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A specific mutation in TBL1XR1 causes Pierpont syndrome

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INTRODUCTION

In 1998 Pierpont and coworkers described two unrelated boys with remarkably similar features (high forehead, underdeveloped mid-face, narrow palpebral fissures and anteverted nares), short stature, hearing loss, developmental delay and distinctive palmar and plantar fat pads.4 Two similar patients were subsequently reported, including one with a choroid plexus papilloma, whereupon the condition was named Pierpont syndrome.2,3 While several patients resembling Pierpont syndrome have been reported,4 clinical re-evaluation and molecular analyses have shown that they had either Coffin–Siris5 or Wiedemann–Steiner syndrome (H. Brunner, personal communication 2013). Initially this caused uncertainty about the phenotype defining Pierpont syndrome, but the characteristics of both Wiedemann–Steiner syndrome and Coffin–Siris syndrome are better known nowadays and allow easy differentiation from the phenotype in the reported patients with Pierpont syndrome.2,3 Until now, the cause of Pierpont syndrome has remained unknown. However, de novo autosomal-dominant mutations were suspected to be the most likely cause.2

For the present study, we collected DNA from two newly identified and four earlier-reported patients with Pierpont syndrome. We performed whole-exome sequencing in four patients and in the parents of one of them, and identified identical de novo missense mutations in the transducin β-like 1 X-linked receptor 1 (TBL1XR1) gene in all four patients with Pierpont syndrome. Sanger sequencing of the two newly diagnosed patients revealed the same mutation. TBL1XR1 is part of the nuclear receptor corepressor (NCoR) complex that plays an essential role in gene transcription.

METHODS

Study cohort

The study cohort consisted of six unrelated patients with Pierpont syndrome (table 1). The clinical diagnosis was based on the combination of the facial characteristics, palmar and plantar fat pads, and global developmental delay (figure 1). The description of patients 1–4 has been published.1–3

Blood samples were obtained from all patients and their (unaffected) parents. Informed consent for the study was obtained from the parents of all patients. The Medical Ethics Committee of the Academic Medical Centre in Amsterdam (NL451117.018.13) approved the study.

Molecular analysis

Targeted enrichment and massive parallel sequencing were performed on genomic DNA extracted from circulating leucocytes of four patients and the...
parents of one of them. Enrichment of the whole exome was performed using the Nimblegen SeqCap EZ Library V3.0 (Roche). Each captured library was then loaded on a SOLiD5500xl platform (Applied Biosystems) (patient 20120174 and unaffected parents 20112227 and 20112228, and patients 20112226, 20121069 and 20121072). Paired-end and single-end sequence reads were aligned to hg19 using the Lifescope aligner (V2.5.1) (Applied Biosystems). Presumed PCR duplicates were discarded using Picard Tools (http://picard.sourceforge.net) and dbSNP (V.137), and predictions were made regarding probabilities of being disease causing. Only variants passing all applied GATK filters, predicted to be a de novo mutation within the trio and disease-causing by KGGSeq were retained.

### Protein characterisation

The structure of the WD40 domain of TBL1XR1 was obtained from the Protein Data Bank (PDB accession code 4LG9). For expression in mammalian cells, constructs of TBL1XR1, HDAC3 and GPS2-SMRT chimaera were cloned into the pcDNA3 vector. Transient transfections and protein purifications were performed as described elsewhere. For the TBL1XR1/HDAC3/GPS2-SMRT chimaera complex, the GPS2-SMRT chimaera contained an N-terminal 10×His-3×Flag tag and a tobacco etch virus (TEV) protease cleavage site. HEK293F cells (Invitrogen) were cotransfected with mixtures of both tagged and untagged constructs using polyethyleneimine (PEI) (Sigma). To transfect 60 mL of cells, 60 mg DNA total was diluted in 6 mL of phosphate-buffered saline (PBS) (Sigma) and vortexed briefly, 240 mL of 0.5 mg/mL PEI was added, then vortexed briefly, and incubated for 20 min at room temperature, then added to 60 mL cells (final density 1×10^6 cells/mL). Cells were harvested 48 h after transfection. For the interaction studies the cells were lysed by sonication in 50 mM Tris/Cl pH 7.5, 100 mM potassium acetate, 5% v/v glycerol, 0.3% v/v

