as already described (18), and p.M406T FOXP2 also showed altered nuclear targeting. HisMax-tag fusion protein FOXP2 was detected using an antibody to the N-terminal XpressTM tag (green). Blue: nuclear DAPI staining. (D) Luciferase reporter assays showing partial loss of down-regulation of the *SRPX2* promoter activity by mutant p.M406T FOXP2. Activity of the *uPAR* promoter remained unchanged. Either of the *SRPX2* (pGL3-SRPX2) and *uPAR* (pGL3-uPAR) promoter constructs was co-transfected into HEK293T cells with FOXP2-containing (pcDNA4/HisMax-FOXP2 or pcDNA4/HisMax-M406T) or with empty (pcDNA4/HisMax) vector and with a β -galactosidase expression vector (pHSV-LacZ) for the normalization of transfection efficiencies. In parallel, an empty (promoterless) vector (pGL3-basic) was transfected with pcDNA4/HisMax-FOXP2 and with pHSV-LacZ. Transcriptional activity was determined by quantifying the luciferase activity of cellular extracts prepared 48 h after transfection. Data show the mean \pm SD relative activity from three experiments done in triplicate. Statistical significances were determined by two-tailed unpaired *t*-test. NS, not significant ($P > 0.05$), *** $P < 0.001$ and **** $P < 0.0001$.

an SRPX2-related phenotype. The proband's father carried the mutation but did not manifest the disease and had normal MRI; this obviously argues against a simple causal role. Two other sibs also carried the mutation but their phenotype could not be ascertained and no MRI was done. This situation is reminiscent of that observed in other genetic cases: for instance, *SHANK3* mutations can be found in patients with autistic syndrome disorder and their unaffected relatives (31). Nevertheless, several arguments account for a likely participation of the p.M406T FOXP2 mutation as a susceptibility factor to the polymicrogyria reported here. First, functional MRI abnormalities of various regions, including Broca's area in the inferior frontal gyrus, have already been detected in people carrying FOXP2 mutations (32). The aforementioned FOXP2 target, *CNTNAP2*, shows enriched expression in frontal gray matter of human fetal brain tissue (33), and FOXP2 itself displays high levels of expression in the developing perisylvian cortex (34). Secondly, p.M406T was neither found in 222 control individuals nor in available databases of human variation. Furthermore, the methionine residue that is mutated is highly conserved across evolution, and p.M406T occurred in a leucine zipper domain that is crucial for the dimerization and subsequent DNA binding of FOXP2 (20). Thirdly, the present study showed that there is a direct functional link between FOXP2 and the SRPX2/uPAR complex that in turn has been implicated in bilateral perisylvian polymicrogyria that predominated on the left side (16,17). More importantly, p.M406T was shown here to have functional consequences *in vitro*: it not only led to altered pattern of nuclear localization of FOXP2, but also to significantly reduced, albeit still active, regulation of the *SRPX2* promoter activity; interestingly also, the promoter activity of *uPAR* remained unchanged. Hence, the effect of p.M406T was more subtle than that of p.R553H. This is consistent with the difference in the genetic influences associated with those two mutant FOXP2 proteins. p.M406T retained partial functionality and the effects of this risk factor are likely to be modulated by genomic background, environmental influences and stochastic developmental events, hence leading to incomplete penetrance in the pedigree reported here. Although the existence of compound heterozygous mutation cannot be firmly excluded, no other *FOXP2* mutation was found in the patient and her mother and conventional as well as molecular cytogenetics did not reveal any gene rearrangement. Moreover, homozygous and compound heterozygous mice lacking functional *Foxp2* not only have severe developmental delays and general motor dysfunction but also do not survive beyond 3 weeks after birth (10).

FOXP2 shows evidence of recent positive selection (35). *FOXP2* underwent two non-synonymous changes since the human–chimpanzee split and cell-based studies suggest that several FOXP2 target genes may be differentially regulated by human and chimpanzee versions of the protein (36). *SRPX2* also exhibited an accelerated rate of non-synonymous substitutions in the human lineage; although there was insufficient statistical power to demonstrate the action of positive selection (37), the human-specific *SRPX2* evolutionary change was associated with modified interaction with uPAR (17). From this evolutionary viewpoint, differences in the regulation of expression of *SRPX2* and of *uPAR* by FOXP2

in different species might have been important. The SRP2 consensus site is conserved in the mouse and chimpanzee *Srpx2* promoters. In contrast, SRP1, while being slightly different between the human and chimpanzee promoters, was not detected in the mouse *Srpx2* promoter. Interestingly, the UP2 and UP6 FOXP2-binding sites of the human *uPAR* promoter region were conserved between human and chimpanzee but did not exist at all in the promoter region of murine *Upar*. Although preliminary, these data may suggest that the regulations by FOXP2 of *uPAR* and of *SRPX2* have appeared or have been modified during evolution since the rodent–primate split.

