European Thyroid Journal (2024) **13** e240162
https://doi.org/10.1530/ETJ-24-0162

Received 24 May 2024
Accepted 24 September 2024
Available online 24 September 2024
Version of Record published 14 October 2024

RESEARCH

Disturbed function of TBL1X has a differential effect on T3-regulated gene expression in two human liver cell models

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Abstract

Background: Mutations in *TBL1X*, part of the NCOR1/SMRT corepressor complex, were identified in patients with hereditary X-linked central congenital hypothyroidism and associated hearing loss. The role of TBL1X in thyroid hormone (TH) action, however, is incompletely understood. The aim of the present study was to investigate the role of *TBL1X* on T3-regulated gene expression in two human liver cell models.

Methods: A human hepatoma cell line (HepG2) wherein TBL1X was downregulated using siRNAs, and human-induced pluripotent stem cell-derived hepatocytes (iHeps) generated from individuals with a TBL1X N365Y mutation. Both cell types were treated with increasing concentrations of T3. The expression of T3-regulated genes was measured by qPCR.

Results: *KLF9*, *CPT1A,* and *PCK1* mRNA expression were higher upon T3 stimulation in the HepG2 cells with decreased *TBL1X* expression compared to controls, while *DIO1* mRNA expression was lower. Hemizygous TBL1X N365Y iHeps exhibited decreased expression of *CPT1A*, *G6PC1*, *PCK1*, *FBP1,* and *ELOVL2* compared to cells with the heterozygous TBL1X N365Y allele, but *KLF9* and *HMGCS2* expression was unaltered.

Conclusion: Downregulation of TBL1X in HepG2 cells and the TBL1X N365Y variant in iHeps have differential effects on T3-regulated gene expression. This suggests that TBL1X may play a gene context role in TH action.

Keywords: coregulators; TBL1X; thyroid hormone action; WD40 repeat-containing proteins

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https://doi.org/10.1530/ETJ-24-0162

Introduction

Isolated central congenital hypothyroidism (CH) is characterized by decreased serum thyroid hormone (TH) concentrations in combination with a normal, low or slightly elevated serum thyroid stimulating hormone (TSH) due to a defect at the level of the pituitary and/or the hypothalamus (1). Isolated central CH is associated with pathogenic variants in a small number of genes involved in signaling pathways of the hypothalamus-pituitary-thyroid (HPT) axis (2, 3, 4, 5). In 2016, we described mutations in Transducin Beta Like X-Linked (*TBL1X*, encoding Transducin-Beta-Like Protein 1, X-Linked (TBL1X), a WD40 repeat-containing protein) in families with isolated central CH and hearing loss (1). More recently García M *et al.* reported a truncating *TBL1X* mutation in a patient with central hypothyroidism, hypoacusia, attention-deficit/hyperactivity disorder (ADHD), gastrointestinal dysmotility, and a Chiari malformation type I (CMI) (6). All mutations (N365Y, W369R, H453Y, R339X) reported to date are located in the highly conserved WD40-repeat domain of the protein, influencing its expression and thermal stability (1, 6). The molecular mechanism behind the association between central CH and *TBL1X* mutations remains unexplained.

TBL1X is a WD40 repeat-containing protein (1, 7) and part of the nuclear receptor corepressor (NCOR1)/silencing mediator of retinoic and thyroid hormone receptors (SMRT) corepressor complex that interacts with nuclear hormone receptors (NRs) and other transcription factors (TFs) (8). Critically, the NCOR1/SMRT corepressor complex is recruited to the thyroid hormone receptor (TR) isoforms to suppress the expression of positively regulated target genes via histone deacetylation in the absence (or presence of low levels) of triiodothyronine (T3, the active TH) (9). NCOR1 is the major TR corepressor involved in hepatic and systemic T3-regulated gene expression (10). Indeed, the expression of a mutant NCOR1 allele that cannot interact with the TRs leads to the de-repression of positively regulated targets in the hypothyroid state (11, 12). NCOR1 is also thought to be important in determining TH sensitivity and the set point of the HPT-axis (13, 14, 15). In contrast, SMRT (or NCOR2) seems to play a minor role in TH action. This notion was based on the observation that the expression of positively regulated T3 target genes in liver-specific SMRT knockout mice remained unchanged compared to control animals. Moreover, postnatal global disruption of SMRT in mice did not dysregulate the HPT axis (10). Interestingly, our group demonstrated that a postnatal deletion strategy to disrupt both NCOR1 and SMRT in mice is rapidly lethal anticipated by metabolic abnormalities including hypoglycemia, hypothermia, and weight loss (16).

