FOXG1 Is Responsible for the Congenital Variant of Rett Syndrome

Francesca Ariani, Giuseppe Hayek, Dalila Rondinella, Rosangela Artuso, 1 Maria Antonietta Mencarelli, Ariele Spanhol-Rosseto, Marzia Pollazzon, Sabrina Buoni, Ottavia Spiga,³ Sara Ricciardi,⁴ Ilaria Meloni,¹ Ilaria Longo,¹ Francesca Mari,¹ Vania Broccoli,⁴ Michele Zappella,2 and Alessandra Renieri1,*

Rett syndrome is a severe neurodevelopmental disease caused by mutations in the X-linked gene encoding for the methyl-CpG-binding protein MeCP2. Here, we report the identification of FOXG1-truncating mutations in two patients affected by the congenital variant of Rett syndrome. FOXG1 encodes a brain-specific transcriptional repressor that is essential for early development of the telencephalon. Molecular analysis revealed that Foxg1 might also share common molecular mechanisms with MeCP2 during neuronal development, exhibiting partially overlapping expression domain in postnatal cortex and neuronal subnuclear localization.

In the classic form of Rett syndrome (RTT [MIM 312750]), females are heterozygous for mutations in the X-linked MECP2 gene (MIM 300005) and the few reported males have an XXY karyotype or MECP2 mutations in a mosaic state. A number of variants have been described including the congenital, the early-onset seizures, and the preserved speech variant.² Soon after the discovery of MECP2 as the RTT gene, we demonstrated that the preserved speech variant is allelic to the classic form.³ More recently, we and others showed that CDKL5 (MIM 300203) is responsible for atypical RTT, namely the early-onset seizures variant.^{4,5} The congenital variant was initially described by Rolando in 1985.⁶ In this form, girls are floppy and retarded from the very first months of life. The majority of congenital variants do not bear MECP2 or CDKL5 mutations, 7,8 with only four cases being reported with MECP2 mutations. 9-11

Using oligo array CGH, we recently identified a de novo 3 Mb interstitial deletion of chromosome 14q12 in a 7 year-old girl. 12 She showed dysmorphic features and a Rett-like clinical course, including normal perinatal period, postnatal microcephaly, seizures, and severe mental retardation. The deleted region was gene poor and contained only five genes. Among them, FOXG1 (MIM 164874) turned out to be a very interesting gene because it encodes a brain-specific transcriptional repressor. We analyzed this gene with a combination of both DHPLC and real-time quantitative PCR in a cohort of 53 MECP2/ CDKL5 mutation-negative RTT patients, including seven classic, 21 preserved speech, seven early-onset seizures, one "forme fruste," two congenital variants and 15 Rettlike cases.¹³ For real-time qPCR analysis, we designed primers and TaqMan probe complementary to a segment located in the middle of the single exon of the gene using Primer Express software (Applied Biosystems). Sequences

of primers and probe (FAM labeled) are listed in Table S1 available online. We used an RNAase P kit as an internal reference (VIC-labeled probe, Applied Biosystems). PCR was carried out as previously described. 14 The starting copy number of the unknown samples was determined with the comparative Ct method, as reported by Livak. 15 By DHPLC, we identified a different de novo FOXG1 truncating mutation in the two congenital variant patients. Real-time qPCR failed to identify any microdeletion in the 53 patients.

FOXG1 encodes forkhead box protein G1, FoxG1 (formerly brain factor 1 [BF-1]), a transcriptional factor with expression restricted to fetal and adult brain and testis. FoxG1 interacts with the transcriptional repressor JARID1B and with global transcriptional corepressors of the Groucho family. The interaction with these proteins is of functional importance for early brain development. 16,17 Like MeCP2, FoxG1 also indirectly associates with the histone deacetylase 1 protein.^{1,17} Both mutations disrupted the protein at different levels (Figure 1). In case 1, a stop-codon mutation p.W255X (c.765 $G \rightarrow A$) impaired the DNA binding because of the disruption of the forkhead domain (Figure 1D, left). Case 2 showed a 1 bp deletion c.969 delC (p.S323fsX325) causing the loss of JARID1B-interacting domain and the misfolding of the motif responsible for the Groucho binding (Figure 1D, right). Lastly, both FOXG1 mutations affected all the four brain fetal isoforms that lack the last 37 amino acids and have different C-terminal domains.18

The two mutated individuals, aged 22 (case 1) and 7 years (case 2), fulfilled the international criteria for RTT variants. 19 Pregnancy, delivery, and auxological parameters at birth were normal. Neurological and behavioral neonatal evaluations were reported as normal, but at three

¹Medical Genetics, Molecular Biology Department, University of Siena, 53100 Siena, Italy; ²Child Neuropsychiatry University Hospital AOUS, 53100 Siena, Italy; ³Biochemistry and Molecular Biology, Molecular Biology Department, University of Siena, 53100 Siena, Italy; ⁴San Raffaele Scientific Institute, Dibit, 20132 Milano, Italy

