

ARTICLE

Deletion of protein tyrosine phosphatase, non-receptor type 4 (*PTPN4*) in twins with a Rett syndrome-like phenotype

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Rett syndrome (RTT), a neurodevelopmental disorder that predominantly affects females, is primarily caused by variants in *MECP2*. Variants in other genes such as *CDKL5* and *FOXG1* are usually associated with individuals who manifest distinct phenotypes that may overlap with RTT. Individuals with phenotypes suggestive of RTT are typically screened for variants in *MECP2* and then subsequently the other genes dependent on the specific phenotype. Even with this screening strategy, there are individuals in whom no causative variant can be identified, suggesting that there are other novel genes that contribute to the RTT phenotype. Here we report a *de novo* deletion of protein tyrosine phosphatase, non-receptor type 4 (*PTPN4*) in identical twins with a RTT-like phenotype. We also demonstrate the reduced expression of *Ptpn4* in a *Mecp2* null mouse model of RTT, as well as the activation of the *PTPN4* promoter by MeCP2. Our findings suggest that *PTPN4* should be considered for addition to the growing list of genes that warrant screening in individuals with a RTT-like phenotype.

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INTRODUCTION

Although variants in *MECP2* account for the majority of Rett syndrome (RTT) cases, diagnosis is still based on clinical criteria.^{1,2} Typical RTT is characterised by a period of developmental regression usually commencing in infancy, which is followed by a recovery or stabilisation phase. Affected individuals exhibit impairment or complete loss of language and hand skills, have gait abnormalities, and display stereotypic hand movements. The current clinical criteria are outlined in Neul *et al*² and take into account the variable nature of the RTT phenotype and allow a better description of the variant forms of RTT that are typically difficult to define clinically.

Despite intensive investigations of both RTT patients and *Mecp2* mutant mouse models, the biological functions of MeCP2 and mechanisms of disease are still not fully understood. What has become apparent is that MeCP2 has multifaceted roles that influence normal brain development and function.^{3,4} Initially, MeCP2 was thought to be a transcriptional regulator of a subset of genes key to the modulation of synaptic plasticity; however, defining these genes has proven somewhat problematic.^{3–7} Although it is apparent that MeCP2 binds to the promoters of a number of genes such as *BDNF*⁸ and *JUNB*,⁹ it is also postulated that MeCP2 may also act in a more global fashion by modulating chromatin architecture.¹⁰

In addition to variants in *MECP2*, other molecular causes of RTT and related phenotypes have been identified, including *CDKL5*,^{11–13} *FOXG1*¹⁴ and *TCF4*,¹⁵ although currently there is growing support to re-categorise individuals with *CDKL5* and *FOXG1* variants as being distinct clinical entities,^{16,17} and *TCF4* variants generally cause a discrete phenotype, Pitt–Hopkins syndrome.¹⁸ These genes may share biological pathways with *MECP2*, thereby potentially underpinning

the overlapping phenotype. In this study, we identify a novel deletion of protein tyrosine phosphatase, non-receptor type 4 (*PTPN4*) in twins with an atypical form of RTT.

MATERIALS AND METHODS

CGH array

Analysis of copy number variation (CNV) of the twins and their parents was carried out by Sydney Genome Diagnostics, the Children's Hospital at Westmead, using an Agilent Sureprint G3Hmn CGH 400K array, 60mer oligo probes, effective resolution 0.08 Mb (Agilent Technologies, Mulgrave, VIC, Australia). The identity panel PowerPlex 16 (Promega, Alexandria, NSW, Australia) was also used to confirm that the twins were monozygous.

Quantitation of *Ptpn4* expression

Brains were harvested from *Mecp2^{tm1Tam}* mice (*Mecp2^{+/-}* and *Mecp2^{-/-}*) at 5 weeks (presymptomatic) and 8–10 weeks (symptomatic), and the hippocampus and cerebellum was dissected from each brain. RNA extraction, cDNA synthesis, and quantitative PCR (qPCR) were performed as described previously;¹⁹ for primers, see Supplementary Methods. The expression levels of *Ptpn4* were calculated relative to *Gapdh* using the $\Delta\Delta C_T$ method.²⁰

Chromatin immunoprecipitation

Differentiated SH-SY5Y cells were used for chromatin immunoprecipitation (ChIP) assays. Chromatin fragments were prepared by sonication and used to perform MeCP2 immunoprecipitation (Supplementary Methods). The ability of MeCP2 to bind to target DNA regions was then measured by qPCR; 1 μ l of eluted DNA (as 5 μ l of a 1:5 dilution) was added, used as a template, and reactions were performed in triplicate. For primers, see Supplementary Methods. Fold enrichment was calculated as fold enrichment relative to the

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input sample, and the ΔC_T was calculated using the average C_T of the INPUT sample as the calibrating sample (sample $C_T - C_T$ of the INPUT sample).

