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The role of transducin β-like 1 X-linked receptor 1 (TBL1XR1) in thyroid hormone metabolism and action in mice

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Abstract

Transducin β-like 1 X-linked receptor 1 (TBL1XR1) is a WD40 repeat-containing protein and part of the corepressor complex SMRT/NCoR that binds to the thyroid hormone receptor (TR). We recently described a mutation in *TBL1XR1* in patients with Pierpont syndrome. A mouse model bearing this Tbl1xr1 mutation (*Tbl1xr1*$^{Y446C/Y446C}$) displays several aspects of the Pierpont phenotype. Although serum thyroid hormone (TH) concentrations were unremarkable in these mice, tissue TH action might be affected due to the role of TBL1XR1 in the SMRT/NCoR corepressor complex. The aim of the present study was to evaluate tissue TH metabolism and action in a variety of tissues of *Tbl1xr1*$^{Y446C/Y446C}$ mice. We studied the expression of genes involved in TH metabolism and action in tissues of naïve *Tbl1xr1*$^{Y446C/Y446C}$ mice and wild type (WT) mice. In addition, we measured deiodinase activity in liver (Dio1 and Dio3), kidney (Dio1 and Dio3) and BAT (Dio2). No striking differences were observed in the liver, hypothalamus, muscle and BAT between *Tbl1xr1*$^{Y446C/Y446C}$ and WT mice. Pituitary TRα1 mRNA expression was lower in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT, while the mRNA expression of Tshβ and the positively T3-regulated gene *Nmb* were significantly increased in mutant mice. Interestingly, Mct8 expression was markedly higher in WAT and kidney of mutants, resulting in (subtle) changes in T3-regulated gene expression in both WAT and kidney. In conclusion, mice harboring a mutation in TBL1XR1 display minor changes in cellular TH metabolism and action. TH transport via MCT8 might be affected as the expression is increased in WAT and kidney. The mechanisms involved need to be clarified.

Key Words

- thyroid hormone
- TBL1XR1
- co-repressor complex
- thyroid hormone action

Introduction

Transducin β-like 1 X-linked receptor 1 (TBL1XR1) is a WD40 repeat-containing protein and part of NCoR and SMRT corepressor complexes. These complexes interact with nuclear hormone receptors, a family of ligand-dependent transcription factors that play a central role in the regulation of gene transcription. We recently

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identified a mutation c.1337A>G; p.Y446C (rs878854402) in *TBL1XR1* in six male patients with Pierpont syndrome, a condition characterized by a dysmorphic face, developmental delay, altered fat distribution and hearing loss but without hypothyroidism (1, 2, 3, 4). A mutation at the same position in the highly conserved WD40 domain of its close homolog transducin β-like 1X (TBL1X) was found in patients with the combination of hearing loss and isolated central congenital hypothyroidism (CH), a shortage of thyroid hormone (TH) that was already present at birth in affected individuals (5).

To understand the functional consequences of the mutation at the tissue level, we developed an animal model bearing the *Tbl1xr1* Y446C mutation using CRISPR-Cas9 technology. The mice displayed delayed growth, changed body composition and impaired hearing (6). As in humans with Pierpont syndrome, also in the animal model, no differences in serum thyroxine (T4), triiodothyronine (T3) and thyroid-stimulating-hormone (TSH) concentrations were observed between *Tbl1xr1*$^{Y446C/Y446C}$ and wild type (WT) mice (6).

There were no serum TH abnormalities reported in Pierpont patients or *Tbl1xr1*$^{Y446C/Y446C}$ mice. However, circulating TH concentrations are not always consistent with tissue TH availability and action as, for example, seen in patients with resistance to thyroid hormone receptor-α (RTHα) (7). Two highly homologous thyroid hormone receptors (TRs) are known, i.e., TRα and TRβ, with a markedly different tissue distribution. Mutations in these homologous genes lead to markedly different phenotypes reflecting altered TH action in many tissues, but only mutations in *TR*β lead to clearly abnormal serum TH concentrations (8). Both TBL1XR1 and TBL1X are being part of the NCoR/SMRT corepressor complex and have overlapping functions, it is reasonable to assume that a defective TBL1XR1 may have an effect on TH signaling via the affected SMRT/NCoR corepressor complex as observed in patients with mutations in TBL1X. Indeed, we observed changes in some T3-responsive genes in white adipose tissue of *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to the WT (6).

We therefore aimed to get more insight into the functionality of TBL1XR1 and TBL1X in relation to T3-regulated gene expression hypothesizing that TBL1XR1 and TBL1X may differ in their preference for a specific TR. In the present study, we focus on the expression of genes involved in TH metabolism and regulation and on T3-responsive genes in both predominantly TRβ organs (liver, kidney, pituitary and hypothalamus) and TRα organs (muscle, white and brown adipose tissue) in WT mice having a mutation in *TBL1XR1* (*Tbl1xr1*$^{Y446C/Y446C}$).

Materials and methods

Generation of the *Tbl1xr1*$^{Y446C/Y446C}$ mice

Tbl1xr1$^{Y446C/Y446C}$ mice were generated using CRISPR-Cas9 technology as previously described (6). Mice at the age of 12 weeks were sacrificed by exsanguination. Hypothalamus, pituitary, liver, kidney, WAT, BAT and gastrocnemius muscle were collected and immediately frozen on dry ice and stored at −80°C until further processing.

In vitro knockdown of TBL1XR1

The human hepatocellular carcinoma cell line HepG2 (ATCC) was cultured in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin–streptomycin–neomycin (Sigma). The cells were cultured in the medium with low T3 concentrations (Dulbecco's modified Eagle's medium, 10% charcoal-stripped FBS and 1% penicillin–streptomycin–neomycin) for 3 days prior to the experiment reaching 80% confluence. Knockdown of TBL1XR1 was performed by introducing small interference RNA (siRNA) using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's protocol. After transfection, cells were incubated for 24 h at 37°C/5% CO_2 until T3 administration. Three specific siRNAs of TBL1XR1 (siRNA1:IDs36171, siRNA2:IDs36172, siRNA3:IDs36173) and negative control siRNAs (scrambled siRNAs with matching GC content) (Ambion) were tested. Knockdown efficiency was determined by measuring *Tbl1xr1* mRNA expression by qPCR. siRNA2 gave the best knockdown (61%) and was used for further studies. After 24 h of transfection, increasing concentrations T3 (Sigma; 0 nM, 0.1 nM, 1 nM, 10 nM and 100 nM) were added for 24 h. Subsequently, cells were harvested for RNA isolation. Three independent experiments were performed and each containing of a technical triplicate.

RNA isolation and qPCR

Total RNA from the hypothalamus, pituitary, liver, kidney, WAT, BAT and gastrocnemius muscle was isolated as previously described (6). Total RNA from HepG2

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cells was isolated using the High Pure RNA isolation kit (Roche). RNA yield was determined using the Nanodrop (Nanodrop) and cDNA was synthesized with equal RNA input with the Transcriptor First Strand cDNA Synthesis Kit (Roche) for qPCR using oligo-d (T) primers (Roche Molecular Biochemicals). As a control for genomic DNA contamination, a cDNA synthesis reaction without reverse transcriptase was included. Quantitative PCR was performed using the SensiFAST SYBR No-ROX Kit (Bioline). Quantification was performed using the LinReg software. PCR efficiency was checked individually and samples with a deviation of more than 5% of the mean were excluded from the analysis. Calculated values were related to the geometric mean expression of the reference genes *EEF1A1*, *TBP* and *HPRT*, all showing stable expression under the experimental conditions. The primers used for qPCR are listed in Table 1.