The role of TBL1X in the NCOR1/SMRT corepressor complex and thus TH action is incompletely understood. However, the presence of central CH in patients carrying the various *TBL1X* mutations suggests an essential role of TBL1X in TH signaling. Currently, patients with central CH with a *TBL1X* mutation are treated with levothyroxine, although an increased sensitivity to TH could be hypothesized. The liver is an important T3 target organ with known effects on beta-oxidation, gluconeogenesis, and cholesterol metabolism (17). Interestingly, mice that lack TBL1X in hepatocytes have increased steatosis and decreased beta-oxidation more consistent with hypothyroidism, but also consistent with the effects of NCOR1 deletion in the liver (18).

The aim of our study was therefore to clarify the effects of i) knockdown of *TBL1X;* and ii) the mutation *TBL1X* N365Y on the expression of T3-regulated genes in liver cells as a first step in understanding the role of TBL1X in TH-signaling *in vivo*. To this end, we used both a human hepatoma cell line (HepG2) wherein *TBL1X* was knocked down, and human-induced pluripotent stem cell-derived hepatocytes (iHeps) developed from a hemizygous affected father and heterozygous daughter carrying the X-linked *TBL1X* N365Y mutation (1).

Materials and methods

Statement regarding sex as a biological variable

Our study examined male and female human samples from father and daughter carrying the X-linked *TBL1X N365Y* mutation, and different effects were reported. The father is hemizygous and diagnosed with central hypothyroidism, while the daughter is heterozygous and presents normal TSH and fT4 levels. mRNA expression of T3 responsive genes was done in both male and female human iPSCs-derived hepatocytes samples and different findings associated with T3 sensitivity were identified for both sexes investigated. Our study examined mRNA expression of T3-responsive genes in HepG2 derived from the liver tissue of a 15-year-old Caucasian American male (19).

In vitro experiments with the human hepatoma cell line HepG2

The human hepatoma cell line HepG2 (ATCC, Rockville, USA) was cultured in Dulbecco's modified Eagle's medium with glucose (1 g/L) (Gibco), supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin- streptomycin-neomycin (Sigma). The cells were cultured in a medium with low T3 concentrations (Dulbecco's modified Eagle's medium, 10% charcoal-stripped FBS, and 1% penicillin-streptomycin-neomycin) prior to the experiment for 3 days reaching 80% confluence. Knockdown of *TBL1X* was performed by introducing small interference RNA (siRNA) using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's protocol. Three specific siRNAs of *TBL1X* (siRNA1:IDs13823, siRNA2:IDs13824, siRNA3:IDs13825)

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https://doi.org/10.1530/ETJ-24-0162

and negative control siRNAs (scrambled siRNAs with matching GC content) were predesigned by Ambion. The knockdown efficiency of the *TBL1X* gene was determined by measuring *TBL1X* mRNA expression in HepG2 cells by qPCR.

Approximately 2.9 × 10^6 cells/well were seeded in 6-well plates in a total volume of 2 mL. After transfection, cells were rested for 24 h at 37°C and 5% CO_2 until T3 administration.

Twenty four hours after transfection, the medium was changed and T3 (Sigma) was added in increasing concentrations (0 nM, 0.1 nM, 1 nM, 10 nM, 100 nM) for 24h and subsequently cells were harvested for RNA isolation. Three independent experiments were performed each containing a technical triplicate.