Luciferase assay

The human *PTPN4* promoter (g.119758859-119759770), NC_00002.12, was PCR amplified using Platinum Pfx DNA Polymerase (Life Technologies, Mulgrave, VIC, Australia). The PCR product was cloned into the Luciferase reporter vector pcBG99-Basic vector (Promega) using HindIII producing the pcBG99-*PTPN4* luciferase reporter construct, and the sequence and orientation of the *PTPN4* promoter was confirmed by sequencing. SH-SY5Y cells were plated into six-well plates (2×10^6 cells per well) and transfected the following day using Lipofectamine LTX (Life Technologies). The ability of MeCP2 to act as a transcriptional regulator of the *PTPN4* promoter was tested by transfecting $4 \mu\text{g}$ of the pcBG99-*PTPN4* or pcBG99-basic and $4 \mu\text{g}$ of a MeCP2 expression vector (pCMV-Tag2C-MeCP2) or the empty expression vector (pCMV-Tag2) simultaneously into the SH-SY5Y cells. In addition, all cells were transfected with $2 \mu\text{g}$ of the red luciferase vector pcBR99, as a transfection control. Twenty-four hours after transfection, the cells were harvested and lysed with Glo Lysis buffer (Promega). Equal volumes ($50 \mu\text{l}$) of the lysate and the luciferase substrate, Chroma-Glo Luciferase Assay System (Promega), were combined in a 96-well white/black plate, and the luminescence of the red and green luciferases was measured immediately using the VICTOR³ plate reader (Perkin Elmer, Melbourne, VIC, Australia). The red luciferase was measured with the filter 610AGLP while the green was measured with the filter 510DF60, and the raw red and green luminescence values were corrected as recommended by the manufacturer (see Supplementary Methods). For each sample, the relative amount of green luminescence was calculated by dividing the green luminescence by the red luminescence. Two independent experiments with triplicate transfections and duplicate readings were performed. In order to compare the results of the two independent experiments, the relative green luminescence of the pcBG99-*PTPN4* vector in the presence of MeCP2 was divided by the average of the relative green luminescence of the pcBG99-*PTPN4* vector without MeCP2.

Statistical analyses

qPCR data of *Ptpn4* transcripts and ChIP enrichment and relative luminescence values of the Luciferase reporter system were analysed for statistical significance using the Mann-Whitney test and Graph Pad Prism, version 5.03 (GraphPad Software, San Diego, CA, USA).

Database submission

The novel *PPNT4* deletion identified in this study was submitted to the DECIPHER database, with the accession numbers being 294047 for Twin 1 and 294048 for Twin 2; <https://decipher.sanger.ac.uk/>.

RESULTS

Case study

Identical twins (confirmed using the PowerPlex 16 identity panel) were born at 36 weeks gestation after a normal pregnancy and delivery. Twin A's birth weight was 2670 gm (50th percentile), length was 44.5 cm (10th–50th percentiles) and head circumference was 33.5 cm (50th percentile). Twin B's birth weight was 2575 gm (10th–50th percentile), length was 44.5 cm (10th–50th percentile) and head circumference was 33 cm (10th–50th percentile). There were no perinatal or postnatal problems, but they were described as being quiet and placid babies. Both were poor feeders and slow to gain weight. First concerns about developmental progress were raised at 9 months, as neither twin had rolled or started to crawl, and they were described as being floppy.

In general, Twin A's development has always been ahead of Twin B. She sat without support at 12 months, walked unaided at 3 years and 4 months, developed a pincer grip at 2 years and started to say single words between 5 and 6 years of age. She has never lost any speech, could talk in short sentences and could imitate words; however, speech was often difficult to comprehend. She had some stereotypic hand

movements, putting her hands to her head, previously to her mouth, flexing and extending her fingers and rubbing and twisting her fingers. There was no loss of fine motor skills. At the age of 17 years, Twin A could throw, catch and kick a ball.