Deiodinase activity

The deiodinase activity was measured as previously described (3, 9, 10, 11). Briefly, tissue (BAT, liver and kidney) was homogenized on ice in PED50 buffer (0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 50 mM dithiothreitol (DTT)) using a Polytron (Kinematica, Luzern, Switzerland) and directly used for analysis (BAT) or snap-frozen and stored at −80°C until use. Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard following the manufacturer's instructions (Bio-Rad Laboratories).

Liver and kidney D1 activity was measured using homogenate incubated for 30 min at 37°C with 0.1 µM rT3 and approximately 1 × 10^5 cpm (3,3′,5′-^{125}I) rT3 in PED10 (0.1 M sodium phosphate, 2 mM EDTA pH 7.2, 10 mM DTT). One sample of each group was incubated in the presence of 500 µM PTU to inhibit D1 activity representing a tissue blank. Liver D3 activity was measured using homogenate incubated for 2 h at 37°C with 1 nM T3 or 500 nM T3 and approximately 2 × 10^5 cpm (3,3′,5-^{125}I) T3 in PE buffer. For each group, we included one sample with 500 nM T3 to saturate D3 representing a tissue blank. After deiodinase and tissue-specific incubations, reactions were stopped by adding ice-cold ethanol. After centrifugation and addition of 0.02 M ammonium acetate (pH 4), the mixture was applied to 4.6 × 250 mm Symmetry C18 column connected to a Waters HPLC system (Model 600E pump, Model 717 WISP autosampler, Waters, Etten-Leur, The Netherlands). The activity in the eluate was measured online using a Radiomatic 150 TR flow scintillation analyzer (Perkin Elmer). D1 activity was calculated by subtracting the activity measured in the tissue blank from the activity measured without PTU and expressed as pmol 3,3′ T2 generated per minute per mg protein. D3 activity was calculated by subtracting the activity measured in the tissue blank from the activity with 1 nM T3 and expressed as fmol generated 3,3′ T2 per minute per mg tissue.

D2 activity was measured using fresh tissue homogenate incubated for 2 h at 37°C in the presence of 0.25 M sucrose, 1 nM T4, and approximately 1.10^5 cpm ^{125}I-T4 (in-house, single-labeled tracer according to Wiersinga *et al.* (12)) in phosphate-EDTA buffer (PE buffer, 0.1 M sodium phosphate, 2nM EDTA, pH 7.2). For each group, we included one sample with 0.5 µM T4 incubation to saturate D2 representing a tissue blank. The reaction was stopped by adding bovine serum and 20% trichloroacetic acid (TCA). After centrifugation, released ^{125}I was counted in the supernatant using the 2470 Automatic γ Counter Wizard2 (Perkin-Elmer). The D2 activity measured with the incubation with 1 nM T4 minus the incubation with 500 nM T4 represents true D2 activity. D2 activity was expressed as ^{125}I fmol released per minute per gram protein. The assay is based on the protocol previously described by Werneck-de-Castro *et al.* (13).

Statistics

Data are expressed as mean ± standard error of the mean (S.E.M.). Variations between *Tbl1xr1*$^{Y446C/Y446C}$ and WT mice were evaluated by two-way analysis of variance (ANOVA) using GraphPad Prism 9.0 software with two grouping factors (sex and strain) followed by Tukey's post hoc analysis. If the magnitude and order of the sex differences were similar in mutant and WT mice, we presented male and female results together. The PCR results from HepG2 data were normalized to the mean value of the control-transfected group without T3 stimulation (T3 0nM) per experiment. Data from three repeated experiments were combined and presented in the graph, each dot represents the average value of the technical replicate. The effects of knockdown and T3 administration were analyzed by two-way ANOVA using GraphPad Prism 9.0 software with two grouping factors (knockdown and T3 administration) followed by Tukey's post hoc analysis. Statistical significance was defined at a level of $P < 0.05$.

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Table 1 List of primers used for qPCR.

Gene name	Symbol	Forward (5′-3′)	Reverse (5′-3′)	Product length (bp)
Mouse				
Eukaryotic translation elongation factor 1 alpha 1	Eef1a1	AGTCGCCTTGGACGTTCTT	ATTTGTAGATCAGGTGGCCG	174
Ribosomal protein, large, P0	Rplp0	GGCCCTGCACTCTCGCTTTC	TGCCAGGACGCGCTTGT	124
Hypoxanthine guanine phosphoribosyl transferase	Hprt	GCAGTACAGCCCCAAAATGG	AACAAAGTCTGGCCTGTATCCAA	84
Deiodinase, iodothyronine, type I	Dio1	GAGCAGCCAGCTCTACGC GG	TGGGGAGCC TTCCTGCTG GT	186
Deiodinase, iodothyronine, type II	Dio2	GCTTCCTCC TAGATGCCT ACAA	CCGAGGCAT AATTGTTAC CTG	105
Deiodinase, iodothyronine type III	Dio3	CCAACTCTAGCAGTTCCGCA	GCCTCCCTGGTACATGATGG	83
Solute carrier family 16 (monocarboxylic acid transporters), member 2	Slc16a2 (Mct8)	GTGCTCTTGGTGTGCATTGG	GGGACACCCGCAAAGTAGAA	386
Solute carrier family 16 (monocarboxylic acid transporters), member 10	Slc16a10 (Mct10)	TGATTCCCCTGTGCAGCGCC	CCACGTCGTAGGTGCCCAGC	228
Solute carrier organic anion transporter family, member 1c1	Slco1c1	ATCACAGAACAAAATAAGTCACGAA	GATTTCCCAGGAAGACATAAACC	77
Thyroid hormone receptor alpha	Thra	CATCTTTGA ACTGGGCAA GT	CTGAGGCTT TAGACTTCC TGATC	347
Thyroid hormone receptor beta1	Thrb1	CACCTGGAT CCTGACGAT GT	ACAGGTGAT GCAGCGATA GT	167
Thyroid hormone receptor beta2	Thrb2	GTGAATCAG CCTTATACC TG	ACAGGTGAT GCAGCGATA GT	255
Thyroid-stimulating hormone receptor	Tshr	GCTCATTCT GCTAACCAG CC	GCGAAAACA GTGAAGAAG CC	204
Thyrotropin-releasing hormone receptor	Trhr	CAACAGATGCTTCAACAGCAC	TTACAACCACTGCGAGCATC	68
Thyroid-stimulating hormone beta subunit	Tshb	TCAACACCA CCATCTGTG CT	TTGCCACAC TTGCAGCTT AC	196
TRH-degrading enzyme	Trhde	TGGCCTTGAACACAACTGGT	GCGCAAAACTGCCATCTCAA	131
Neuromedin B	Nmb	CTTCGCATTGTTCGCTTCCG	CTAGAGCTTTCTTTCGCAGGAG	248
Kruppel-like factor 9	Klf9	CCACCGAATCTGGGTCGAG	TCCGAGCGCGAGAACTTTT	265
ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	Serca1	GGAATGCAGAGAACGCTATCG	TCCTTTGCACTGACTTTCGGT	90
ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Serca2a	AATCTGACCCAGTGGCTGATG	AGAGGGCTGGTAGATGTGTTG	197
Myosin, heavy polypeptide 1, skeletal muscle, adult	Myh1	CGGAGTCAGGTGAATACTCACG	GAGCATGAGCTAAGGCACTCT	153
Myosin, heavy polypeptide 2, skeletal muscle, adult	Myh2	TGGAGGGTGAGGTAGAGAGTG	TTGGATAGATTTGTGTTGGATTG	220
Myosin, heavy polypeptide 4, skeletal muscle	Myh4	CACCTGGACGATGCTCTCAGA	GCTCTTGCTCGGCCACTCT	150
Myosin, heavy polypeptide 7, cardiac muscle, beta	Myh7	ACTGTCAACACTAAGAGGGTCA	TTGGATGATTTGATCTTCCAGGG	114
Myogenin	Myog	TTGCTCAGCTCCCTCAACCAGGA	TGCAGATTGTGGGCGTCTGTAGG	193
Lysine demethylase and nuclear receptor corepressor	Hr	CGGAGACAATCATAGGAAGCAAG	CCGGTCAGTACCCCTACCT	184
Glycoprotein hormones, alpha subunit	Cga	GATCGACAATCACCTGCCCA	GTTTACATTCTGGGCAACCCTG	178
Phosphoenolpyruvate carboxykinase 1, cytosolic	Pck1	ATGTTCGGGCGGATTGAAG	TCAGGTTCAAGGCGTTTTCC	81
Thyroid hormone responsive	Thrsp	CATCCTTACCCACCTGACCC	TGTCCAGGTCTCGGGTTGAT	157
Carnitine palmitoyltransferase 1a, liver	Cpt1a	AAA GAT CAA TCG GAC CCT AGA CA	CAG CGA GTA GCG CAT AGT CA	123
Glucose-6-phosphatase, catalytic	G6pc	TCA ACC TCG TCT TCA AGT GGA TT	TGT AGT AGT CGG TGT CCA GGA CC	71
Human				
TBL1X/Y related 1	TBL1XR1	CCATGGCCAGTCCACTACAG	TCCAGCACTTGGTGAACAGA	126
Hypoxanthine phosphoribosyltransferase 1	HPRT	CCTGCTGGATTACATCAAAGCACTG	TCCAACACTTCGTGGGGTCCT	289