### Table 1  Main clinical features of presently described individuals with Pierpont syndrome, including updates of the published patients 1 and 2,1 32 and 43

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Growth parameters at birth</th>
<th>Hypotonia</th>
<th>Brain imaging</th>
<th>Intellectual disability</th>
<th>Hypertonia</th>
<th>Microcornea</th>
<th>Flat malleae</th>
<th>Short fingers/toes</th>
<th>Subcalcanal fat pads</th>
<th>Protein characterisation</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>M</td>
<td>45.7 (&lt;P3)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>2</td>
<td>20</td>
<td>M</td>
<td>50.0 (P50)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>+</td>
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<tr>
<td>3</td>
<td>12</td>
<td>M</td>
<td>48.5 (P10)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4</td>
<td>5.7</td>
<td>M</td>
<td>43.0 (&lt;P3)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>M</td>
<td>51 (P75)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>6</td>
<td>19</td>
<td>F</td>
<td>90.5% and 28.0 (&lt;P3)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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*Centiles between brackets.
†IQ 50–60 ++ IQ 35–50 +++ IQ <35 (IQ data from publications,1–3 formal testing in patient 5, and estimated in patient 6).
‡Cornea diameter<10.0 mm.
§Hearing loss was evaluated by audiometry.
+, abnormality present; –, abnormality not present; A, central atrophy; AE, abnormal dental eruption; CP, choroid plexus papilloma; EV, enlarged ventricles; OFC, occipital frontal circumference; W5, widely spaced; X, no data available.
Triton X-100 and Roche complete protease inhibitor (buffer A); insoluble material was removed by centrifugation. The complex was bound to Flag resin (Sigma), washed three times with buffer A, three times with buffer B (50 mM Tris/Cl pH 7.5, 300 mM potassium acetate, 5% v/v glycerol) and three times with buffer C (50 mM Tris/Cl pH 7.5, 50 mM potassium acetate, 5% v/v glycerol, 0.5 mM tris (carboxyethyl) phosphine (TCEP)). The complex was eluted from the resin by overnight cleavage at 4°C with TEV protease in buffer C.

mRNA expression
We analysed TBL1XR1 mRNA expression in brain tissue (hypothalamus; pituitary gland), in white and brown adipose tissue, in muscle tissue and in liver tissue. In addition we analysed TBL1XR1 RNA expression in lymphocytes of two patients (aged 13 and 20 years, respectively) and four controls (age between 25 and 30 years).

Tissues
Three hypothalami and pituitaries were obtained from the Netherlands Brain Bank in accordance with permission for brain autopsy and the use of human brain material and clinical information for research purposes. Three unfixed, frozen (~80°C) hypothalamus–pituitary specimens were used for mRNA expression. The paraventricular nucleus (PVN) region was cut in serial, coronal 50 μm sections from unfixed frozen hypothalami on a

Figure 1  Face and extremity features in individuals with Pierpont syndrome. Note (A) the high forehead, narrow palpebral fissures, flat malae, broad nasal ridge and tip, thin upper vermilion and large ears (upper row, left to right: patients 1, 2 and 3; lower row, left to right: patients 4, 5 and 6); (B) marked grooves and pillowing of hands and feet, and subcalcaneal fat pads (upper row, left to right: patients 2, 4 and 6; lower row, left to right: patients 2, 4 and 6).
cryostat and the PVN area macroscopically dissected, collected, and stored at −80°C until processing, as previously reported.10 RNA was extracted from the PVN and from homogenised pituitaries using TriReagent (Sigma) per manufacturer’s instructions, followed by DNase treatment (Qiagen GmbH, Germany). cDNA was synthesised with an Applied Biosystem Kit. White adipose tissue cDNA was kindly provided by Drs M Serlie and M Kilicarslan (Department of Endocrinology, AMC, Amsterdam) and synthesised from RNA isolated from subcutaneous, periumbilical adipose tissue biopsies from healthy lean men under local anaesthesia (approved by the Medical Ethics Committee of the Academic Medical Centre in Amsterdam). Liver tissue cDNA was kindly provided by Drs M Serlie and P Gilijamse (Department of Endocrinology, AMC, Amsterdam) and synthesised from RNA isolated from liver tissue biopsies obtained during gastric bypass surgery (approved by the Medical Ethics Committee of the University Medical Centre Maastricht). Muscle cDNA was commercially available and obtained from Clontech, Takara (Mountain View, California, USA). RNA from whole blood of four healthy controls and two patients was isolated using the High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol. cDNA synthesis was performed using the Transcriptor cDNA Synthesis Kit for RT-PCR with oligo d(T) primers (Roche Molecular Biochemicals, Mannheim, Germany). From every sample a −RT reaction was performed in order to check for genomic DNA contamination.