*Correspondence: renieri@unisi.it

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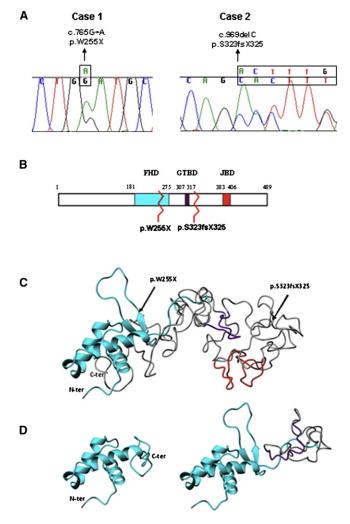


Figure 1. FOXG1 Mutations and Alterations of the Functional **Domains**

- (A) Sequence tracing of *FOXG1* mutations in the two patients. Mutated bases are indicated above the line.
- (B) Schematic representation of FoxG1 protein. The three main functional domains are shown: the DNA binding fork-head domain in light blue (FHD), the Groucho-binding domain in violet (GTBD), and the JARID1B binding domain in red (JBD). The numbers at the top refer to the amino acid positions. Mutations are indicated by zigzag lines.

(C and D) Ribbon representation of the tertiary structure obtained with Phyre v.0.2 software. (C) shows the structure of the region containing the three functional domains of wild-type protein (amino acids 180–489). Arrows highlight the two mutations. The FHD domain (cyan) consists of three alpha helices and one beta hairpin (two beta strands and one loop), whereas the GTBD (violet) and JBD (red) domains are random coiled. (D) shows structural modification after p.W255X (left) and p.S323fsX325 (right) mutations. The p.W255X mutation determines a protein truncation just after the second beta strand leading to the loss of the beta hairpin and thus preventing DNA binding. The p.S323fsX325 mutation leaves the FHD domain intact and truncates the protein just after GTBD, inducing conformational changes that lead to its misfolding.

months, an abnormal head-circumference growing was noticed in the patients. These patients appeared to weep inconsolably, and they did not respond when called and were unable to lift their heads. Case 1 was never able to sit unaided and laid permanently in bed, whereas case 2 was barely able to sit. They were always apraxic and from 1 year of age, they showed peculiar jerky movements of the upper limbs and midline stereotypic activities, typical of RTT syndrome (Figure 2). They never acquired spoken language. Generalized convulsions appeared at 14 years in case 1 and at 2 1/2 years in case 2. Ever since cases 1 and 2 were 3 and 5 years old, respectively, an EEG showed features often found in RTT patients: a multifocal pattern with spikes and sharp waves and occasional paroxysmal activity. In both patients brain MRI showed corpus callosum hypoplasia, a finding which has already been reported in RTT.²⁰ Currently, they show microcephaly (OFC of 49 cm in case 1, and 47 cm in case 2). They have occasional periods of deep breathing with exaggerated inspirations. Sialorrhoea, bruxism, scoliosis, and cold lower extremities as well as stypsis are present in both patients who are currently fed by mouth.

These two girls show neurological and neurovegetative symptoms as well as somatic features consistent with a diagnosis of congenital RTT variant. It should only be noted that a retrospective assessment concerning the possible presence of a regression was not feasible. We attempted to compare their phenotype with the four other MECP2mutated girls described as congenital variants. 9-11 However, they have been reported with very little detail, thereby hampering a posteriori clinical re-evaluation according to the revised criteria. 19 According to the new criteria, in the classic form, psychomotor development may have been delayed from birth; thus, a re-evaluation of these four patients would have lead to their reclassification as classic form. Alternatively, the disruption of either MeCP2 or FoxG1 may lead to a phenotype, namely the congenital variant, indistinguishable at the level of the clinical and instrumental investigations performed.

A translocation with inversion affecting fetal isoforms of FOXG1 was recently described in a 7-year-old girl. 18 She had acquired microcephaly, alalia, inability to sit and walk, and epilepsy in common with the present cases. Corpus callosum was absent, whereas in our cases, it was hypoplasic. Stereotypic hand activities were not mentioned, and tetraplegia was described.¹⁸ The clinical features of this patient have something in common with a RTT phenotype. The impairment of only fetal FOXG1 isoforms and the possible contribution of genes at the other two breakpoints of the complex rearrangement might explain the phenotypic differences.

The mouse ortholog *Foxg1* has a restricted expression domain in the central nervous system coinciding with the emergence of the telencephalic structures of the brain. Its function has been extensively characterized and found to promote telencephalon development by sustaining proliferation of the progenitor pool and preventing





Figure 2. Pictures of the Two Congenital RTT Patients

Case 1 (#156) is shown on the left; case 2 (#868) is shown on the right. They show peculiar jerky movements of the upper limbs frequently pushed in different directions accompanied by continuous flexionextension, wringing movements of the fingers of the hands. The hands were brought together in hand-washing and hand-mouth stereotypic activities, which were intense and present all the time they were awake. Similar flexion-extension movements of the toes were noticed in the feet. The double scoliosis of case 1 is clearly evident, whereas the other girl maintained a straight vertebral column as often occurs in RTT in the first decade. Teeth grinding was present, and the tongue often protruded out from the mouth.

premature cortical neural differentiation. 21,22 In agreement, FoxG1 expression is found in the proliferating neuroepithelium starting from early development onward.²³ This expression profile might explain the particular early onset of the neurological symptoms displayed by the patients.

