An animal model for Pierpont syndrome: a mouse bearing the Tbl1xr1 Y446C/Y446C mutation

Yalan Hu1, Peter Lauffer2, Michelle Stewart3, Gemma Codner3, Steffen Mayerl4, Heike Heuer4, Lily Ng5, Douglas Forrest5, Paul van Trotsenburg2, Aldo Jongejans6, Eric Fliers7, Raoul Hennekam8 and Anita Boelen1,∗

1Endocrine Laboratory, Department of Clinical Chemistry, Amsterdam Gastroenterology, Endocrinology & Metabolism, Amsterdam UMC, University of Amsterdam, Amsterdam 1105AZ, The Netherlands
2Department of Pediatric Endocrinology, Emma Children’s Hospital, Amsterdam Gastroenterology, Endocrinology & Metabolism, Amsterdam UMC, University of Amsterdam, Amsterdam 1105AZ, The Netherlands
3The Mary Lyon Centre, MRC Harwell, Harwell Campus, Oxfordshire OX11 0RD, UK
4Department of Endocrinology, Diabetes and Metabolism, University Hospital Essen, University of Duisburg-Essen, Essen 45122, Germany
5Laboratory of Endocrinology and Receptor Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA
6Bioinformatics Laboratory, Department of Epidemiology and Data Science, Amsterdam Public Health, Methodology Amsterdam, Amsterdam UMC, University of Amsterdam, Amsterdam 1105AZ, The Netherlands
7Department of Endocrinology, Amsterdam Gastroenterology, Endocrinology & Metabolism, Amsterdam UMC, University of Amsterdam, Amsterdam 1105AZ, The Netherlands
8Department of Pediatrics, Emma Children’s Hospital, Amsterdam UMC, University of Amsterdam, Amsterdam 1105AZ, The Netherlands
∗To whom correspondence should be addressed at: Endocrine Laboratory, Amsterdam UMC, Academic Medical Center, Meibergdreef 9, 1105AZ Amsterdam, The Netherlands. Tel.: +31 207325749. Email: a.boelen@amsterdumumc.nl

Abstract

Pierpont syndrome is a rare disorder characterized mainly by global developmental delay, unusual facial features, altered fat distribution in the limbs and hearing loss. A specific mutation (p.Tyr446Cys) in TBL1XR1, encoding a WD40 repeat-containing protein, which is a component of the SMRT/NCoR (silencing mediator retinoid and thyroid hormone receptors/nuclear receptor corepressors), has been reported as the genetic cause of Pierpont syndrome. Here, we used CRISPR-cas9 technology to generate a mutant mouse with the Y446C mutation in Tbl1xr1, which is also present in Pierpont syndrome. Several aspects of the phenotype were studied in the mutant mice: growth, body composition, hearing, motor behavior, thyroid hormone state and lipid and glucose metabolism. The mutant mice (Tbl1xr1Y446C/Y446C) displayed delayed growth, altered body composition with increased relative lean mass and impaired hearing. Expression of several genes involved in fatty acid metabolism differed in white adipose tissue, but not in liver or muscle of mutant mice compared to wild-type mice (Tbl1xr1+/+). No difference in thyroid hormone plasma concentrations was observed. Tbl1xr1Y446C/Y446C mice can be used as a model for distinct features of Pierpont syndrome, which will enable future studies on the pathogenic mechanisms underlying the various phenotypic characteristics.

Introduction

Pierpont syndrome is a rare disorder with autosomal dominant inheritance that manifests with global developmental delay, variable degrees of intellectual disability, distinct facial characteristics, impaired hearing, motor defects and altered fat distribution in the distal limbs (1–4). It was first reported in 1998 by Dr Mary Ella Mascia Pierpont et al. in two boys with global developmental delay, facial abnormalities, microcephaly, bilateral congenital fat pads anteromedial to the heels and palmar and plantar fat accumulations (1). In 2005, Oudesluis et al. (2) reported another boy with a similar phenotype and suggested to call this combination of symptoms Pierpont syndrome. In Pierpont’s initial report, there was no description of hearing loss in the initial patient, and the hearing of the second patient was initially described as normal. However, after a follow-up period of 6 years the second patient showed unilateral hearing loss (2). In a more recent paper, all five reported patients had (audiometry proven) hearing loss (4).