PCR Primers were designed to amplify TBL1XR1 transcript (NM_024665.4, F: 5'-CCATGGCCACTCCAGTAACG-3', R: 5'-TCCAGGCACTTGGTGACACAGA-3'), product size 126 bp, and annealing temperature 65°C. Real-time PCR was performed using the Lightcycler480 and Lightcycler480SybrGreen I Master mix (Roche Molecular Biochemicals, Mannheim). Melting curve analysis was performed and product size was determined by DNA gel analysis. All samples contain mRNA as checked by HPRT expression (hypoxanthine phosphoribosyl transferase, a housekeeping gene).10 Expression levels in whole blood were quantified using the LinReg software.11 The mean efficiency was calculated for each assay and samples that had a deviation of more than 5% were excluded. Calculated values were normalised by HPRT expression.
wise had no signiﬁcant health problems. She developed pectus excavatum and thoracic scoliosis, but other than that she was normal in body weight and little subcutaneous fat tissue. She gradually increased growth in height, a relatively large head, low muscle mass and delayed puberty. Her head circumference was estimated to be below 35 at age 18 years. She had increased body fat from age 12 years. Formal cognitive testing was not possible, but her IQ to be 45. Hearing loss was detected in the first year of life. She had bilateral talipes. Her development was markedly delayed from birth to the age of 1 year. Both her unusual face and unusual feet were evident at birth. She has always had mild fat pads anteromedial to both heels.

RESULTS

Study cohort

Patient 5 was the term, ﬁrst-born child of healthy, non-consanguineous parents. She had intrauterine growth retardation, was hypotonic at birth, and had bilateral hip dislocations. She experienced feeding diffculties with gastrostomy placement in infancy, and followed a markedly delayed motor and cognitive development. She was able to walk with assistance, and had no speech. Formal cognitive testing at age 7 years showed her IQ to be 35. Hearing loss was detected in the ﬁrst year of life. She gradually developed a progressive thoracolumbar scoliosis requiring rod placement at age 10 years. Her postnatal growth in height and of skull circumference was decreased. Onset of menarche was at the age of 11 years. Both her unusual face and abnormal creases of palms and soles were evident at birth. She has always had mild fat pads anteromedial to both heels.

Patient 6 was the ﬁrst-born child of healthy, non-consanguineous parents. She was remarkably hypotonic at birth. At that time it was noticed that she had unusual facial morphology, broad thumbs, deep pillowing of palms and soles, and bilateral talipes. Her development was markedly delayed from early on: she never developed any speech and had no sphincter control. Formal cognitive testing was not possible, but her IQ was estimated to be below 35 at age 18 years. She had increasingly decreased growth in height, a relatively large head, low body weight and little subcutaneous fat tissue. She gradually developed pectus excavatum and thoracic scoliosis, but otherwise had no signiﬁcant health problems.

Molecular analysis

Whole-exome sequencing yielded a single missense mutation c.1337A>C in TBL1XR1 located at 3q26.32, resulting in the amino acid substitution p.Tyr446Cys (Y446C) in all four patients studied, but not in the parents of one of them (see online supplementary table S1). No other potentially pathogenic variant in the same gene was present in all four patients, although the equivalent residue is a phenylalanine in the homologous Sif2 protein from Saccharomyces cerevisiae. The structure of the WD40 domain from Sif2 has also been reported. Despite relatively low sequence identity between TBL1XR1 and Sif2, the structures of their WD40 rings itself are similar. Furthermore, the yeast residue equivalent to Y446, f446, adopts a very similar conformation (ﬁgure 2B) suggesting a conserved function for this largely non-polar amino acid. To conﬁrm that the Y446C mutation does not grossly perturb the fold and behaviour of TBL1XR1 we examined the ability of the TBL1XR1 to assemble correctly with the GPS2:SMRT:HDAC3 complex. As predicted, the mutant protein was readily expressed and puriﬁed and assembled correctly into the TBL1XR1:GPS2:SMRT:HDAC3 complex. As predicted, the mutant protein was readily expressed and puriﬁed and assembled correctly into the TBL1XR1:GPS2:SMRT:HDAC3 complex (ﬁgure 2C and D), suggesting that the molecular pathology of the Y446C mutation is an impaired protein–protein interaction with an as yet unidentified molecular partner rather than a failure to fold correctly.