(Continued)

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Table 1 Continued.

Gene name	Symbol	Forward (5′-3′)	Reverse (5′-3′)	Product length (bp)
Eukaryotic translation elongation factor 1 alpha 1	EEF1A1	TTTTCGCAACGGGTTTGCC	TTGCCCGAATCTACGTGTCC	120
TATA-box binding protein	TBP	CCCGAAACGCCGAATATAATCC	AATCAGTGCCGTGGTTCGTG	80
KLF transcription factor 9	KLF9	CCTCCCATCTCAAAGCCCATT	CGCCTTTTTCGATCGCTTGAT	248
Iodothyronine deiodinase 1	DIO1	TGGTTCGTCTTGAAGGTCCG	AAATTCAGCACCAGTGGCCT	149
Thyroid hormone responsive	THRSP	CGAGAAAGCCCAGGAGGTGA	AGCATCCCGGAGAACTGAGC	204
Carnitine palmitoyltransferase 1A	CPT1A	TGTGCTGGATGGTGTCTGTCTC	CGTCTTTTGGGATCCACGATT	100
Glucose-6-phosphatase catalytic subunit 1	G6PC1	GACTGGCTCAACCTCGTCTT	CGTAGTATACACCTGCTGTGCC	181
Phosphoenolpyruvate carboxykinase 1	PCK1	GCTGGTGTCCCTCTAGTCTATG	GGTATTTGCCGAAGTTGTAG	166

Results

Thyroid hormone metabolism in TRα tissues

To investigate TH metabolism, we determined mRNA expression of *Dio1, Dio2* and *Dio3*, *Mct8*, *Mct10*, and *Thra1* and *Thrb1* in the gastrocnemius muscle, BAT and WAT, all known as TRα tissues (Fig. 1). D2 activity was measured in BAT. No differences in expression levels of genes involved in TH metabolism were observed in the gastrocnemius of *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT mice. In BAT, *Dio2* mRNA expression was significantly decreased in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT mice, which agrees with the trend in D2 activity in *Tbl1xr1*$^{Y446C/Y446C}$ mice. We therefore measured *Tshr* mRNA expression in BAT which was also decreased in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to the WT mice. The expression of genes involved in TH metabolism in WAT was similar in *Tbl1xr1*$^{Y446C/Y446C}$ mice and WT mice, except for the expression of *Mct8,* which was markedly higher in WT mice.

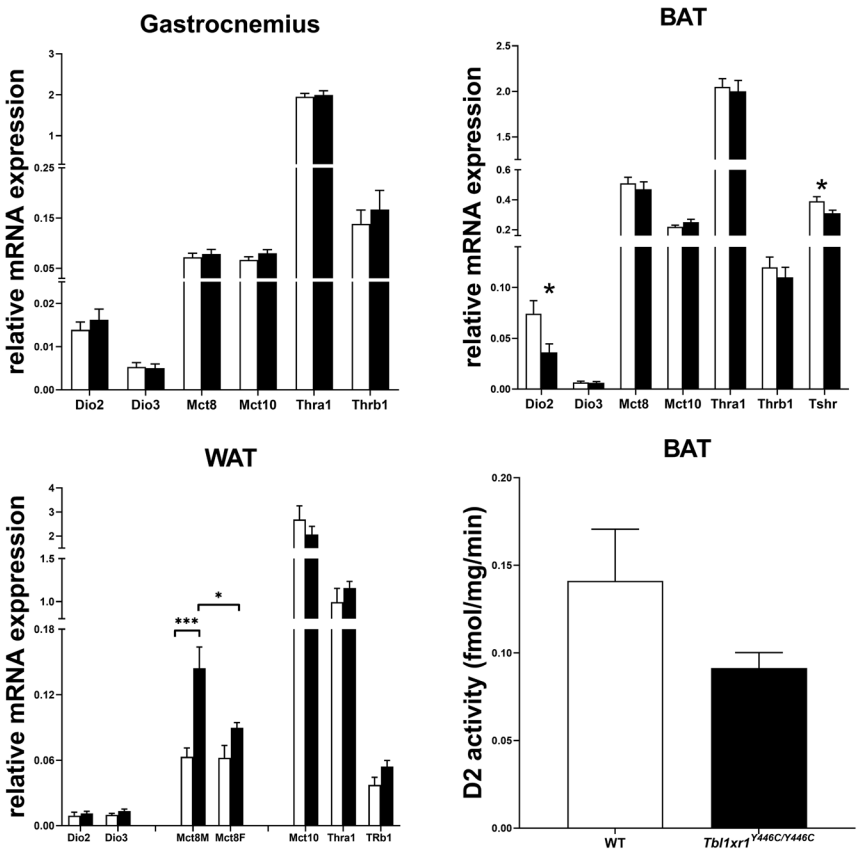

Figure 1
Relative RNA expression of TH metabolism genes and deiodinase activity in TRα tissues (gastrocnemius muscle, BAT and WAT) in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to age- and sex-matched *Tbl1xr1*$^{+/+}$ mice. Relative RNA expression is measured by qPCR analysis. White bars indicate *Tbl1xr1*$^{+/+}$ and black bars indicate *Tbl1xr1*$^{Y446C/Y446C}$ mice. Four groups in total: male-WT, male mutant, female-WT, female-mutant. There were 6–8 animals per group. Combined data of male and female are shown (expect for Mct8) since there is no sex difference or the sex differences were similar in both mutant and WT mice. Data are expressed as mean ± S.E.M.; differences between the groups were analyzed using two-way ANOVA; *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