We identified a single heterozygous missense variant, c.1337A>G (p.Tyr446Cys), in transducin β-like 1 X-linked receptor 1 (TBL1XR1) in six patients with Pierpont syndrome (4). TBL1XR1 is a member of the WD40 repeat-containing gene family. The WD40 domain consists of 4–8 WD40 repeat and folds into a β-propeller structure, providing a platform for the interaction and assembly of several proteins into a signalosome. The WD40 domain interacts with proteins and DNA and is involved in a wide range of cellular functions (5). In general, WD40 domain proteins coordinate ubiquitination, histone methylation (6) and acetylation (4).

TBL1X, short for transducin beta like 1 X-linked, is another member of the WD40 repeat-containing gene family. (6)
family and shares high sequence similarity with TBL1XR1 (86%). Notably, a mutation in TBL1X at an identical position in the WD40 domain as observed in TBL1XR1 results in a different phenotype in patients, i.e. isolated central congenital hypothyroidism [CH-C (4)]. The term central CH refers to a congenital shortage of thyroid hormone as a result of insufficient thyroid-stimulating hormone (TSH) production and/or release at the level of the pituitary and/or impaired stimulation of the pituitary by thyrotropin-releasing hormone (TRH) in the hypothalamus. Thyroid hormone is indispensable for growth, development and auditory function (7). Interestingly, patients with a mutation in TBL1X showed also impaired hearing (8), a symptom observed in patients with Pierpont syndrome too. Moreover, a mutation in TBL1Y, a homolog at chromosome Y, has been reported to be associated with hearing loss as well (9).

TBL1XR1 is expressed in various brain areas including the hypothalamus and pituitary, white and brown adipose tissue (WAT and BAT), muscle and liver (4). At present, it is unknown which genes and molecular mechanisms are involved in the pathogenesis of the Pierpont phenotype. In the present study, we developed a mouse model bearing the Tbl1xr1 Y446C mutation in both alleles in order to study its effects on growth, hearing, psychomotor development and a variety of target tissues including WAT, BAT, liver and muscle. To this end, we measured body composition, the auditory evoked brainstem response (ABR) and gene expression of a variety of genes involved in lipid metabolism in liver, muscle and adipose tissue, both in mice carrying the mutation and wild-type (WT) mice. Psychomotor development was assessed by measuring locomotor coordination. As the identical mutation in TBL1X resulted in central-CH (CH-C), we also studied plasma thyroid hormone concentrations as well as hypothalamic Trh mRNA expression in these mice.

Results

Generation of the Tbl1xr1 Y446C/Y446C mice

Using CRISPR-Cas9 technology, we generated a homozygous mouse model of Pierpont Syndrome containing a Y446C point mutation in exon 14 of Tbl1xr1 resulting in an amino acid change from tyrosine to cysteine (Fig. 1A). The mutation is located in the WD40 domain of the protein. The F0 generation was mated with WT C57BL/6J mice of the same age to obtain a F1 generation. Subsequently, mature F1 mice were backcrossed to obtain a F2 generation Tbl1xr1 Y446C/+ mouse and the F2 generation was intercrossed to obtain homozygous Tbl1xr1 Y446C/Y446C mice (Fig. 1B).

Body composition

To evaluate the effect of the Tbl1xr1 mutation on growth, body weight was recorded weekly between postnatal week 4 and 10. Body weight of male Tbl1xr1 Y446C/Y446C mice was lower as compared to male Tbl1xr1+/+ mice at all time points (Fig. 2A). No differences were observed between female Tbl1xr1 Y446C/Y446C and Tbl1xr1+/+ mice.

Tbl1xr1 Y446C/Y446C mice displayed higher lean mass and lower fat mass compared to Tbl1xr1+/+ mice (Fig. 2B and C). No sex difference was observed in percentage lean and fat mass (data not shown).

Bone

To evaluate the effect of the mutation on bone, we measured bone mineral density (BMD) and bone mineral composition (BMC). BMD was lower in both male and female Tbl1xr1 Y446C/Y446C mice compared to Tbl1xr1+/+ mice (Fig. 3A). BMC was lower only in male Tbl1xr1 Y446C/Y446C mice (Fig. 3B).

Hearing

Hearing was assessed by testing the ABR, an overall indicator of auditory function. The ABR thresholds were moderately elevated in Tbl1xr1 Y446C/Y446C mice compared to Tbl1xr1+/+ mice in response to a range of stimuli (8, 16, 32 kHz and a click stimulus), as measured in combined groups of males and females (Fig. 4A). Histological analysis of the cochlea of adult mice did not reveal obvious morphological abnormalities. Figure 4B shows representative sections in the region of the organ of Corti with no overt defects observed in mutant mice compared to control mice.