Generally, the functioning and development of brain circuits underlying human speech result from complex sequential processes that must be tightly regulated. The emerging picture arising from the study of various speech-related disorders (RE, DVD, BPP) is that of a complicated and intertwined network of regulation and interaction comprising *FOXP2*, *uPAR*, *SRPX2* as well as *CNTNAP2*. Moreover, the situation is likely to be considerably more complex: FOXP2 certainly has many more functional targets (13,14), and *uPAR* (38,39) as well as *SRPX2* (40,41) expression can be modulated by other transcription factors. In addition to uPAR, SRPX2 itself has many more possible partners (17), some of which such as FBN1 (Fibrillin-1) or PCSK6 (Subtilisin-like proprotein convertase 4, PACE4) also showed significant enrichment after ChIP-chip FOXP2 experiments (14). It is thus expected that alterations of several other molecular pathways will be found in future studies of speech-related syndromes. As such, it will be crucial to identify and study each of them, and then try to integrate how and when they may interfere with each other and with the development/functioning of speech-related areas and networks. From this viewpoint, the identification of the FOXP2-*SRPX2*/*uPAR* functional and genetic link, and its alteration by a FOXP2 pathogenic mutation, represent important entry points for deciphering the complicated regulatory networks of molecules that go awry in speech-related disorders. Together with recently described FOXP2-*CNTNPA2* genetic and SRPX2/uPAR proteomic links, the present findings make novel genetic and molecular links between distinct phenotypes that share clinical, epidemiological and neurobiological features, including autism, epilepsy of speech-related areas and developmental speech and language disorders.

MATERIALS AND METHODS

In silico analyses and 5'-RACE experiments

The promoter regions of the human *SRPX2* (GenBank accession no. NM_014467) and *uPAR* (GenBank accession no. NM_002659) genes were taken from the UCSC human genome browser web site (March 2006 human reference sequence, NCBI Build 36.1; <http://genome.ucsc.edu/>) and were screened *in silico* for the presence of FOX, FOXP and FOXP2 consensus-binding sites within 1.5 kb of DNA sequence 5' to each canonical TSS and using the DNA Pattern Find program of the Sequence Manipulation Suite (42) at http://bioinfo.unice.fr/software/sms2/dna_pattern.html. The possible existence of alternative transcripts with more distal

5' TSS was tested *in silico* and by RACE performed from human whole-brain total RNA using FirstChoice[®] RLM-RACE Kit (Ambion) according to the manufacturer's instructions and using the following primers: 5'-RACE Outer Primer and SRPX2-Race1 (5'-tagaacgagtggtcctctgtctgt) or uPAR-Race1 (5'-gacgcaggtgtggagcagca) for the first PCR and 5'-RACE Inner Primer and SRPX2-Race2 (5'-cggcctccgggattctgttaacactgc) or uPAR-Race2 (5'-tgcgcggggctcctgcacgtctctctct) for the nested PCR. Consensus-binding sites were as defined previously (14): for the general FOX family, TRTTKRY, where R = A or G, K = T or G and Y = T or C; for members of the FOXP subfamily, TATTTT; for FOXP2, AATTTG and ATTTGT. For evolutionary comparisons, the promoter regions of *Pan troglodytes SrpX2* (GenBank accession no. NM_001135656) and *Upar* (GenBank accession no. NM_001009031) genes and of *Mus musculus SrpX2* (GenBank accession no. NM_026838) and *Upar* (GenBank accession no. NM_011113) genes were taken from the corresponding UCSC chimpanzee [Chimp Mar. 2006 (panTro2) assembly, Build 2 Version 1, October 2005] and mouse [Mouse July 2007 (mm9) assembly, Build 37] genome browsers at <http://genome.ucsc.edu/>, respectively.