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Thyroid hormone metabolism in TRβ tissues

To investigate TH metabolism in TRβ tissues, we determined mRNA expression of *Dio1, Dio2* and *Dio3*, *Mct8, Mct10, Slco1c1*, and *Thra1* and *Thrb1* in the hypothalamus, pituitary, liver and kidney (Fig. 2). D1 activity was measured in the liver and kidney, and D3 activity in the liver. No differences in the expression of genes involved in TH metabolism were observed in the hypothalamus and the liver of *Tbl1xr1*$^{Y446C/Y446C}$ and WT mice. In line, liver D1 and D3 activity were similar.

In the pituitary, *Thra1* expression was significantly lower in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT mice, while *Trhr* expression was higher. No differences in *Dio1*, *Thrb1, Mct10* expression were observed in the kidneys of *Tbl1xr1*$^{Y446C/Y446C}$ and WT mice, while *Mct8* expression was markedly higher in the kidney of *Tbl1xr1*$^{Y446C/Y446C}$ mice.

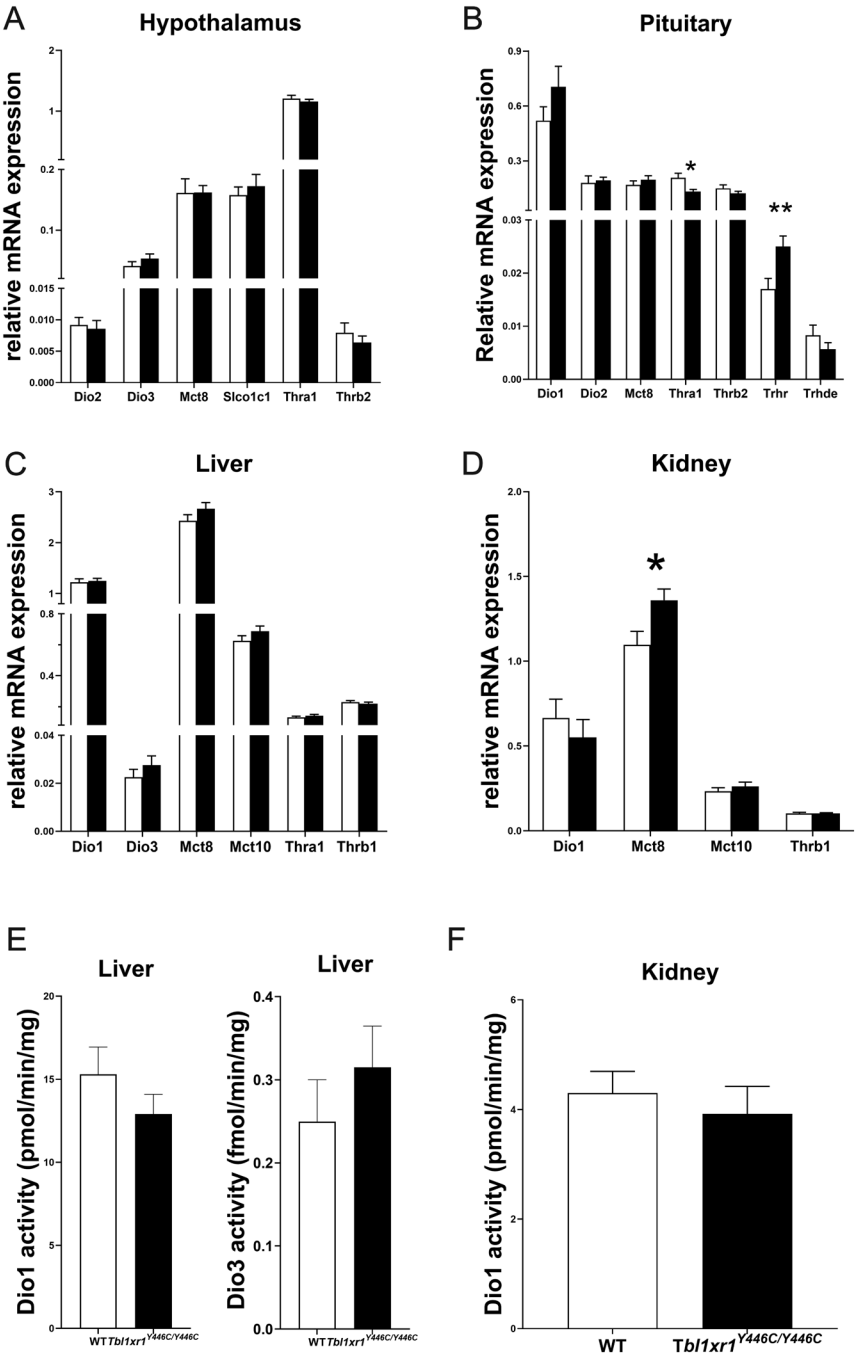

Figure 2
Relative RNA expression of TH metabolism genes and deiodinase activity in TRβ tissues (hypothalamus, pituitary, liver and kidney) in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to age- and sex-matched *Tbl1xr1*$^{+/+}$ mice. Relative RNA expression is measured by qPCR analysis. White bars indicate *Tbl1xr1*$^{+/+}$ and black bars indicate *Tbl1xr1*$^{Y446C/Y446C}$ mice. Four groups in total: male-WT, male mutant, female-WT, female-mutant. There were 6–8 animals per group. Combined data of male and female are shown since there is no sex difference or the sex differences were similar in both mutant and WT mice. Data are expressed as mean ± S.E.M.; differences between the groups were analyzed using two-way ANOVA; *($P < 0.05$), **($P < 0.01$).

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Thyroid-hormone-responsive gene expression in TRα tissues

Thyroid hormone action in the gastrocnemius was assessed by measuring mRNA expression of the following TH-responsive genes: *Klf9, Serca1, Serca2a, Myh1, Myh2, Myh4, Myh7* and *Myog* (Fig. 3). No differences in mRNA expression were observed between *Tbl1xr1*$^{Y446C/Y446C}$ and WT mice, except for *Serca1* which was slightly higher in the gastrocnemius of *Tbl1xr1*$^{Y446C/Y446C}$ mice. The TH action was assessed in BAT and WAT by measuring mRNA expression of *Klf9*. This was higher in WAT of *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT mice, while no difference was observed in BAT.