Locomotor activity

Mice underwent the Locomtronic® test, which was used to evaluate motor coordination and activity. No differences were observed in locomotor activity between Tbl1xr1 Y446C/Y446C and Tbl1xr1+/+ mice (Fig. 5).

Hypothalamus–pituitary–thyroid axis

No differences in serum T4, T3, and TSH concentrations or hypothalamic Trh mRNA were observed between Tbl1xr1 Y446C/Y446C and Tbl1xr1+/+ mice. We did find gender differences with lower serum T3 and TSH and higher absolute hypothalamic Trh mRNA in female mice (Fig. 6). However, the gender-related differences were similar in Tbl1xr1 Y446C/Y446C mice and Tbl1xr1+/+ mice.

Genes involved in fat metabolism in BAT, WAT, liver and muscle

The mutated Tbl1xr1 did not affect genes involved in lipid metabolism in BAT, liver or gastrocnemius muscle. Three genes displayed a gender difference; Ucp1 in BAT, Ppara and Fasn in liver. The gender differences did not differ between Tbl1xr1 Y446C/Y446C and WT mice. However, increased expression of three genes (Ppara, Ucp1 and Fasn) in WAT was observed in the Tbl1xr1 Y446C/Y446C mice compared to WT mice (Fig. 7). No difference was observed in the expression of genes involved in glucose metabolism in any of the four organs (Supplementary Material). To further explore the differentially expressed genes in WAT of WT and Tbl1xr1 Y446C/Y446C mice, RNA-seq was performed in WAT. Gene Set Enrichment Analysis
Figure 1. Generation of the Tbl1xr1\(^{Y446C/Y446C}\) mouse line. (A) Representative view of the CRISPR/Cas9 targeting strategy used for generating Tbl1xr1\(^{Y446C/Y446C}\) mice. Using CRISPR/Cas9, a tyrosine-to-cysteine missense mutation (Y446C) was generated in exon 14 of the mouse sequences of the mouse Tbl1xr1 locus. A silent mutation (G>C) was generated to remove the PAM site without changing the protein sequence. The nucleotide and amino acid of WT and mutant alleles (in bold) are shown. Exons are indicated with gray boxes and introns are noted by gray lines. The targeting site is indicated by a black arrow. (B) Breeding strategy to obtain Tbl1xr1\(^{Y446C/Y446C}\) mice. Founder mice were crossed to C57BL/6J to generate F1s, then F1s crossed to C57BL/6J to generate F2s. F2s were intercrossed for generating the respective cohorts.

(GSEA) revealed 11 gene sets (using the search terms ‘ADIPOGENESIS’ and ‘ADIPOGENIC’) related to adipogenesis that showed significant changes in expression [false discovery rate (FDR) < 0.05] between WT and Tbl1xr1\(^{Y446C/Y446C}\) mice (see Table 2). We excluded one of these as it focused on adipogenesis in BAT (M8364). All remaining 10 gene sets were established in 3T3-L1 cells that were differentiated into adipocytes using a variety of inducers [such as thiazolidinediones (TZDs) and IDX (insulin, dexamethasone, isobutylmethylxanthine)]. The direction of differential expression of four of the ten gene sets (M1579, M1577, M1626 and M2271—containing genes related to either positive or negative regulation of adipogenesis) was indicative of increased adipogenesis in WAT of Tbl1xr1\(^{Y446C/Y446C}\) mice (Table 2). However, the remaining gene sets (M2432, M2270, M1645, M2420, M1675 and M2182—all containing genes related to positive regulation of adipogenesis) were downregulated in WAT of Tbl1xr1\(^{Y446C/Y446C}\) mice, indicating reduced adipogenesis.

Discussion
In this study, we used CRISPR-Cas9 technology to generate mice bearing the Y446C mutation in Tbl1xr1 to establish an animal model for Pierpont syndrome. We
evaluated various aspects of the human phenotype (growth, motor development, hearing and WAT) in these mice in order to study the functional consequences of the mutation at the tissue level. As an identical mutation in the highly homologous gene TBL1X results in CH-C, we also studied thyroid function in the mice. The main finding of the study is that the homozygous Y446C mutation in mice recapitulates several of the main features of Pierpont syndrome, i.e. growth retardation, hearing loss and altered body composition. This may guide future studies on the molecular mechanisms underlying Pierpont syndrome and the main phenotype characteristics.