Constructs and site-directed mutagenesis

For luciferase gene reporter experiments, the promoter regions of *SRPX2* and of *uPAR* as defined above were amplified by PCR from genomic DNA and subcloned into the pGL3 vector (Promega), 5' to the coding sequence of the firefly luciferase reporter gene. Primers used were as follows: PROM_SRPX2.F: 5'-aaaaggtacctaaactctgggagctggaga; PROM_SRPX2.R: 5'-aaaaaagctttttatgtattttgtgcccttt; PROM_UPAR.F: 5'-aaaaactcgagctggcgtgccctgttaa; PROM_UPAR.R: 5'-aaa aagcttggctcctgcacgtctctct. Direct sequencing was used to confirm the integrity of each construction. The pcDNA4/HisMax-FOXP2 vector has been used previously (18). Quick-Change Site-Directed Mutagenesis kit (Stratagene) was used to generate the pcDNA4/HisMax-R553H and pcDNA4/HisMax-M406T vectors allowing the expression of the mutant forms (p.R553H and p.M406T) of FOXP2. The following primers were used: FOXP2.R553H-F: 5'-gcaacttgaagaatcagtcacataatcttagcctgc and FOXP2.R553H-R: 5'-gcaggctaagattatgatgactgacttctccaagtgc; FOXP2.M406T-F: 5'-cgtcttc aagcaatgacgaccacttgcacatgc and FOXP2.M406T-R: 5'-gcatgtgcaagtgggtcgtcattgcttgaagacg. The presence of the desired mutations (c.G2032A and c.T1591C, corresponding to p.R553H and to p.M406T, respectively, with nucleotide positions as in NM_014491) and the absence of any other unwanted mutation were confirmed by direct sequencing.

Cell cultures and transfections

HEK293T cells were grown in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (Lonza) supplemented with 2 mM L-of glutamine, 100 U/ml of penicillin, 100 mg/ml of streptomycin and 10% fetal calf serum. One day before transfection, cells were seeded in six-well plates with a concentration of 10⁵ cells/well. When cells reached 70–80% confluence, they were transiently transfected using the Lipofectamine Plus[™] reagent (Invitrogen) according to the manufacturer's instructions.

Real-time RT-PCR

HEK293T cells were transfected with expression vectors for either wild-type FOXP2 (pcDNA4/HisMax-FOXP2) or mutant FOXP2 (pcDNA4/HisMax-R553H), or with an empty vector (pcDNA4/HisMax). Cells were harvested 48 h after transfection and total RNAs were extracted in TRIzol reagent. One microgram of total RNA was reverse-transcribed using random hexamers and the Superscript^(R) II RNase H reverse transcriptase (Invitrogen), according to the manufacturer's instructions.

PCR primers were designed for the *SRPX2* and *uPAR* genes as well as for the *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *B2M* (beta-2-microglobulin) control genes, using the Universal Probe Library Assay Design Center at <http://www.roche-applied-science.com/>. Primers were: for *SRPX2*: forward 5'-agggcacaataaccataaaaca and reverse 5'-gaagtcagccatcaattaacttctaa; for *uPAR*: forward 5'-cctctgc aggaccacgat and reverse 5'-tggtctctctgagtggtgaca; for *GAPDH*: forward 5'-tccactggcgtcttacc and reverse 5'-ggcag agatgatgaccctttt; for *B2M*: forward 5'-taggagggtgctgcaacttag, and reverse 5'-cttatgcacgcttaactatcttaaca. Quantitative PCR was carried out using LightCycler[®] 480 SYBR Green I Master (Roche) in the LightCycler[®] 480 system (Roche). All primer pairs were optimized to ensure specific amplification of the PCR product and the absence of any primer dimer. Quantitative PCR standard curves were set up for all. Quantification was calculated using the comparative C_T method. Fold changes were reported for cells transfected with either of wild-type (pcDNA4/HisMax-FOXP2) or mutant (pcDNA4/HisMax-R553H) FOXP2-containing expression vector and when compared with cells transfected with empty (pcDNA4/HisMax) vector. Relative quantification was performed using *GAPDH* as reference gene; the other control gene *B2M* did not show any significant variation (data not shown). For each gene, fold changes were reported as the mean of comparisons between five cDNA preparations. Data were expressed as mean ± SEM. Statistical significance was assessed using unpaired *t*-tests (two-tailed).