Induced pluripotent stem cells (iPSCs) and the generation of iHeps

Peripheral blood mononuclear cells (PBMCs) of a male patient (case ID A.III.8) with the *TBL1X* N365Y variant (c.1246A>T, N365Y) and his daughter (case ID A.IV.2), who carries the *TBL1X* N365Y variant but does not have central hypothyroidism, were collected and frozen until further use. Clinical details of the family have been reported elsewhere (1). Both patients provided written informed consent before enrollment. iPSCs lines MTSTH001-1 and MTSTH002 were generated by reprogramming PBMCs of the male patient (case ID A.III.8) and his daughter (case ID A.IV.2). PBMCs were reprogrammed using the Sendai virus Cyto Tune2 Reprogramming Kit (Thermo Fisher Scientific) as we have previously detailed (20, 21). iPSCs were maintained on growth factor reduced Matrigel (Corning) in mTeSR-1 media (StemCell Technologies) and verified to be free of mycoplasma and karyotypically normal as determined by G-band karyotyping analysis from 20 metaphases. Detailed protocols for iPSC derivation and culture are available for download at https://crem.bu.edu/cores-protocols/.

Directed differentiation into the hepatic lineage (iHeps)

iPSCs-directed differentiation to iHeps was performed using the previously published protocol (21, 22, 23). In brief, undifferentiated iPSCs were passaged at 1 × 10^6 cells per well of a Matrigel-coated 6-well plate, and placed into hypoxic conditions (5% O_2, 5% CO_2, 90% N_2) for the remainder of the differentiation. Cells were patterned into definitive endoderm using the STEMdiff Definitive Endoderm Kit per manufacturer's instructions (StemCell Technologies) for 4 days with endoderm efficiency confirmed via cell surface staining for CXCR4 and cKit. Cells were subsequently grown in serum free base media supplemented with stage-specific growth factors to specify the hepatic lineage and induce maturation. At day 19 iHeps were treated for 24h with escalating concentrations of T3 (Sigma; 0 nM, 0.1 nM, 1 nM, 10 nM, and 100 nM). A detailed protocol for the derivation of hepatocytes from iPSCs is available for free download from https://crem.bu.edu/cores-protocols/. The iHep experiment was repeated 4 times and the total number of samples per group in each line (TBL1X 002, and TBL1X 001) was combined as T3 0 nM n = 8, T3 0.1 nM n = 4, T3 1 nM n = 8, T3 10 nM n = 4, and T3 100 nM n = 8. However, some samples were omitted from the analysis secondary to technical failure with RNA quality or PCR amplification.

Flow cytometry

Definitive endoderm (DE) efficiency was quantified at day 5 using anti-human CD184 (CXCR4)-PE (StemCell Technologies) and anti-human CD117(CKIT)-APC (Thermo Fisher Scientific) conjugated monoclonal antibodies. Cells were harvested, then the pellet was resuspended in PBS+ (2% FBS in PBS), and incubated with CKIT and CXCR4 antibodies for 30 min on ice. To confirm the cells differentiated into hepatocytes at day 20 we analyzed alpha-fetoprotein (AFP) presence through flow cytometry. iHEPs were fixed in 1.6% paraformaldehyde (VWR) for 20 min at 37°C, then permeabilized for 5 min in permeabilization wash buffer (BioLegend). Cells were incubated for 30 min at room temperature with AFP (Abcam) antibody, followed by incubation with anti-rabbit IgG-AlexaFluor488 (Thermo Fisher Scientific) secondary antibody for 30 min at room temperature. Data analysis was performed using FlowJo software (Becton, Dickinson & Company). For all flow cytometry analysis, gating was determined using isotype-stained controls.

RNA isolation and qPCR

Total RNA from HepG2 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). The primers used for qPCR are listed in Table 1. 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 eukaryotic translation elongation factor 1 alpha 1 (EEF1A1), TATA-box binding protein (TBP), and hypoxanthine phosphoribosyltransferase 1 (HPRT), all showing stable expression under the experimental conditions.

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Table 1 Primers used in HepG2 cells.