mRNA expression

TBL1XR1 mRNA expression was well visible in the pituitary and the PVN area of the hypothalamus, as well as in liver and muscle tissue and both white and brown adipose tissue, ﬁtting the clinical symptomatology of Pierpont syndrome (ﬁgure 3A, B).

Stability of mutated complexes

TBL1XR1 is a highly conserved protein found in all eukaryotes (see online supplementary ﬁgure S1). It contains two structured domains: an amino-terminal domain that mediates tetramerisation of the protein and a carboxy-terminal WD40 domain. The TBL1XR1 tyrosine-to-cysteine mutation identiﬁed here is located in the WD40 domain on one side of the inner surface of the WD40 ring (ﬁgure 2A). Y446 is largely exposed to solvent, and mutation to cysteine would not be expected to signiﬁcantly perturb the structure of the domain. A tyrosine in this position is found in nearly all TBL1XR1 proteins, although the equivalent residue is a phenylalanine in the homologous Sif2 protein from Saccharomyces cerevisiae. The structure of the WD40 domain from Sif2 has also been reported. Despite relatively low sequence identity between TBL1XR1 and Sif2, the structures of their WD40 rings itself are similar. Furthermore, the yeast residue equivalent to Y446, f446, adopts a very similar conformation (ﬁgure 2B) suggesting a conserved function for this largely non-polar amino acid. To conﬁrm that the Y446C mutation does not grossly perturb the fold and behaviour of TBL1XR1 we examined the ability of the TBL1XR1 to assemble correctly with the GPS2:SMRT:HDAC3 complex. As predicted, the mutant protein was readily expressed and puriﬁed and assembled correctly into the TBL1XR1:GPS2:SMRT:HDAC3 complex (ﬁgure 2C and D), suggesting that the molecular pathology of the Y446C mutation is an impaired protein–protein interaction with an as yet unidentified molecular partner rather than a failure to fold correctly.
TBL1XR1 mRNA expression in whole blood was lower in patients compared with controls (figure 4). The small number of patients available for analysis precludes statistical analysis to determine whether this difference is significant.

DISCUSSION
In this study, we found a single TBL1XR1 missense mutation in six patients with Pierpont syndrome that was absent in their unaffected parents.

TBL1XR1, a member of the WD40 repeat-containing protein family, is composed of 18 exons. The product of TBL1XR1, TBL1XR1 (or TBLR1; 55 595 Da; 514 amino acids), contains a carboxy-terminal WD40 domain containing eight WD40 repeats and an amino-terminal LisH domain that mediates tetramerisation of the protein and its interactions with NCoR/SMRT and GPS2. TBL1XR1 is an essential component of the NCoR/SMRT corepressor complex (figure 2C), which interacts with nuclear hormone receptors, a family of ligand-dependent transcription factors involved in regulation of gene transcription (figure 5). When bound to unliganded nuclear hormone receptors, corepressors mediate silencing of gene transcription by recruiting chromatin-modifying enzymes. When a nuclear hormone receptor is liganded, corepressors dissociate to relieve repression of transcription. In negatively regulated target genes, corepressors are essential for activation of transcription. The WD40 repeats in TBL1XR1 are thought to be involved in the interaction of the corepressor complex with histones stabilising the association of the complex with the chromatin.

TBL1XR1 may play a regulatory role in the NF-kB pathway and Wnt-mediated transcription. NF-kB transcription requires IKKα to phosphorylate SMRT on chromatin, which recruits TBL1XR1 to the gene promoter. During depletion of TBL1XR1, NF-kB transcription and cell survival are compromised. TBL1XR1 also recruits β-catenin to the Wnt target-gene promoter. In the presence of TBL1XR1, β-catenin is able to remove corepressors from the promoter of Wnt target genes by competitive binding, thereby activating transcription.