Luciferase reporter assays

HEK293T cells were cotransfected in six-well plates with (i) 150 ng of the appropriate reporter construct (pGL3-basic for negative control, pGL3-promoter for positive control, pGL3-SRPX2 or pGL3-uPAR construct for the analysis of each corresponding promoter activity), (ii) 150 ng of pHSV-LacZ for normalization and (iii) 1.2 µg of a construct for either the expression of FOXP2 wild-type (pcDNA4/HisMax-FOXP2), or the expression of mutant FOXP2 proteins (pcDNA4/HisMax-R553H or pcDNA4/HisMax-M406T), or no FOXP2 expression (empty pcDNA4/HisMax). Forty-eight hours after transfection, cells were lysed with 150 µl of Reporter Lysis Buffer (Promega). Firefly luciferase activity was quantified using the Luciferase Assay System (Promega) on a LB9507 Luminometer (Lumat). LacZ activity was measured using the β-Galactosidase Enzyme Assay System (Promega) on a NanoDrop[™] 1000 (Thermo Fisher Scientific). All transfections were performed in triplicate and repeated in three independent experiments (nine biological replicates in total).

The relative luciferase activities were then calculated with correction for transfection by β -galactosidase activity. Data were expressed as mean \pm SEM. Statistical significance was assessed using unpaired *t*-tests (two-tailed).

EMSA experiments

HEK293T nuclear extracts (transfected with FOXP2, FOXP2.R553H or the pcDNA4 empty vector control) were prepared as described previously (18). Probes were designed as 24–30 nucleotide oligomers (Supplementary Material, Table S1) based around the predicted binding sites in the *SRPX2* and *uPAR* promoters (Table 1). Mutant promoters were the same, however, with the core predicted binding site replaced (Supplementary Material, Table S1). A consensus probe previously shown to be efficiently bound by FOXP2 was used as a positive control (5'-agcttaacaagacaacacaataa) and an irrelevant promoter sequence (corresponding to an NFK binding site) was used as a negative control (5'-agctccgggggtgatttactcccgc). Oligonucleotide labeling and DNA-binding reactions were performed as described previously (18). Where unlabeled competitor probes were used to confirm specificity of DNA binding, they were added in 10-fold excess and pre-incubated at room temperature for 15 min, before the addition of labeled probe. Supershift assays were performed via pre-incubation of an N-terminal FOXP2 antibody (N-16; Santa Cruz) or a normal goat IgG negative control antibody (Santa Cruz) with nuclear lysates for 15 min at room temperature prior to the binding reaction. Protein–DNA interactions were resolved on a 5% polyacrylamide Tris/borate/EDTA gel.

Immunocytochemistry experiments

HEK293T cells were cultured on cover slips (LabTek I, Dutcher) and transfected with either a wild-type FOXP2-containing vector (pcDNA4/HisMax-FOXP2) or with a mutant FOXP2-containing vector (pcDNA4/HisMax-R553H or pcDNA4/HisMax-M406T) or with an empty vector (pcDNA4/HisMax). Forty-eight hours after transfection, cells were fixed using 4% paraformaldehyde solution at room temperature. HisMax-tag fusion protein FOXP2 was detected using an antibody to the N-terminal XpressTM tag and nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were captured with a fluorescence microscope (Leica, DMR) equipped with a CoolSnap camera.

Genetic screening

Thirty-two patients presenting with various related disorders, such as RE with DVD, perisylvian polymicrogyria, CSWS and Landau–Kleffner syndromes, were subjected to careful clinical, neuropsychological and electroencephalogram recordings, as well as to neuroimaging examinations whenever appropriate. All patients had given informed consent for the collection of blood samples and DNA extractions. In particular, one girl with the p.M406T FOXP2 mutation had polymicrogyria of the left rolandic operculum as shown by MRI; she had focal epilepsy with CSWS and cognitive and language impairments. Pregnancy, birth and initial development were

normal. Language acquisition was delayed with no clear regression. She had mental retardation with verbal, performance and full-scale IQs around 40 that remained unchanged from 7 to 16 years old. CSWS were detected at 6 years old and disappeared at the age of 10 years old. She also had right hemiparesia. At 25 years old, the patient was under 50 mg/day of phenobarbital and had rare nocturnal focal seizures; she still had cognitive and language deficits. The patient was born from unaffected parents. As her father also carried the mutation, he was submitted to MRI examination which did not reveal any abnormality (data not shown). The proband's sister and brother also carried the mutation; they did not show any obvious neurological disorder upon preliminary examination and no MRI was done. The whole coding sequence of the *FOXP2* gene was screened for mutations by direct sequencing of each exon and its surrounding intronic sequences. Both strands of each corresponding PCR product (list of primers upon request) were sequenced with an ABI3700 DNA Analyzer (Applied Biosystems) and the data were analyzed with the Genalys 3.0 software (43).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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