Gene	Accession number	Sequences (5'-3')		Product length
		Forward	Reverse	
TBL1X	NM_005647.4	AACAGGCTCTGTGATGGCTG	GGGATTACAAAGTTGCGCGT	216
TBL1XR1	NM_024665.7	CCATGGCCAGTCCACTACAG	TCCAGCACTTGGTGAACAGA	126
HPRT	NM_000194.3	CCTGCTGGATTACATCAAAGCACTG	TCCAACACTTCGTGGGGTCCT	289
EEF1A1	NM_001402.6	TTTTCGCAACGGGTTTGCC	TTGCCCGAATCTACGTGTCC	120
TBP	NM_001172085.2	CCCGAAACGCCGAATATAATCC	AATCAGTGCCGTGGTTCGTG	80
KLF9	NM_001206.4	CCTCCCATCTCAAAGCCCATT	CGCCTTTTTCGATCGCTTGAT	248
DIO1	NM_001039716.3	TGGTTCGTCTTGAAGGTCCG	AAATTCAGCACCAGTGGCCT	149
THRSP	NM_003251.4	CGAGAAAGCCCAGGAGGTGA	AGCATCCCGGAGAACTGAGC	204
CPT1A	NM_001876.4	TGTGCTGGATGGTGTCTGTCTC	CGTCTTTTGGGATCCACGATT	100
G6PC1	NM_000151.4	GACTGGCTCAACCTCGTCTT	CGTAGTATACACCTGCTGTGCC	181
PCK1	NM_002591.4	GCTGGTGTCCCTCTAGTCTATG	GGTATTTGCCGAAGTTGTAG	166

Total RNA from iHeps was isolated at day 20 using the miRNeasy kit (Qiagen) according to the manufacturer's instruction. 250 ng of RNA was reverse transcribed into cDNA using a kit (SuperScript VILO; Invitrogen). qPCR was performed in duplicate using QuantStudio 6 Pro system (Thermo Fisher). TaqMan assays for KLF transcription factor 9 (*KLF9)*, carnitine palmitoyltransferase *1A (CPT1A)*, glucose-6-phosphatase catalytic subunit 1 *(G6PC1)*, phosphoenolpyruvate carboxykinase 1 *(PCK1)*, fructose-bisphosphatase 1 *(FBP1)*, ELOVL fatty acid elongase 2 *(ELOVL2), and* 3-hydroxy-3-methylglutaryl-CoA synthase 2 *(HMGCS2)* were used to quantify the transcripts. The Taqman assays used are listed in Table 2. Relative mRNA levels were calculated using standard-curve methods and normalized to the expression level of Eukaryotic 18S rRNA Endogenous Control (VIC™/TAMRA Probe – Applied Biosystems™).

Statistical analysis

Results are expressed as mean ± S.D. Data from three repeated HepG2 experiments were combined. Data from the HepG2 experiments was normalized to the mean value of the control transfected group without T3 stimulation (T3 0 nM) per experiment. 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 *post-hoc* analysis. Statistical analysis of iHeps data was performed by two-way ANOVA using GraphPad Prism 10 software with two grouping factors (mutation and T3 administration) followed by Tukey's *post-hoc* test. *P* values < 0.05 were considered statistically significant.

Table 2 Taqman primers used in Iheps.

Gene symbol	Reference
CPT1A	Hs00912671_m1
ELOVL2	Hs00214936_m1
FBP1	Hs00983323_m1
G6PC	Hs02802676_m1
HMGCS2	Hs00985427_m1
KLF9	Hs00230918_m1
PCK1	Hs00159918_m1

Study approval

The study was approved by the Medical Ethical Committee of the Academic Medical Centre, Amsterdam UMC (number NL66178.018.18). The experiments involving the differentiation of human iPSCs lines were performed with the approval of Boston University Institutional Review Board (BUMC IRB protocol H33122).