Twin B sat unsupported at 15 months, walked unaided at 4 years and 4 months, developed at pincer grip at 2 years and started saying single words at 6 years. She lost the ability to speak from approximately 9 years of age. She had learnt to say approximately six words but, at age 17 years, did not articulate any words. She had good receptive language skills and could follow simple commands.

Both twins had a seizure disorder from approximately 2½ years of age. Twin A's EEG at the time showed symmetric background 8–10 Hz activity posteriorly. Epileptiform activity was present with frequent sharp/spike discharges noted in the left occipital region. An EEG performed at 10 years showed poorly formed background activity and multi-focal sharp discharges bilaterally. There were frequent spike and wave generalised paroxysmal discharges, most without clinical accompaniments. She was seizure free from 13 years of age. Anticonvulsant medications were subsequently stopped.

Twin B's EEG performed at 12 years showed frequent polyspike discharges, which alternated from the right to left hemisphere. At 13 years, video telemetry documented partial seizures with an onset from the left frontotemporal region. The interictal EEG showed continuous generalised slowing of the background activity, which was more prominent on the left. At 17 years, Twin B continued to have generalised seizures, some associated with cyanosis lasting up to 2 min every 6–8 weeks. This was managed with sodium valproate and lamotrigine.

The twins had ECGs performed at 17 years of age. Twin A had a prolonged corrected QT value but no cardiac arrhythmia seen on 24-h Holter monitoring, while twin B's ECG was normal.

Both twins had a normal breathing pattern. They had a mildly disturbed but relatively good sleep pattern, with occasional walking at night, laughing, talking and singing. They used to grind their teeth, but this subsequently spontaneously ceased. They had some difficulty chewing, in that they chewed with an open mouth, and Twin B rolled food around with her tongue. They were described as fussy eaters but did not require their food to be softened, pureed or mashed. Both had constipation.

When reviewed at age 17 years, Twin A appeared in good health. She was alert and cooperative throughout the consultation and at times spoke in sentences. Her weight was 36.5 kg (10th percentile), height 140.3 cm (< 1st percentile) and head circumference was 53 cm (~10th percentile). She had mild trigonocephaly, a high nasal bridge, almond shaped eyes, prominent lips, thickened gums, widely spaced teeth and a narrow palate. She had shortened 4th metatarsals. Her feet were cool to palpation and mottled. Her back was straight and hirsute. She could walk without assistance with a slightly wide-based gait. She repetitively twisted an object in her hand. She drooled but did not grind her teeth.

When last reviewed with her sister, Twin B also appeared in good health. Her weight was 26.1 kg (< 3rd percentile), height was 132.1 cm (< 1st percentile) and head circumference was 51 cm (2nd percentile). Like her sister, she had mild trigonocephaly, a high wide nasal bridge, almond shaped eyes, thickened gums and widely spaced teeth. Her back was straight and hirsute. She had small cold hands and feet with shortened 4th metatarsals bilaterally. She demonstrated stereotypic hand movements in the form of putting her hands to the side of her head, flexing and extending her fingers continuously and flapping her hands. She was able to walk unaided with a wide base of support.

Overall, although the twins have many of the features suggestive of RTT they did not fulfil the clinical criteria for the typical form of the disorder. Twin B could be classified as having variant RTT according

to the Neul criteria;² however, Twin A did not fulfil the clinical criteria for variant RTT.

De novo deletion at chromosome 2q14.2

CGH array analysis of the twins identified a deletion (arr[hg19] 2q14.2 (120,584,760-120,726,563) × 1 dn), with a minimum size of 90 Kb (Chr2.hg19:g. 120,584,760_120,726,563del) and a maximum size of 160 Kb (Chr2.hg19:g. 120,579,294_120,734,950del), Figure 1. This deletion was not seen in their healthy parents or their two healthy siblings. This region contains a single gene, protein tyrosine phosphatase, non-receptor type 4 (*PTPN4/PTPMEG*), GenBank ref NC_000002. This phosphatase is expressed predominately in the brain and testis²¹