Thyroid-hormone-responsive genes in TRβ tissues

TH action in the hypothalamus was assessed by measuring mRNA expression of the TH-responsive gene Hairless (*Hr*) which was not changed in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT mice. TH action in the pituitary was assessed by measuring mRNA expression of the negatively T3-regulated genes *Tshb* and *Cga* and the positively T3-regulated gene *Nmb*. Pituitary *Tshb* and *Nmb* expression was higher in *Tbl1xr1*$^{Y446C/Y446C}$ mice than in WT mice (Fig. 4).

In the liver and kidney, TH action was assessed by measuring mRNA expression of the following TH-responsive genes *Klf9*, *Thrsp*, *G6pc*, *Cpt1a* and *Pck1* (liver *Pck1* data is published in (6)). No differences in mRNA expression were observed in the liver while *Pck1* mRNA expression in the kidney was higher in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT mice (Fig. 4).

Sex difference of thyroid hormone metabolism gene and responsive genes

Sex-specific differences in thyroid homeostasis can occur (14, 15, 16), which is confirmed in the present study (Table 2). Sex differences were observed in *Mct8* expression in the kidney and liver, *Mct10* expression in the kidney, liver and gastrocnemius and *Oatp1c1* expression in the hypothalamus. Deiodinase expression differed between male and female mice in the kidney (*Dio1*), WAT (*Dio2*) and liver *Dio3*. TR expression also differed between males and females: *TRα1* in the hypothalamus and liver, *TRβ1* in BAT and *TRβ2* in the pituitary. As a result, sex differences were also observed in some TH-responsive genes in the tested organs: *Myog* in the gastrocnemius, *Cpt1a* in the liver and kidney, and *Klf9*, *Thrsp* and *Pck1* in the kidney (Table 3). However, the magnitude and order of the sex differences were similar in mutant and WT mice.

Effect of TBL1XR1 knockdown on T3-regulated gene expression in HepG2 cells

To evaluate the effect of *TBL1X* knockdown on T3-regulated gene expression in HepG2 cells, mRNA expression of *DIO1*, *KLF9*, *THRSP*, *CPT1A*, *G6PC* and *PCK1* was measured (Fig. 5). All genes were responsive to T3. The T3-induced increase in *DIO1*, *THRSP,G6PC* and

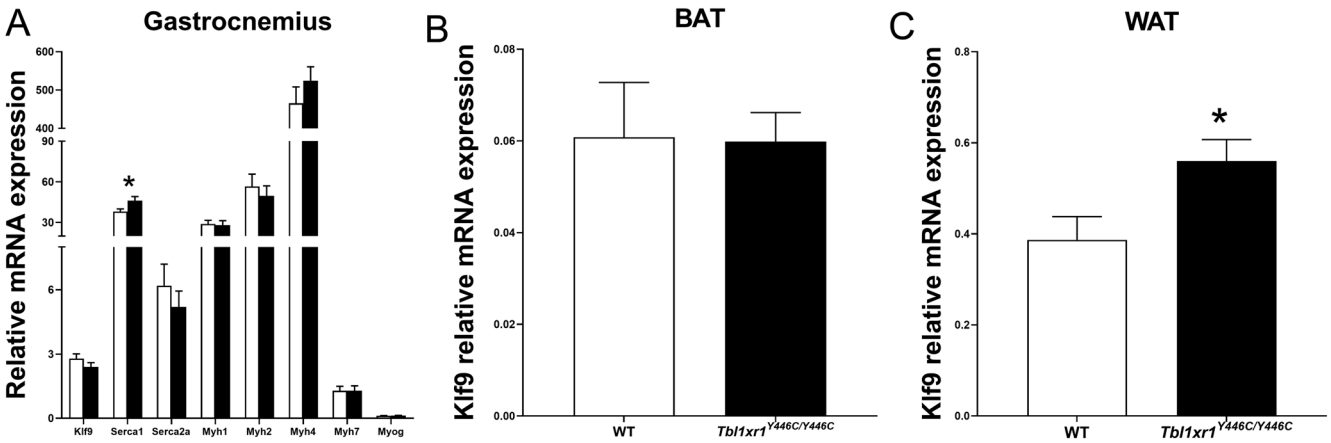

Figure 3
Relative RNA expression of TH-responsive genes in TRα tissues (gastrocnemius muscle, BAT and WAT) in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to age- and sex-matched *Tbl1xr1*$^{+/+}$ mice. Relative RNA expression is measured by qPCR analysis. White bars indicate *Tbl1xr1*$^{+/+}$ and black bars indicate *Tbl1xr1*$^{Y446C/Y446C}$ mice. Four groups in total: male-WT, male mutant, female-WT, female-mutant. There were 6–8 animals per group. Combined data of male and female are shown since there is no sex difference or the sex differences were similar in both mutant and WT mice. Data are expressed as mean ± S.E.M .; differences between the groups were analyzed using two-way ANOVA; *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

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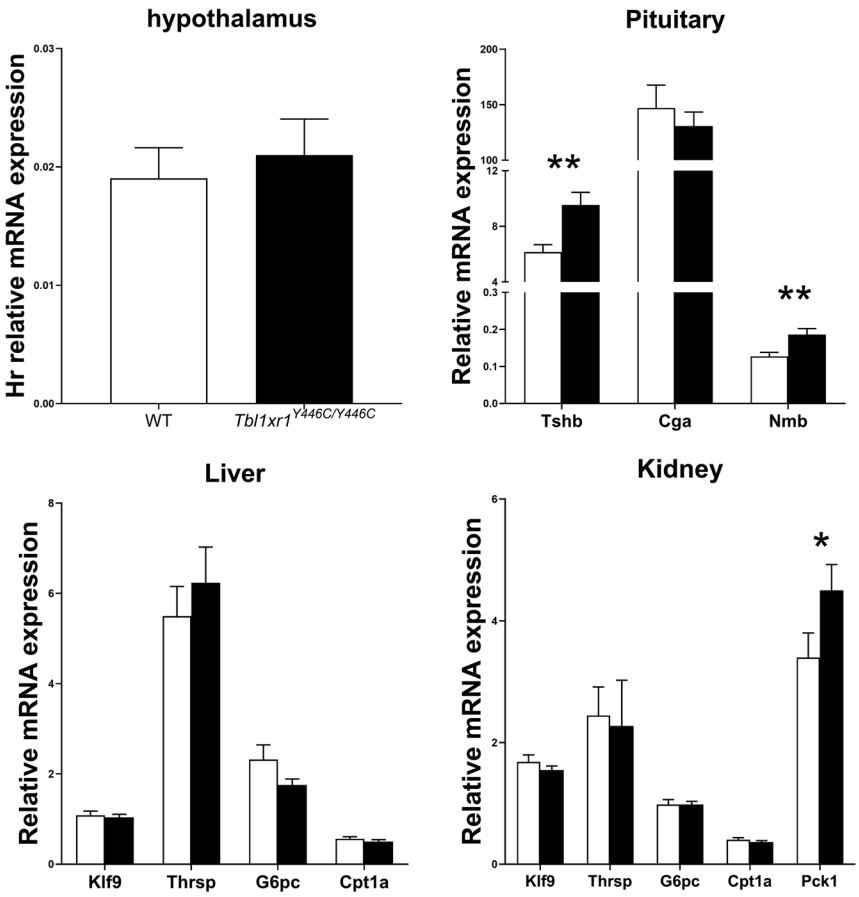