Despite its early expression in telencephalon development, in this study we found that Foxg1 expression is detectable in the differentiating cortical compartment in the postnatal stages, although at lower levels with respect to the early embryonic phases (Figure 3A). This expression profile overlaps with the described MeCP2 expression

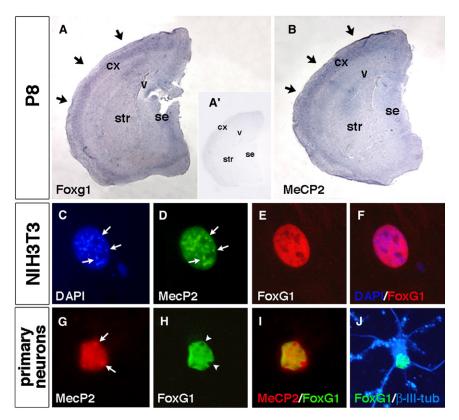


Figure 3. FoxG1 and MeCP2 Expression **Domain in Postnatal Cortex and Neuronal Subnuclear Localization**

(A and B) Expression analysis by in situ hybridization of Foxq1 and MeCP2 on P8 postnatal forebrain tissue. As shown in (A), Foxq1 expression is found in differentiating and mature cortical neurons in the definitive cortical plate (indicated by arrows in [A]) similar to the MeCP2 expression pattern (indicated by arrows in [B]). In (A'), the inset shows background staining with a sense cRNA for Foxg1 in the same in situ hybridization conditions used for (A) and (B).

(C-J) FoxG1 and MeCP2 sub-cellular localization in non-neural and primary neurons. As shown in (C)-(F), in NIH 3T3 cells, MeCP2-GFP exogenous protein has a diffuse nuclear localization with accumulation in the heterochromatic foci (indicated by arrows in [D]) as identified when compared with DAPI staining (indicated by arrows in [C]). (E) and (F) show that conversely, FoxG1-flag exogenous protein displays a widespread nuclear localization without enrichment in heterochromatic sites. (G)-(J) show FoxG1 and MeCP2 localization in

12DIV (days in vitro) primary hippocampal neurons. In (G), MeCP2 endogenous protein is accumulated in heterochromatic foci (indicated by arrows). As shown in (H), FoxG1-flag exogenous nuclear localization is excluded from heterochromatic puncta (indicated by arrowheads). As shown in (I), MeCP2 and FoxG1-flag colocalize in the nuclear compartment outside the heterochromatic foci. As shown in (J), Nuclear FoxG1-flag localization is detected in a differentiated β-III-tubulin-positive neuron. The following abbreviations are used: cx, cerebral cortex; se, septum; str, striatum; and v, ventricle.

domain in cortical tissues, in differentiating and mature neurons (Figures 3A and 3B). *Foxg1* homozygote-mutant mice die shortly after birth with severe brain defects. ^{24–26} Unfortunately, the severe compromised development of *Foxg1* mutant telencephali has prevented the analysis of its function in more differentiated neurons. At the singlecell level, FoxG1 localizes in the nuclear compartment but is excluded from the MeCP2-positive heterochromatic foci both in nonneural and primary neurons (Figures 3C–3J). These findings suggest that, differently from MeCP2, FoxG1 is not a transcriptional repressor stably associated with heterochromatin. However, both proteins have a large colocalization domain in other nuclear compartments (Figure 3I).

Overall, these data suggest that FoxG1 may exert some additional functions in differentiating and mature neurons, thus sharing similarities with those described for MeCP2. These findings may provide some biological evidence for the development of similar clinical manifestations in disorders affecting the two genes. However, it is also possible that the two transcriptional regulators act on different stages of the process that leads to proper cortical development, from early cell-fate decisions to later circuit connectivity and dendritic development.

FoxG1 shares some interesting analogies with MeCP2 in its molecular functions, raising the question whether the two protein networks may interact in some circumstances and on selective common targets. Future studies will address this intriguing hypothesis. Recently, heterozygous $Foxg1^{+/-}$ mice were found to display subtler defects including a reduction in size of the corpus callosum together with specific patterning defects. Furthermore, heterozygous $Foxg1^{+/-}$ exhibit learning deficits based on fear-condition behavioral tests associated with a loss of postnatal neurogenesis in the hippocampus. These mice represent a very interesting animal model for further investigation about how Foxg1 haploinsufficiency may impact on brain development and neuronal maturation and function.

In conclusion, we demonstrated that *FOXG1* is a previously unrecognized gene responsible for variant Rett syndrome. It is worth noting that in the revised criteria for Rett syndrome the female sex is no longer present as inclusion criteria. ¹⁹ This seemed to open the door to the discovery of an autosomal gene.

Supplemental Data

One table listing primers and probes for real-time qPCR is available at http://www.ajhg.org/.

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Web Resources

The URLs for data presented herein are as follows:

Italian Rett database and biobank, http://www.biobank.unisi.it/ Online Mendelian Inheritance in Man (OMIM), http://www.ncbi. nlm.nih.gov/Omim/

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