Results

Effects of TBL1X knockdown on T3 regulated gene expression in HepG2 cells

The knockdown efficiency of *TBL1X* in the three consecutive experiments was 42%, 46%, and 66% respectively and a combined knockdown efficiency of *TBL1X* was shown in Supplementary Figure 1 (see section on supplementary materials given at the end of this article). To evaluate the effect of *TBL1X* knockdown in HepG2 cells on T3-regulated gene expression, mRNA expression of *DIO1*, *KLF9*, *THRSP*, *CPT1A*, *G6PC1*, and *PCK1* was measured (Fig. 1). The mRNA expression of all these genes increased to varying extent upon increasing T3 concentrations. *TBL1X* knockdown increased the expression of *KLF9*, *CPT1A*, and *PCK1* compared to the expression in cells of the control transfected groups. Interestingly, the knockdown of *TBL1X* markedly decreased the expression of *DIO1* (P_{ANOVA} < 0.0001). Knockdown of *TBL1X* did not have an effect on *THRSP* and *G6PC1* expression. The interaction between T3 stimulation and *TBL1X* knockdown on the expression of *KLF9*, *DIO1*, *G6PC1*, and *THRSP* was not significant while there is an interaction effect for *CPT1A* and *PCK1* (both *P* < 0.05). In addition, we measured mRNA expression of

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https://doi.org/10.1530/ETJ-24-0162

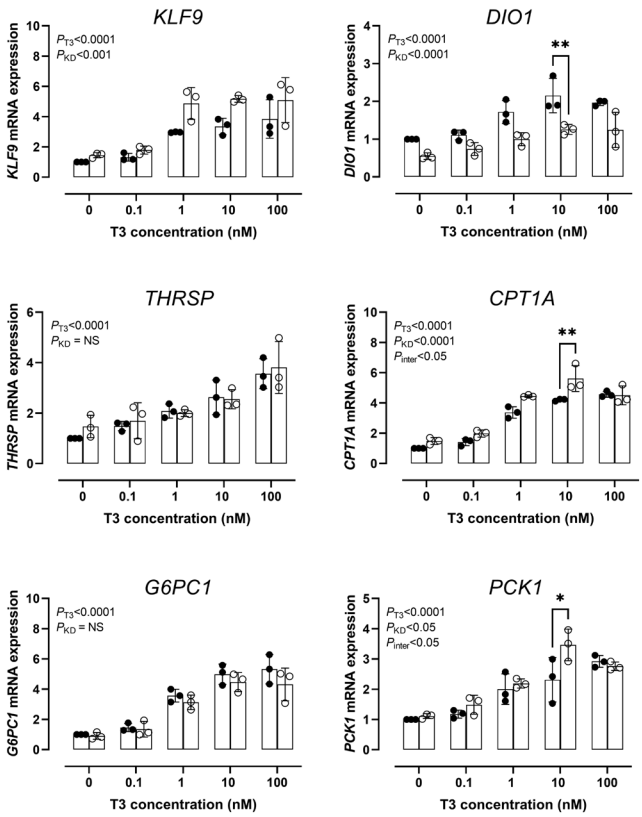

Figure 1

Effects of TBL1X knockdown on T3 regulated genes in HepG2 cells. Controls are represented by black dots and TBL1X knockdown by open circles. Cells are stimulated with increasing concentration of T3. mRNA expression is normalized to the control group without T3 which is set at 1. Mean values ± S.D. of three independent experiments are shown, and each experiment consists of 3 values per group (technical triplicates). *P*-values for two-way analysis of variance are indicated on the top-left corner of each figure panel. *Post hoc* analysis (Tukey) *P*-values are indicated by asterisks above the corresponding bars: *($P < 0.05$), **($P < 0.01$).

FBP1, *ELOVL2*, and *HMGCS2* (all T3 negatively regulated genes) in HepG2 cells, *FBP1* and *HMGCS2* were not expressed in HepG2 cells (data not shown) while *ELOVL2* was not responsive to T3 (Supplementary Figure 2).