Figure 4
Relative RNA expression of TH-responsive genes in TRβ tissues (hypothalamus, pituitary, liver and kidney) in *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to age- and sex-matched *Tbl1xr1*$^{+/+}$ mice. Relative RNA expression is measured by qPCR analysis. White bars indicate *Tbl1xr1*$^{+/+}$ and black bars indicate *Tbl1xr1*$^{Y446C/Y446C}$ mice. Four groups in total: male-WT, male mutant, female-WT, female-mutant. There were 6–8 animals per group. Combined data of male and female are shown since there is no sex difference or the sex differences were similar in both mutant and WT mice. Data are expressed as mean ± S.E.M.; differences between the groups were analyzed using two-way ANOVA; *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

PCK1 expression was impaired by knocking down *TBL1XR1* of which *DIO1* mRNA expression was severely affected (P_{kd} < 0.0001). Knockdown of *TBL1XR1* did not affect *KLF9* and *THRSP* mRNA expression upon T3 stimulation.

Discussion

The present study aimed to evaluate tissue TH metabolism and TH target gene expression in predominantly TRα organs (muscle, WAT and BAT) and TRβ organs (liver, kidney, pituitary and hypothalamus) of mice harboring a mutation in *Tbl1xr1*, a gene encoding a WD40 repeat-containing protein. TBL1XR1 is part of the SMRT/NCoR corepressor complex that binds to nuclear receptors and controls gene transcription. Previous work performed on two patient cohorts (Pierpont syndrome and isolated central CH with impaired hearing) unraveled two identical mutations in the two close homologs *TBL1XR1* and *TBL1X* (4, 5). TBL1X is also part of NCoR and SMRT corepressor complexes and is involved in TH signaling. Despite the fact that TBL1XR1 is part of the same corepressor complex, hypothyroidism was not reported in Pierpont patients, and the mice harboring the *Tbl1xr1* mutation did not show any abnormalities in serum TH concentrations. The striking differences in the clinical phenotypes of central CH and Pierpont patients are difficult to reconcile with overlapping functions of nearly identical proteins and suggests a differentiation in the interaction with other proteins in the corepressor complex. An attractive candidate for this is the TR. Three bonafide (T3 binding) TR isoforms can be distinguished, the TRα1, TRβ1 and TRβ2, with a clearly differential tissue distribution. Mutations in these genes are known to lead to different phenotypes reflecting altered TH action in many tissues, whereas only mutations in TRβ lead to clearly abnormal serum thyroid hormone concentrations, reflecting the important role of selective TRβ2 expression in the hypothalamus and anterior pituitary in negative feedback regulation (17). Although *Thra1* expression in the hypothalamus and pituitary is substantial, we consider both organs as typically TRβ because of the abovementioned reason.

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Table 2 Differences in mRNA expression of genes involved in the TH metabolism genes in TRα and TRβ tissues of *Tbl1xr1*$^{Y446C/Y446C}$ (mutant) and WT mice analyzed by two-way ANOVA. Symbols indicate difference between groups.

Tissue	Gene	Genotype difference	Sex difference	Interaction	WT vs mutant male	WT vs mutant female	Male vs female WT	Male vs female mutant
Gastrocnemius	Dio2	ns	ns	**	ns	ns	ns	ns
	Dio3	ns	ns	ns	–	–	–	–
	Mct8	ns	ns	ns	–	–	–	–
	Mct10	ns	**	ns	ns	ns	ns	*
	Thra1	ns	ns	ns	–	–	–	–
	Thrb1	ns	ns	ns	–	–	–	–
BAT	Dio2	*	ns	ns	ns	ns	ns	ns
	Dio3	ns	ns	ns	–	–	–	–
	Mct8	ns	ns	ns	–	–	–	–
	Mct10	ns	ns	*	ns	ns	ns	ns
	Thra1	ns	ns	ns	–	–	–	–
	Thrb	ns	*	ns	ns	ns	ns	ns
	Tshr	*	ns	ns	ns	ns	ns	ns
WAT	Dio2	ns	*	ns	ns	ns	ns	ns
	Dio3	ns	ns	ns	–	–	–	–
	Mct8	***	*	*	***	ns	ns	*
	Mct10	ns	ns	ns	–	–	–	–
	Thra1	ns	ns	ns	–	–	–	–
	Thrb1	ns	ns	ns	–	–	–	–
Tissue	**Gene**	**Genotype difference**	**Sex difference**	**Interaction**	**WT vs mutant male**	**WT vs mutant female**	**Male vs female WT**	**Male vs female mutant**
Pituitary	Dio1	ns	ns	ns	–	–	–	–
	Dio2	ns	ns	ns	–	–	–	–
	Mct8	ns	ns	ns	–	–	–	–
	Thra1	*	ns	ns	ns	ns	ns	ns
	Thrb2	ns	**	ns	ns	ns	*	ns
	Trhr	**	ns	ns	ns	ns	ns	ns
	Trhde	ns	ns	ns	–	–	–	–
Hypothalamus	Dio2	ns	ns	ns	–	–	–	–
	Dio3	ns	ns	ns	–	–	–	–
	Mct8	ns	ns	ns	–	–	–	–
	Slco1c1	ns	**	ns	ns	ns	ns	*
	Thra1	ns	*	ns	ns	ns	ns	ns
	Thrb2	ns	ns	*	ns	ns	ns	ns
Kidney	Dio1	ns	***	ns	ns	ns	***	***
	Mct8	*	*	ns	ns	ns	ns	ns
	Mct10	ns	***	ns	ns	ns	ns	*
	Thrb1	ns	ns	ns	–	–	–	–
Liver	Dio1	ns	ns	ns	–	–	–	–
	Dio3	ns	***	ns	ns	ns	***	***
	Mct8	ns	***	ns	ns	ns	**	**
	Mct10	ns	**	ns	ns	ns	ns	*
	Thra1	ns	*	ns	ns	ns	ns	ns
	Thrb1	ns	ns	ns	–	–	–	–

*P < 0.05; **P < 0.01; ***P < 0.001.

