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

Patrice Roll1,2, Sonja C. Vernes3,†, Nadine Bruneau2,4,5, Jennifer Cillario2,4,5, Magali Ponsole-Lenfant2,4,5, Annick Massacrier2,4,5, Gabrielle Rudolf6, Manal Khalife1,2,‡, Edouard Hirsch6, Simon E. Fisher3 and Pierre Szepetowski2,4,5,*

1INSERM Unité 910, Marseille, France, 2Université de la Méditerranée, Aix-Marseille 2, France, 3Wellcome Trust Centre for Human Genetics, University of Oxford, UK, 4INSERM Unité 901, Marseille, France, 5Institut de Neurobiologie de la Méditerranée (INMED), Marseille, France and 6Département de Neurologie, Hôpitaux Universitaires de Strasbourg, France

Received August 2, 2010; Revised and Accepted September 16, 2010

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 brain is an important challenge and may offer a new perspective on developmental disorders.

†Present address: IMP, Dr. Bohr-Gasse 7, 1030 Vienna, Austria.
‡Present address: INRA, Domaine de Vilvert, 78352 Jouy en Josas, France.
*To whom correspondence should be addressed at: Institut de Neurobiologie de la Méditerranée (INMED), Inserm UMRS901, ‘Génétique des Epilepsies Isoles et Associées’ (GEIA) Group, Parc Scientifique de Luminy, BP 13-13273 Marseille Cedex 09, France. Tel: +33 491828111; Fax: +33 491828101; Email: szepetowski@inmed.univ-mrs.fr
© The Author 2010. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oxfordjournals.org
mutations in the such processes (1,2). In particular, rare loss-of-function
rises such key entry points into the molecular networks underlying
fashion. It has thus been possible to pinpoint genes that consti-
tution 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).

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 (rolan-
dic/sylvian epilepsy), functional (DVD) and developmental
(bilateral perisylvian polymicrogyria) disorders of the speech
areas of the cortex (OMIM 300643, 300388) (16). Several pro-
teins 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 plasmino-
gen activator receptor of the urokinase type (also known as
PLUR), 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 dis-
orders 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 function-
ing/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 (FOX sites: TATTTTG and ATTTGT). The FOXP2 consensus sequence ATTTGT is contained within the FOXP sites and the FOXP sites also
permute 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
SRPX2 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,
SRPX2) 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 identi-
ied 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, compara-
tive 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 dra-
matic differences with their human promoter counterparts,
with the most obvious evolutionary modification being the
lack of any of the UP1 to UP6 consensus forhead 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 tran-
script of FOXP2 (isoform 1; GenBank accession no.
NM_014491). When HEK293T cells were transected with a
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) 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, FOX2 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 (EMSAs) 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). By comparison, no supershift was

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

<table>
<thead>
<tr>
<th>Sequence a</th>
<th>Consensus-binding site b</th>
<th>Position (nt) c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SRPX2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRP1 d</td>
<td>TGGTTATGagTGTATTTAT</td>
<td>−110; −120</td>
</tr>
<tr>
<td>SRP2</td>
<td>GAAAACA</td>
<td>−305</td>
</tr>
<tr>
<td>SRP3</td>
<td>AATTGG</td>
<td>−664</td>
</tr>
<tr>
<td>SRP4</td>
<td>TATTTGT</td>
<td>+767</td>
</tr>
<tr>
<td>SRP5</td>
<td>TGGTGGC</td>
<td>−919</td>
</tr>
<tr>
<td>SRP6</td>
<td>TATTTGT</td>
<td>−1260</td>
</tr>
<tr>
<td>SRP7</td>
<td>TGTGGAT</td>
<td>−1373</td>
</tr>
<tr>
<td><strong>$uPAR$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UP1</td>
<td>TATTTATC</td>
<td>−131</td>
</tr>
<tr>
<td>UP2 e</td>
<td>ACAAACAAAACAAAACAC</td>
<td>−693; −697; −701</td>
</tr>
<tr>
<td>UP3</td>
<td>ATCAATA</td>
<td>−746</td>
</tr>
<tr>
<td>UP4</td>
<td>ATAAATA</td>
<td>−832</td>
</tr>
<tr>
<td>UP5 f</td>
<td>TATTTATTTATTTAT</td>
<td>−1160; −1164; −1168</td>
</tr>
<tr>
<td>UP6 f</td>
<td>ATAAATATATATATATATATATTA</td>
<td>−1319; −1323; −1327; −1331</td>
</tr>
</tbody>
</table>

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.
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...
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 forkhead 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...
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...
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 rolандic area; interestingly, the centrotemporal electroencephalographic hallmark of RE has shown increased frequency in patients with DVD (30). To the best of our knowledge, no rolандic 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 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 transcription 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.
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 Xpress™ 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 ± 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).

FOX2 shows evidence of recent positive selection (35). FOX2 underwent two non-synonymous changes since the human–chimpanzee split and cell-based studies suggest that several FOX2 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 SRPX2 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-CNTNAP2 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://bioinf.unice.fr/softwares/sms2/dna_pattern.html. The possible existence of alternative transcripts with more distal...
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), S′ to the coding sequence of the firefly luciferase reporter gene. Primers used were as follows: PROM_SRPX2.F: 5′-aaaaagcttcaatacctgccggtgcgga; PROM_SRPX2.R: 5′-aaaaagcttcaatacctgccggtgcgga; PROM_UPAR.F: 5′-aaaaagcttcaatacctgccggtgcgga; PROM_UPAR.R: 5′-aaaagcttcaatacctgccggtgcgga. Direct sequencing was used to confirm the integrity of each construct. 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′-ggcaggttctggtttggtctgttg; FOXP2.R553H-R: 5′-ggcaggttctggtttggtctgttg; FOXP2.M406T-F: 5′-ctatcggtcctggtttggtctgttg; FOXP2.M406T-R: 5′-ctatcggtcctggtttggtctgttg. The presence of the desired mutations (c.G2032A and c.T1591C, corresponding to mutation M406T and R553H, respectively) was 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-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® 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′-aggctcagcatactcctaattc; reverse 5′-gtttcttcttttcttttctttc; for uPAR: forward 5′-ctctctctctctctctctct; reverse 5′-tttctctctctctttttttc; for GAPDH: forward 5′-ctctctctctctctctctct; reverse 5′-tttctctctctctttttttc.

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 LKB 9507 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 ± 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′-agttaaacaagacaacaaataa) and an irrelevant promoter sequence (corresponding to an NFK binding site) was used as a negative control (5′-agctccgggggtgatctccccg). 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, 1.5 cm, Dutchers) 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 Xpress™ 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 prior to the study, according to the appropriate ethical committees. We thank H. Gara-Ksouri and F. Schaller-Marly at INMED for technical help and R. Leventer at Royal Children’s Hospital, Victoria, Australia, for expertise in MRI interpretation.

Conflict of Interest statement. None declared.

FUNDING
This work was supported by INSERM (Institut National de la Santé et de la Recherche Médicale) and by grants from ANR (Agence Nationale de la Recherche), FRC (Fédération pour la Recherche sur le Cerveau), the Wellcome Trust (project grant 080971 and core award 075491) and Autism Speaks. S.E.F. is a Royal Society Research Fellow. S.C.V. was funded by a French MRT (Ministry of Research and Technology) PhD fellowship.

REFERENCES


19. A humanized version of Foxp2 affects cortico-basal ganglia circuits in mice. Cell, 137, 961–971.