Characterization of iPSC derived hepatic cells derived from TBL1X N365Y mutation iPSC lines

Flow cytometry was used to analyze the expression of definitive endoderm-specific markers such as CKIT and CXCR4, and the hepatocyte maker AFP. Flow cytometric results demonstrated no differences in endodermal (Supplementary Figure 3A and 3B) or hepatocyte specification (Supplemental Figure 3C and 3D) between the father and the daughter carrying the *TBL1X N365Y* mutation lines. Additionally, we confirmed the presence of iHeps through mRNA expression of *AFP* and Hepatocyte Nuclear Factor 4 (*HNF4*). The expression of these hepatocyte-specific markers was similar between father and daughter lines, confirming the efficiency of our protocol in differentiating iPSC into fetal hepatocytes (Supplementary Figure 4).

The effect of TBL1X N365Y on T3 regulated genes in iHeps

To evaluate the effect of *TBL1X N365Y* on T3-regulated gene expression in iHeps from the affected father and the daughter carrying the *TBL1X N365Y* mutation (on one X chromosome), mRNA expression of *KLF9*, *CPT1A*, *G6PC1*, and *PCK1* was measured (Fig. 2). The mRNA expression of all these genes increased to varying extent upon increasing T3 concentrations. The mRNA expression of *CPT1A*, *G6PC1*, and *PCK1* is lower in the cells of the father who is hemizygous for *TBL1X N365Y* compared to the expression in iHeps of the daughter who is a carrier. *TBL1X* N365Y did not affect *KLF9* expression which is similar in both father and daughter. Additionally, mRNA expression of three T3 negatively regulated genes, *FBP1*, *ELOVL2*, and *HMGCS2* was measured (Fig. 3). The mRNA expression of all these genes decreased to varying extent upon increasing T3 concentrations. Hemizygous *TBL1X N365Y* decreased the expression of *FBP1* and *ELOVL2* compared to the expression in iHeps of the daughter who is a carrier. *HMGCS2* expression did not differ between the patient and the carrier.

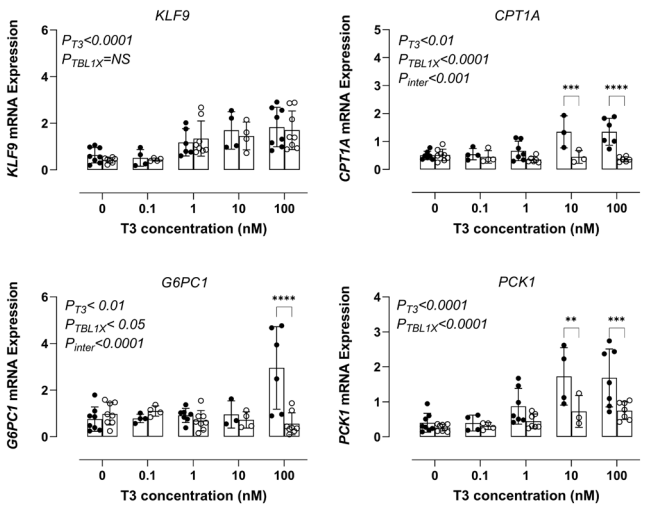

Figure 2

Effects of TBL1X N365Y on T3 positively regulated genes in iHeps cells. The daughter mutation is represented by black dots, the father mutation by open dots. Cells were stimulated with increasing concentration of T3. Mean values ± S.D. consists of T3 0 nM $n = 8$, T3 0.1 nM $n = 4$, T3 1 nM $n = 8$, T3 10 nM $n = 4$, and T3 100 nM $n = 8$ per group. *P*-values for two-way analysis of variance are indicated on the top-left corner of each figure panel. *Post hoc* analysis (Tukey) *P*-values are indicated by asterisks above the corresponding bars: **($P < 0.01$), ***($P < 0.001$), ****($P < 0.0001$).