Recently, we have reported a homozygous mouse model of Pierpont syndrome, carrying the Y446C point mutation. These mice display several phenotypical characteristics of Pierpont syndrome including delayed growth and impaired hearing (6), while serum TH concentrations were unaffected. We decided to extend our observations in these mice by studying TH metabolism and action in selected TRα and TRβ organs in order to unravel the potential role of TBL1XR1. Ideally, one would also perform the same set of experiments in mice harboring the identical mutation in *TBL1X* but unfortunately, the generation of such a model using CRISPR-Cas9

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Table 3 Differences in mRNA expression of thyroid-hormone-responsive genes in TRα and TRβ tissues of *Tbl1xr1*$^{Y446C/Y446C}$ (mutant) and WT mice analyzed by two-way ANOVA. Symbols indicate difference between groups

Tissue	Gene	Genotype difference	Sex difference	Interaction	WT vs mutant male	WT vs mutant female	Male vs female WT	Male vs female mutant
Gastrocnemius	*Klf9*	ns	ns	ns	–	–	–	–
	Serca1	*	ns	ns	ns	ns	ns	ns
	Serca2a	ns	ns	ns	–	–	–	–
	Myh1	ns	ns	ns	–	–	–	–
	Myh2	ns	ns	ns	–	–	–	–
	Myh4	ns	ns	ns	–	–	–	–
	Myh7	ns	ns	ns	–	–	–	–
	Myog	ns	***	ns	ns	ns	ns	**
BAT	*Klf9*	ns	ns	ns	–	–	–	–
WAT	*Klf9*	*	ns	ns	*	ns	ns	ns
Tissue	**Gene**	**Genotype difference**	**Sex difference**	**Interaction**	**WT vs. mutant male**	**WT vs. mutant female**	**Male vs. female WT**	**Male vs. female mutant**
Pituitary	*Cga*	ns	ns	ns	–	–	–	–
	Tshb	**	ns	ns	ns	*	ns	ns
	Nmb	**	ns	ns	ns	ns	ns	ns
Hypothalamus	*Hr*	ns	ns	ns	–	–	–	–
Kidney	*Klf9*	ns	**	ns	ns	ns	ns	ns
	Cpt1a	ns	***	ns	ns	ns	ns	ns
	G6pc	ns	ns	ns	–	–	–	–
	Thrsp	ns	*	ns	ns	ns	ns	ns
	Pck1	*	**	ns	ns	*	ns	**
Liver	*Klf9*	ns	ns	ns	–	–	–	–
	Thrsp	ns	ns	ns	–	–	–	–
	G6pc	ns	ns	ns	–	–	–	–
	Cpt1a	ns	*	ns	ns	ns	ns	ns

*P < 0.05; **P < 0.01; ***P < 0.001.

technology failed (Y Hu, G Codner, M Stewart, SE Fleur, P van Trotsenburg, E Fliers, RC Hennekam & A Boelen, unpublished data).

Overall, no striking differences in genes involved in the TH metabolism were observed in TRα and TRβ organs of *Tbl1xr1*$^{Y446C/Y446C}$ mice compared to WT mice suggesting that TBL1XR1 is not involved in the basal regulation of Dio1 and Dio3. Interestingly, T3-induced *DIO1* mRNA expression in HepG2 cells (human cell line) was markedly impaired if TBL1XR1 was knocked down suggesting a role for TBL1RX1 in the activation of DIO1. *Dio2* expression was lower in BAT of mutant mice just as the expression of *Tshr*. It is known that the TSHR in BAT is involved in the regulation of thermogenesis by increasing uncoupling protein-1 (Ucp1) which is also responsive to T3 (18). However, mRNA expression of *Mct8* and *Mct10* and *Thra1* in BAT did not differ in *Tbl1xr1*$^{Y446C/Y446C}$ mice, and *Klf9* remained stable. Previously, we have shown that a variety of T3-responsive genes involved in fat and glucose metabolism (*Thrsp, Fasn, Ucp1, Me, Glut4, Ppargc1a, Pck1*) were unaltered in BAT of mutant mice (6). Together, these observations suggest that local TH activity in BAT is not affected by TBL1XR1 in spite of decreased *Dio2* and *Tshr* expression.

Mct8 mRNA expression was higher in WAT and kidney of *Tbl1xr1*$^{Y446C/Y446C}$ mice, suggesting a role of TBL1XR1 in the regulation of kidney MCT8. MCT8 is an important TH transporter and is differentially expressed in cell types. Elevated MCT8 expression was found in human placenta associated with severe intrauterine growth restriction (associated with fetal hypothyroxinaemia), in femurs of hypothyroid mice, and in liver and skeletal muscle of critically ill patients (low-circulating TH concentrations) and rabbits (19, 20, 21). However, it is difficult to say that TH status is a modulator of MCT8, as liver Mct8 mRNA expression in mice was not affected by acute or chronic T3 administration (22). Furthermore, MCT8 protein expression was undetectable in the hypothalamus of a hyperthyroid subject (23) and athyroid Pax8 knockout mice showed unaffected Mct8 expression in the brain

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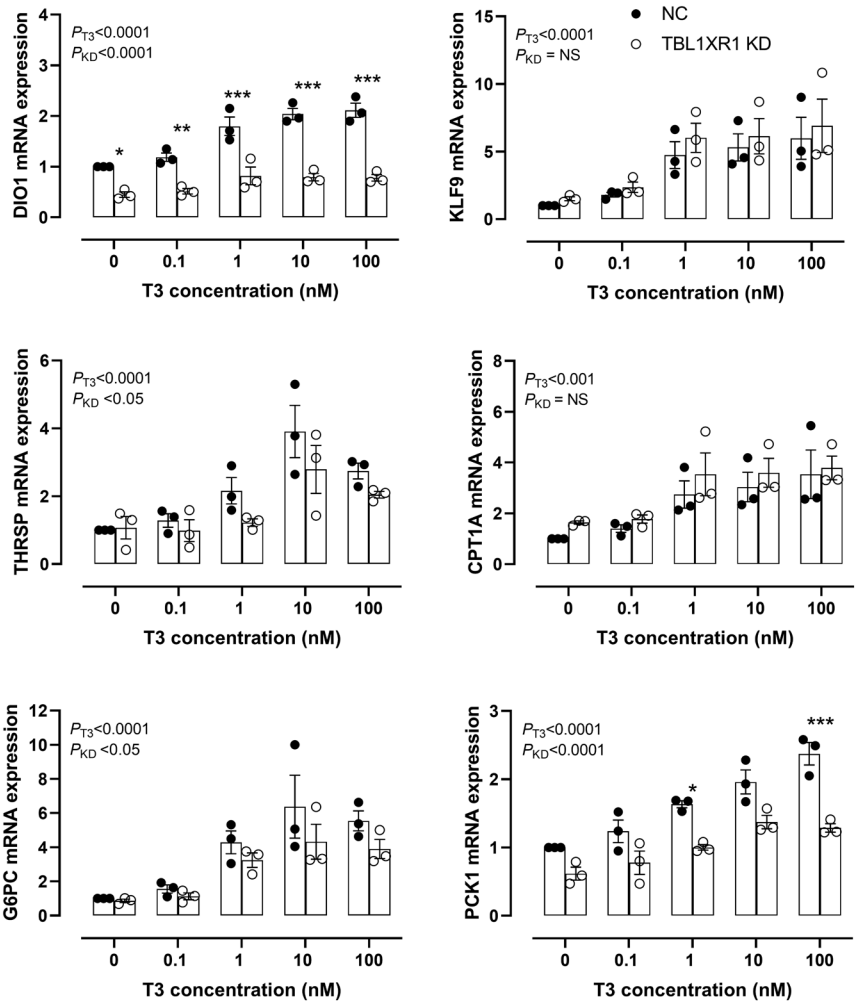

Figure 5
Effects of TBL1XR1 knockdown on T3-regulated gene expression in HepG2 cells. The control situation is represented by the black dots and the TBL1XR1 knockdown is represented by white dots. Cells are stimulated with increasing concentrations of T3. Relative mRNA expression of the genes is normalized to the control group without T3 which is set at 1. Mean values ± S.E.M. of three independent experiments (THRSP only two experiments) are shown, each group of each experiment consists a technical triplicate. *P*-values represent the effect of T3 treatment and TBL1XR1 knockdown analyzed using two-way ANOVA; differences between the groups at a specific T3 concentration were given by symbols: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$).