Our study is the first describing the phenotype of mice bearing a mutation in Tbl1xr1 and linking this to Pierpont syndrome. However, studies have been described wherein TBL1RX1 is either overexpressed or deficient. Overexpression of TBL1R1 is associated with a worse prognosis of gastric cancer (10) while deficiency of Tbl1xr1 causes asthenozoospermia (11).

A major difference between humans and mice is that the heterozygous mouse does not show a detectable phenotype while in humans the heterozygote shows Pierpont syndrome, and only the homozygous mice mimic the heterozygous human phenotype. It is not uncommon that variants in genes act differently in mice and humans and that mutated genes that cause a phenotype in humans do not cause a phenotype in mice. The background varies, several mechanisms exist, often as a result of evolutionary adaptations in humans, paralogous gene duplication events in mice and similar mechanisms. For instance, homologous genes may exist in different copies, evident also in man for TBL1XR1, as at least two other genes (TBL1X and TBL1Y) exist with a high sequence similarity and to some extent a similar function (evident through the hearing loss that occurs in variants of each of these genes). Mechanisms are even more complex as many proteins act in protein complexes and also ligand–receptor interactions are involved, so phenotypes are caused by a combination of proteins. A well-known phenomenon is a dominant-negative effect (in the heterozygote binding of the mutated protein to the normal protein causes inactivation of the complete complex, so of normal protein as well) that may be present in the human and not in the mouse. At present, we do not know what the explanation is for the difference in the functioning of Tbl1xr1 in mice and TBL1XR1 in humans.

**Growth and body composition**

We observed that the weight gain of male Tbl1xr1Y446C/Y446C mice lagged significantly behind that of male Tbl1xr1+/+ mice, but there was no difference in body weight of
Figure 4. (A) ABR in Tbl1xr1Y446C/Y446C mice compared to age-matched Tbl1xr1+/+ animals. Average thresholds are shown for different stimuli (click, 8, 16, 32 kHz) for mice at 3–5 months of age; n = 11 per group including both males and females since there is no gender difference. White bars indicate Tbl1xr1+/+ and black bars indicate Tbl1xr1Y446C/Y446C mice. Data are expressed as mean ± SEM; difference between Tbl1xr1+/+ and Tbl1xr1Y446C/Y446C groups was analyzed using two-way ANOVA for each stimulus; * (P < 0.05), ** (P < 0.01), *** (P < 0.001). (B) Histology of the cochlea. Six μm plastic sections of a representative mid-basal turn of the cochlea showing a lack of obvious abnormalities in inner hair cells (arrow) or outer hair cells (arrowheads) or the organ of Corti in Tbl1xr1Y446C/Y446C mice.

We observed an increased percentage of lean mass and a decreased percentage of fat mass in the Tbl1xr1Y446C/Y446C mice, indicating that the Tbl1xr1Y446C/Y446C mice had a leaner phenotype. It remains unknown at present whether the leaner phenotype of the Tbl1xr1Y446C/Y446C mice is present in humans with Pierpont syndrome since such data are not available. We do know that fat distribution in Pierpont syndrome patients is abnormal in the distal limbs, that both height and weight are below the 50th centile in all patients reported to date except one and that in half of the reported patients body weight is at a clearly lower centile than height; this may be an indication of an altered body composition in humans as well.

Bone
BMC and BMD of Tbl1xr1Y446C/Y446C mice were lower than those of Tbl1xr1+/+ mice. Bone density data have not been reported in humans with Pierpont syndrome. It is, therefore, of interest to measure bone parameters in patients with Piermont syndrome in the future.

Hearing
The higher hearing threshold in Tbl1xr1Y446C/Y446C mice compared to Tbl1xr1+/+ mice reflects moderate hearing loss. This is consistent with the impaired hearing reported in most patients with Pierpont syndrome (4,12). The mouse model may be of value to explore the
Figure 6. Characterization of the hypothalamus–pituitary–thyroid axis in Tbl1xr1Y446C/Y446C mice compared to age- and sex-matched Tbl1xr1+/+ animals. (A) Serum T4 concentrations, (B) serum T3 concentrations, (C) serum TSH concentrations, (D) normalized TRH expression in the hypothalamic PVN in both male and female mice (female and male WT animals are set as 1). White bars indicate Tbl1xr1+/+ and black bars indicate Tbl1xr1Y446C/Y446C mice. Data are expressed as mean ± SEM; 6–7 animals per group; differences between groups were analyzed using two-way ANOVA. The photograph shows representative TRH mRNA expression using FISH in the PVN of female Tbl1xr1+/+ and Tbl1xr1Y446C/Y446C mice.