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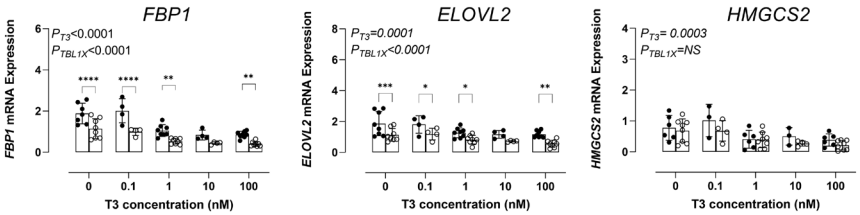

Figure 3

Effects of TBL1X N365Y on T3 negatively regulated genes in iHeps cells. The daughter mutation is represented by black dots, the father mutation by open dots. Cells were stimulated with increasing concentration of T3. Mean values ± S.D. consists of T3 0 nM *n* = 8, T3 0.1 nM *n* = 4, T3 1 nM *n* = 8, T3 10 nM *n* = 4, and T3 100 nM *n* = 8 per group. *P*-values for two-way analysis of variance are indicated on the top-left corner of each figure panel. *Post hoc* analysis (Tukey) *P*-values are indicated by asterisks above the corresponding bars: *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$), ****($P < 0.0001$).

Discussion

Mutations in the WD40 repeat domain of TBL1X have been identified as one of the genetic causes of central CH (1, 6). Central hypothyroidism is defined as TH deficiency due to insufficient stimulation of the thyroid gland by the pituitary and/or hypothalamus (1). Central CH associated with mutations in *TBL1X* is accompanied by impaired hearing (1, 6). TBL1X is a component of the NCOR1/SMRT corepressor complex which represses gene expression mediated by unliganded TR. All known mutations are located at the WD40 repeat domain which is involved in nuclear protein-protein interactions. We used two human cellular models, the HepG2 cell line and iHeps, to investigate the role of TBL1X in TH action.

We found that *TBL1X* knockdown in HepG2 cells results in increased expression of *KLF9*, *CPT1A*, and *PCK1* compared to control cells, which is consistent with the role of TBL1X in the corepressor complex. Disturbed corepressor function is supposed to lead to an increase in T3-regulated gene expression. This is consistent with the observation of Takamizawa *et al.* (24) who showed that *TBL1X N365Y* significantly inhibited the TR/NCOR1 mediated activity of the TRH promoter in *n*-1 cells, even in the presence of wild-type TBL1X. However, the fact that *TBL1X* knockdown markedly repressed the T3-induced expression of *DIO1* in HepG2 cells but did not affect the T3-induced expression of *G6PC1* and *THRSP*, indicates a differential role of TBL1X in T3 action that is gene context-specific. Of note, the knockdown of *TBL1XR1*, a close homolog of *TBL1X*, in HepG2 cells similarly repressed the expression of *DIO1* markedly (25), while an impaired NCOR1 complex significantly activates the expression of liver *DIO1* in mice (12). This indicates that each component of the NCOR1/SMRT corepressor complex has unique roles in the regulation of T3 genes.

Given the importance of T3 in regulating liver metabolic pathways, and to further explore whether *TBL1X* mutations change T3 signaling we analyzed the expression of T3 target genes in human iHeps derived from a father and daughter who both carry the *TBL1X* N365Y mutation. The hemizygous father was diagnosed with central CH at the age of 2 weeks while the heterozygous daughter had normal TSH and fT4 concentrations (1). We showed before that TBL1X N365Y protein was poorly expressed compared with wild-type protein (1). *TBL1X N365Y* markedly repressed the T3-induced expression of *CPT1A*, *G6PC1*, and *PCK1* in the cells derived from the father, suggesting that TBL1X acts as a coactivator. Surprisingly, in the father's iHeps T3 upregulated the expression of *KLF9* to a similar extent as observed in the daughter. The decreased sensitivity of *CPT1A*, *G6PC1*, and *PCK1* to TH is in line with mice with impaired hepatic expression of TBL1X which show increased steatosis and decreased fatty acid oxidation (18), both symptoms of hypothyroidism. We next assessed the expression of genes negatively regulated by T3 such as *FBP1*, *ELOVL2*, and *HMGCS2*. The expression of *FBP1* and *ELOVL2* upon T3 was lower in the father cells compared to the daughter while *HMGCS2* mRNA expression did not differ. Collectively, these results suggest that patients carrying the TBL1X mutation mainly have a decreased sensitivity to T3 which is dependent on the gene evaluated and the sample's features (hemizygous or carrier). While the effects of T3 in the father's iHeps were observed in the context of *CPT1A*, *G6PC1*, and *PCK1*, and in the negatively regulated T3 target genes *FBP1* and *ELOVL2*, the carrier's iHeps displayed more pronounced T3 sensitivity to the genes analyzed. The data suggest that the *TBL1X* mutation could play a role in determining T3 sensitivity in human hepatocytes. Nevertheless, further studies are necessary to explore these differential effects.