(24). Therefore, other mechanisms regulating MCT8 remain to be elucidated. The increase in *Mct8* expression in WAT was associated with increased expression of *Klf9* compared to WT mice. Previously, we showed that the mRNA expression of several other T3-responsive genes (*Pparα*, *Ucp1, Fasn*) was also significantly increased in WAT of mutant mice (6). Our experimental setup does not allow to discriminate between the possibility that TBL1XR1 is involved in the regulation of MCT8, thereby increasing intracellular T3 availability and T3-regulated gene expression or, alternatively, that TBL1XR1 is involved in T3-regulated gene expression by itself, possibly via the TRα in WAT. Either way, a WAT phenotype is present in Pierpont patients (4) and in the mouse model (6), which supports the role of TBL1XR1 in adipose tissue. The molecular mechanism involved remains to be established.

The increase in *Mct8* in the kidney was associated with increased expression of *Pck1* (a T3-responsive gene), while other T3-regulated genes were not affected in the kidney of mutant mice. Increased *Mct8* and T3-responsive gene expression has been observed in both WAT and kidney, while WAT is a TRα organ (25) and the kidney predominantly expresses TRβ1 (8).

In contrast to WAT and BAT, *Dio2, Dio3*, *Thra1*, *Mct8* and *Mct10* expression did not differ in the gastrocnemius of mice harboring the TBL1XR1 mutation. Also, most of the T3-responsive genes (*Klf9*, *Serca2a*, *Myh1*, *Myh2*, *Myh4*, *Myog* and *Myh7*) studied were similar in mutant and WT mice. Only *Serca1* mRNA expression was higher in gastrocnemius of *Tbl1xr1*$^{Y446C/Y446C}$ mice. As none of the other T3-responsive genes were affected it might be possible that TBL1XR1 has an effect on *Serca1* via other nuclear receptors.

In contrast to other TRβ organs, the pituitary of mutant mice displayed significant changes in the expression of T3-regulated genes while *Dio1* and *Dio2* and *Mct8* did not differ. *Tshb* is negatively regulated by

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T3 (17) and was found to be increased in the pituitary of mutant mice, while the expression of *Nmb*, a positively regulated gene (26), also increased. These changes are difficult to reconcile with altered TH action. Pituitary *Trhr* expression was significantly increased in *Tbl1xr1*$^{Y446C/Y446C}$ mice, the expression of which is regulated among others by thyrotropin-releasing hormone (TRH). However, using *in situ* hybridization, we showed earlier that TRH expression is unaltered in the paraventricular nucleus of the mutant mice (6).

We observed the changes of a variety of T3-responsive genes in the tissues of *Tbl1xr1*$^{Y446C/Y446C}$ mice. However, a limitation of our study is that these experiments were performed in the basal state and not after stimulation of T3. To further explore the effect of TBL1XR1 on T3 signaling, we knocked down TBL1XR1 in a human liver cell line (HepG2) and stimulated these cells with increasing concentrations of T3. Six well-known T3-responsive genes were evaluated and we showed that T3-induced mRNA expression of *DIO1*, *THRSP*, *G6PC* and *PCK1* was significantly lower in TBL1XR1 knockdown cells compared to control cells. Although the molecular mechanism is still unknown, it is clear that TBL1XR1 is differentially involved in T3 signaling. Interestingly, TBL1XR1 knockdown resulted in impaired *DIO1*, *THRSP*, *G6PC* and *PCK1* mRNA expression upon T3 stimulation which is in contrast to the expectation that a disturbed corepressor complex leads to T3 hypersensitivity as observed in mice lacking NCOR in the liver (27). Our results suggest that TBL1XR1 function as a coactivator instead as a corepressor. Indeed, TBL1XR1 is required for transcriptional activation by many nuclear receptors (28, 29).

In conclusion, in the present study we evaluated tissue TH metabolism and action in a variety of tissues of *Tbl1xr1*$^{Y446C/Y446C}$ mice (Fig. 6). We speculated that the differences in phenotypes between patients with Pierpont syndrome and isolated central CH based on mutations in *TBL1XR1* and *TBL1X,* respectively, were related to the differential effects of these proteins on TRα and TRβ. However, our results do not allow for the conclusion that TBL1XR1, as part of the corepressor complex, preferably binds to the TRα. This is illustrated by the observation that in WAT of mutant mice, both *Klf9* and *Fasn* expression were upregulated, as Klf9 is positively regulated by T3 via TRα1 (30), while Fasn is positively regulated by T3 via the TRβ1 (31). In addition, knockdown of TBL1XR1 in HepG2 cells resulted in impaired expression of *DIO1*, *THRSP*, *G6PC* and *PCK1* mRNA expression upon increasing T3 concentrations, which suggests that TBL1XR1 may have an effect on T3 signaling via being a coactivator. Therefore, further studies are necessary to unravel the role of TBL1XR1 in T3 signaling.

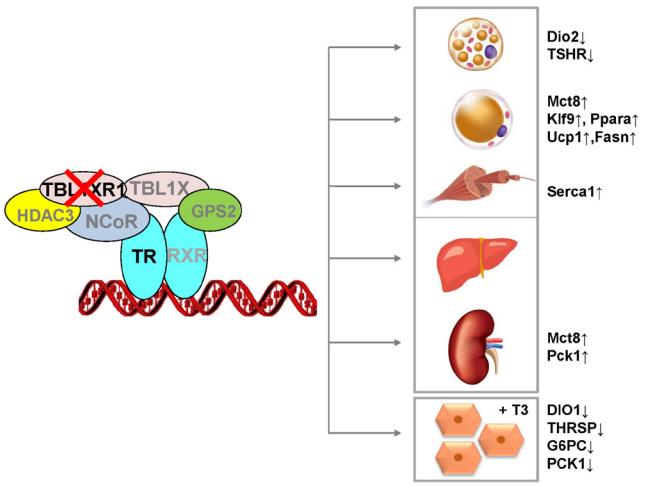

Figure 6
Schematic overview of the effects of a corepressor complex that contains a mutated TBL1XR1 (mice study) or less TBL1XR1 (cells) on T3-regulated gene expression in the peripheral T3 target organs (brown fat, white adipose tissue, muscle, liver, kidney) and HepG2 cells.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
YH performed the analyses and wrote this paper. KF helped with the analyses and reviewed the paper before submission. ASPT and RH reviewed the paper before submission. EF and EB made substantial contributions to the content of the paper and reviewed the manuscript before submission. AB designed the study, made substantial contributions to the content, reviewed/edited the manuscript before submission and supervised. :

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