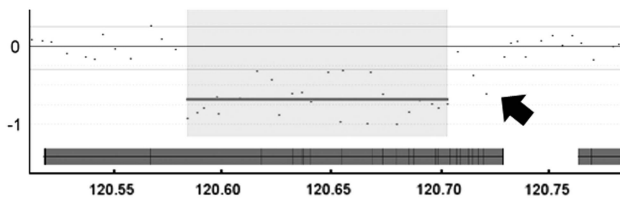


Figure 1 Comparative genomic hybridisation (CGH) array analysis. CGH array testing mapped the approximate position of the deletion, at coordinates 120.58–120.70 Mb, within chromosome 2. The 400K array data shown here is for Twin A. The central grey bar indicates gene *PTPN4*. The x axis shows the hg19 coordinates. This image is derived from the *CGH Fusion* software: *InfoQuant* Ltd (London, UK). Note that the segmentation algorithm used here may have underestimated the distal extent of the deletion (see arrow). Within the ISCN terminology, the deletion was reported as extending from 120,584,760 to 120,726,563.

and knockout of the *Drosophila* and murine orthologues results in neurological abnormalities.^{22,23} This essential role of *PTPN4* in neuronal development and function suggests that *PTPN4* is a good candidate gene for the variant RTT phenotype seen in the twins.

No benign CNVs involving *PTPN4* have been identified in the Copy Number Variation Healthy Control Database (<http://cnv.chop.edu>). Searches of the CNV database DECIPHER²⁴ identified 10 individuals with deletions or duplications involving the *PTPN4* (Table 1). Two of the individuals with *de novo* deletions displayed intellectual disability, and one with a *de novo* deletion was described as having oculomotor apraxia, although there are little additional clinical details for the latter case. However, all of these cases had deletions encompassing multiple genes, and therefore their phenotypes cannot necessarily be attributed to *PTPN4* alone.

Ptpn4 is downregulated in the brain of a mouse model of Rett syndrome

Several *Mecp2* knockout and knock-in mice have been developed to model the RTT phenotype. Although the phenotype of the *Ptpn4* null mouse is mild compared with that of the *Mecp2* null mouse, both models display impairment of motor co-ordination and learning. We therefore examined whether *Ptpn4* expression is dysregulated in the brain of the *Mecp2^{tm1Tam}* mouse. qPCR analysis of the *Ptpn4* transcript (Figure 2) demonstrated a significant reduction of *Ptpn4* levels in both cerebellum and hippocampus of symptomatic *Mecp2^{-ly}* mice, while in the presymptomatic animals a significant reduction was only observed in the cerebellum but not in the hippocampus. A brain region-specific dysregulation of *Ptpn4* in presymptomatic *Mecp2^{-ly}* mice thus became more widespread with progression of the disease phenotype.

Table 1 Phenotype of other deletions/duplications^a affecting 2q14.2

Abnormality	Inheritance	Size (Mb)	Number of genes affected	Sex	Age of presentation (years)	Phenotype
Deletion	<i>De novo</i>	13.7	58	F	3	Behavioural problems Intellectual disability Cystic hygroma of neck
Deletion	<i>De novo</i>	3.28	16	M	Unknown	Feeding problems in infancy Hip, general abnormalities Joint laxity Intellectual disability Sandal gap of toes Short stature, general abnormalities Sleep apnoea Speech delay
Deletion	<i>De novo</i>	6.53	21	M	3	Oculomotor apraxia
Duplication	Unknown	39.87	134	M	Unknown	Unknown, pregnancy terminated
Duplication	<i>De novo</i>	53.36	181	F	1	Atrial septum defect Ectopic/supernumerary kidneys Delayed closure and large fontanelles Short stature, prenatal onset
Duplication	<i>De novo</i>	147.5	732	M	—	Unknown
Duplication	Inherited from normal parent	0.44	5 only partial duplication of <i>PTPN4</i>	F	—	None
Duplication	Inherited from normal parent	0.5	6 only partial duplication of <i>PTPN4</i>	F	4	Intellectual disability Short stature, proportionate
Duplication	Inherited from normal parent	0.53	6 only partial duplication of <i>PTPN4</i>	M	2	Hydrocephalus/large ventricles, non-specific Macrocephaly Intellectual disability Seizures
Duplication	Inherited from normal parent	0.44	5 only partial duplication of <i>PTPN4</i>	—	—	None

Abbreviations: F, female; M, male.