The difference in TH action between HepG2 and iHeps may be related to the difference between a *TBL1X* knockdown and a *TBL1X* mutation. We showed earlier that the latter seems to have a deleterious effect on protein folding (1) while the knockdown of the *TBL1X* gene results in less protein. However, both models show a differential effect on T3-related genes.

Alternatively, the difference in TH action between HepG2 and iHeps could also be explained by the expression levels of TRα and TRβ. TRβ1 is the main TR isoform in hepatocytes (26). *THRB* expression is present at low

levels in HepG2 cells (27) as well as *THRA* (28). iHeps express both *THRA* and *THRB* mRNA, with THRB being the dominant receptor, which is similar to the expression pattern observed in human hepatocytes (29). Therefore, it is possible that iHeps more accurately replicate the *in vivo* hepatocyte environment compared to HepG2 cells. It is tempting to speculate that the differences in the response to T3 between HepG2 and iHeps are the result of differences in the expression of TRα and TRβ. However, the lack of reliable antibodies against TRα and TRβ makes it difficult to determine the expression levels of both receptors in both models. Alternatively, the nature of the cell model (tumor cell line or differentiated iPSC) could be the reason for the differential responses (30, 31). In addition to the role of TBL1X in TR function, TBL1X interacts with other transcription factors such as the estrogen receptor (ER), the androgen receptor (AR), and PPARγ, which may exert an influence on the gene expression as well (8).

In conclusion, we studied the role of TBL1X in TH action in HepG2 cells and iHeps and found a differential effect of T3 on the expression of a variety of T3-responsive genes. TBL1X acts as a corepressor in the regulation of *KLF9*, *CPT1A*, and *PCK1* in HepG2 cells while it acts as a coactivator in the regulation of *DIO1*. In iHeps, TBL1X appears to function as a coactivator as the expression of T3 positively regulated genes was repressed by the mutation in TBL1X. However, the observation that T3 negatively regulated genes in iHeps were repressed by mutated TBL1X indicates that TBL1X indeed acts as a corepressor. Further research is needed to unravel the mechanisms involved, but based on the results of this study it can be concluded that a mutation in TBL1X does not cause general T3 hyper- or hypo-sensitivity while knockdown of *TBL1X* in HepG2 cells seems to strengthen the sensitivity to T3 selectively.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/ETJ-24-0162.

Declaration of interest

EB receives consultancy fees from Madrigal and Aligos.

AB is an editorial board member of the *European Thyroid Journal*. She was not involved in the editorial or peer review process for this paper.

Funding

This work was supported by a grant (to YH) from the Chinese Scholarship Council (CSC) (no. 201907720056), ANH and AW were supported by NIH grant DK117940.

Author contribution statement

YH: conducting experiments, acquiring and analyzing data, writing the manuscript; LSDO: conducting experiments, acquiring and analyzing data, writing the manuscript; KF: conducting experiments, acquiring data; ASPT: collecting patients and reviewing the manuscript; EF: reviewing the manuscript, supervision; JEK: conducting experiments, acquiring data; AAW: designing research studies, reviewing the manuscript; ANH: designing research studies, providing reagents, reviewing the manuscript; EB: designing research studies, reviewing the manuscript, supervision; AB: designing research studies, providing reagents, reviewing the manuscript, supervision.

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