TBL1XR1 mutations have been implicated in several phenotypes. Recurrent TBL1XR1 mutations have been described in DNA of lymphatic malignancies, including primary central nervous system lymphomas, acute lymphoblastic leukaemia and Sézary syndrome. The precise mechanisms by which TBL1XR1 mutations contribute to tumourigenesis remain unclear, but it has been hypothesised that loss of TBL1XR1 could compromise the ability of corepressor complexes to inhibit receptor activity, leading to increased activation of receptor target genes. In addition, it has been suggested that TBL1XR1 might act as a tumour-suppressor gene in the lymphatic system, and upregulates VEGF-C inducing lymphangiogenesis in oesophageal squamous cell carcinoma. Contrarily, TBL1XR1 has been nominated as a novel breast cancer oncogene, indicating that any role of TBL1XR1 in tumourigenesis is tissue-specific.

Whole-exome sequencing of 209 children with autism spectrum disorder (ASD) and their parents showed a de novo p.Leu282Pro mutation in TBL1XR1 in one child, who also had intellectual disability. Subsequently, evaluation of 44 candidate genes in 2494 ASD cases identified two de novo TBL1XR1 mutations (p.Leu282Pro, p.Ile397SerfsX19). Apart from intellectual disability, however, these patients had no features in common with Pierpont syndrome (B O’Roak and E Eichler, personal communication 2013). Similarly, a non-dysmorphic patient with intellectual disability, autism and West syndrome was found to have a de novo p.Gly70Asp mutation in TBL1XR1. Three patients, including a mother and child, have been described with a deletion involving only TBL1XR1 in patients who had mild to moderate intellectual disability without autistic behaviour or manifestations of Pierpont syndrome. In addition, three cases with small deletions involving TBL1XR1 and other genes are included in the Decipher database (http://decipher.sanger.ac.uk/). All had intellectual disability, ASD or ASD-like behaviour but lacked physical signs of
Pierpont syndrome (Z Stark, M Decamp, B Dallapiccola, personal communications, 2014). In one additional published patient the phenotype was attributed to a 2.2 Mb deletion at 3q26.3 involving TBL1XR1, but an updated annotation showed that the deletion did not encompass TBL1XR1. This suggests that the phenotype of individuals with a microdeletion 3q26.3 is caused by a loss of function of TBL1XR1, and consists of intellectual disability and frequently ASD, and a phenotype that shows no resemblance to Pierpont syndrome. It remains at present uncertain whether the abundant hypothalamic and pituitary mRNA expression of wild-type TBL1XR1 reported here is related to the intellectual disability in individuals with deletions.

The differences in phenotype in subjects with the Y446C mutation compared with subjects with whole-gene deletions or other TBL1XR1 mutations suggest different pathogenic mechanisms. The Y446C mutation could act in a dominant negative way, in agreement with our finding that the mutant TBL1XR1 Y446C protein is able to assemble into the HDAC3 corepressor complex. Since TBL1XR1 Y446C can form hetero-tetramers with wild-type TBL1XR1, TBL1XR1 Y446C will likely be present in most HDAC3 corepressor complexes, and the impaired or inappropriate protein–protein interactions with an as yet unidentified molecular partner will likely be present ubiquitously.

In conclusion, one specific TBL1XR1 missense mutation is responsible for the phenotype in individuals with Pierpont syndrome. The difference in phenotype between non-ASD Pierpont patients with the Y446C mutation in TBL1XR1 and individuals with a complete deletion or other mutation of TBL1XR1 suggests mutation-specific mechanisms of pathogenesis for ASD. Further studies of the pathogenesis in individuals with deletions and mutations in TBL1XR1 could yield useful insights into the pathogenesis of ASD.

Contributors CAH and RCH collected and analysed data, and wrote the manuscript. AJ, BR and RCH performed exome sequencing data validation and mutation analysis. PJW, AB, OB-S and JWR performed functional studies. FF, RH, RK, AHO, MEP, GB5, FS, ASPV1 and EF collected patient samples and analysed data. RCH conceived the project and supervised the experiments. All authors revised the manuscript critically and approved the final version for publication.

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Patient consent Obtained.

Ethics approval Medical Ethics Committee of the Academic Medical Centre in Amsterdam.

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