^aDeletions/duplications reported in DECIPHER database (<http://decipher.sanger.ac.uk>), accessed 1 July 2014.

MeCP2 binds to *PTPN4* sequences

MeCP2 is a transcriptional regulator that can both activate and repress gene expression. As *Ptpn4* expression is reduced in the absence of *Mecp2*, it is possible that MeCP2 may function to activate the expression of *PTPN4*. To test whether *PTPN4* is a transcriptional target, we tested for MeCP2 binding to *PTPN4* sequences using ChIP assays in the SH-SY5Y human neuroblastoma cell line. Two PCR amplicons were designed to amplify regions 5' to exon 1 of *PTPN4*, containing the putative RNA polymerase II binding sites and the CpG island, respectively. An additional amplicon in intron 15 of *PTPN4* was also designed as a gene-specific non-promoter sequence control (Figure 3a). As a positive control for MeCP2 binding, an amplicon in the promoter of a known MeCP2 target in SH-SY5Y cells, *JUNB*,⁹ was tested. For a negative control, intron 6 of *PORIN* was used. The MeCP2 ChIP studies revealed enrichment for all amplicons above that of the normal serum and no antibody control background levels (Figure 3b).

MeCP2 enhances the expression of the *PTPN4* promoter *in vitro*

Given the inconclusive results of the ChIP assays, we next investigated

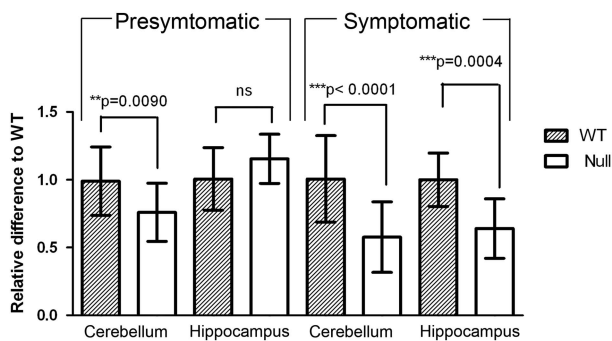


Figure 2 *Ptpn4* expression reduced in the brain of the *Mecp2^{tm1Tam}* mouse model of RTT. Quantitation of *Ptpn4* expression in the cerebellum and hippocampus of presymptomatic and symptomatic *Mecp2^{+/+}* (wild type) and *Mecp2^{-/-}* (null) mice ($n=5$, error bars = SD).

the ability of MeCP2 to mediate the expression *PTPN4* promoter using a luciferase reporter vector containing the *PTPN4* promoter (pcBG99-*PTPN4*). In SH-SY5Y cells, the level of luciferase expression in the presence of MeCP2 was 2.1 ± 0.67 -fold of that of pcBG99-*PTPN4* alone (Figure 4), indicating that MeCP2 is capable of acting as a transcriptional activator to the *PTPN4* promoter.

DISCUSSION

This is the first report of a human disease associated with haplo-insufficiency of *PTPN4*. Evidence from knockout models in other species demonstrates that *PTPN4* is required for neuronal development and function. In *Drosophila*, the null phenotype manifests as a defect of axonal development,²³ while knockout mice show impaired motor learning and cerebellar synaptic plasticity.²² Although essential for neuronal function, the phenotype of the *Ptpn4* knockout mouse is milder than that of *Mecp2* null mice.^{22,25–27}

Similarly, compared with typical RTT, the twins described here have a milder phenotype. It is interesting to note that while the twins exhibited a number of clinical features that overlap with those seen in RTT, including shortening of the 4th metatarsals, only the more severely affected twin fulfilled the clinical criteria for variant RTT. A potentially important distinguishing feature is the facial dysmorphism exhibited by the twins, which is not seen in *MECP2* mutation-positive RTT individuals, but it remains to be seen whether these dysmorphic features are a consequence of *PTPN4* haplo-insufficiency.

PTPN4 is predominantly expressed in the brain, with the strongest expression in the thalamus and the Purkinje cells of the cerebellum.²¹ *PTPN4* is a phosphatase that regulates the phosphorylation of the glutamate receptor GluR1/GRIN2A in Purkinje neurons and is capable of interacting with both GRIN2A and another glutamate receptor GluRdelta2/GRID2.²¹ Variations in *GRIN2A* are associated with autism spectrum disorders, sporadic schizophrenia^{28,29} and epileptic encephalopathy³⁰ while *GRID2* is important for regulating synaptic plasticity and motor co-ordination.³¹ The phenotype seen in the *Ptpn4* knockout mouse could be due to a modulation of the function of these glutamate receptors.

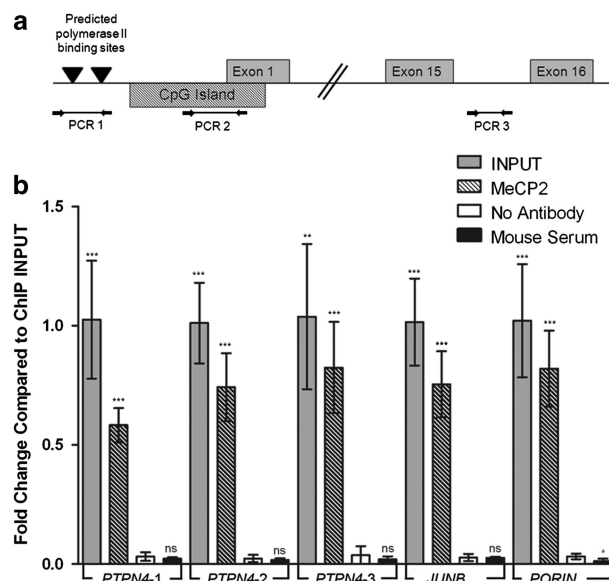


Figure 3 ChIP analysis of MeCP2-binding sites in SH-SY5Y cells. (a) Location of the amplicons in the *PTPN4* gene. (b) Fold enrichment of MeCP2, no antibody and normal mouse serum ChIP from SH-SY5Y lysates across the *PTPN4*, *JUNB* and *PORIN* amplicons. Duplicate biological replicates were analysed in triplicate, P -values for each amplicon relative to the no antibody control (NS = not significant, $**P \leq 0.01$, $***P \leq 0.001$). Error bars = SD.

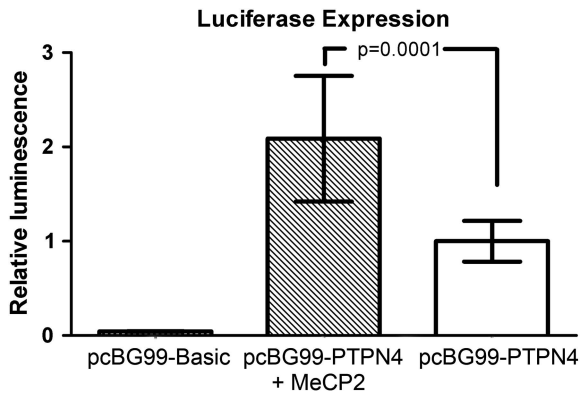


Figure 4 MeCP2 enhances *PTPN4* promoter-driven gene expression in SHSY-5Y cells. Relative luminescence of the promoterless luciferase vector (pcBG99-basic) and the *PTPN4* promoter-driven luciferase vector (pcBG99-PTPN4), with and without MeCP2. Two independent experiments with triplicate transfections and duplicate readings were performed. Error bars = SD.

Although deficiency of MeCP2 function is the major cause of classical RTT, the exact pathological mechanisms have been elusive.^{3,4} However, there are clearly defects of dendritic spine number and structure in the *Mecp2*-deficient brain, which result in a generalised loss of synaptic plasticity.^{32–35} In comparison, *Ptpn4* null mice display a more specific loss of cerebellar synaptic plasticity,²² consistent with the more restricted expression of *Ptpn4*. The observed reduction of *Ptpn4* in the brain of *Mecp2* null mice and the *in vitro* demonstration that MeCP2 enhances the strength of the *PTPN4* promoter in neuronal cells suggests this phosphatase may be a component of the biological pathways that are disrupted by *Mecp2* deficiency.

In conclusion, we describe a *de novo* deletion of the gene *PTPN4* in identical twins with a RTT-like phenotype. Furthermore, we demonstrate reduced expression of *Ptpn4* in the brain of a *Mecp2* null mouse model and the activation of *PTPN4* promoter by MeCP2. Together, these results suggest that haplo-insufficiency of *PTPN4* may be the cause of the phenotype seen in these twins. We suggest that *PTPN4* should be considered as a possible novel candidate gene of variant RTT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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