mechanism behind impaired hearing in the patients. In addition, mutations in two other genes homologous to TBL1XR1, i.e. TBL1X and TBL1Y, have also been found to be associated with hearing impairment (8,9,13). Perhaps, TBL1XR, TBL1X and TBL1Y play an overlapping but independent role in the process of auditory development. If these related factors can compensate for each other in vivo, this may in part account for the variable observation of hearing loss in Pierpont syndrome. The hearing loss is unlikely to be associated with the mild hypothyroidism observed in patients with mutations in TBL1X, as mutations in TBL1XR1 and TBL1Y are unrelated to central hypothyroidism and the mouse models do not have altered thyroid hormone concentrations. The underlying defects may be subtle as histological analysis showed no obvious morphological abnormalities of the cochlea, presumably consistent with the moderate degree of hearing loss.

Locomotor activity

We did not observe impaired locomotor behavior in the Tbl1xr1Y446C/Y446C mice. This contrasts with patients who show significant motor retardation as part of their global development delay (1–4). Still, the number of locomotor errors in the mutant mouse group was slightly higher than that in the WT group but the difference was not statistically significant. In future studies, additional and perhaps more sensitive locomotor tests including
the balance beam test and hanging tail test should be used to investigate this aspect in more detail and to detect more subtle locomotor deficits in this mouse model.

**Genes involved in fat metabolism in WAT, liver and muscle**

To explore whether the mutation affects glucose and fatty acid metabolism in key metabolic organs, we analyzed several genes involved in glucose and fatty acid metabolism in liver, muscle, BAT and WAT. The mutation had no detectable effect on glucose and lipid metabolism in most tissues. However, three key genes in fatty acid metabolism (*Ppara*, *Ucp1*, *Fasn*) showed increased mRNA expression in WAT.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that belong to the superfamily of nuclear receptors. Activation of PPARα reduces triglyceride level and increases FA uptake and oxidation (14). UCP1 is a protein mediating cold-, sympathetic nervous system- and thyroid hormone-stimulated BAT thermogenesis (15–17). Activation of PPARα by lipolysis-derived fatty acids contributes to the coordination of *Ucp1* gene transcription (thermogenesis) (18).

The increased expression of *Ppara* (18–21) and *Ucp1* mRNA suggests that WAT has a tendency of browning (22,23), which may be partly responsible for the reduced weight gain of *Tbx15* mice and the leaner phenotype of *Tbx15* mice since browning was found to be beneficial in terms of reduction of body weight and improvement of insulin sensitivity (24).

Fatty acid synthase (*FASN*), encoded by *Fasn*, is the key enzyme for *de novo* lipogenesis (24,25). Cellular FASN expression is physiologically upregulated in a state of energy excess (25) and increased FASN gene expression in adipose tissue is linked to visceral fat accumulation and impaired insulin sensitivity (26), which is contradictory to the effect of browning. Increased FASN expression, however, fits with the phenotype of the patients as they have fat pads in the limbs suggesting fat accumulation.

**Figure 7.** Relative mRNA expression of genes involved in fatty acid metabolism in peripheral tissues (BAT, WAT, liver and gastrocnemius muscle) in *Tbx15* mice compared to age- and sex-matched *Tbx15* mice. Measurements by qPCR analysis. Since there is no gender difference in most of the genes except *Ucp1* in BAT and *Ppara* and *Fasn* in liver, combined data of male and female are shown. White bars indicate *Tbx15* and black bars indicate *Tbx15* mice. Data are expressed as mean ± SEM; 6–7 animals per group; differences between groups were analyzed using two-way ANOVA; *(P < 0.05), **(P < 0.01), ****(P < 0.001).
Fat pads in hands and feet, observed in Pierpont patients but not in the mutant mice, are compatible with an enhanced local adipogenesis while a leaner phenotype may indicate a reduced adipogenesis. However, a lean phenotype might also be due to other mechanisms, especially an enhanced metabolism, which we were not able to study in the mice. RNA-seq analysis revealed gene sets that were downregulated in Tbl1xr1\*Y446C/Y446C mice fitting with less adipogenesis but also gene sets with changes suggesting increased adipogenesis. Adipose development is an extremely complex developmental process, which is regulated by a large number of factors (27). Environmental factors such as temperature or diet affect adipocyte gene expression by epigenetic events (28). In Pierpont syndrome, fat pads are solely located at hands and feet. This suggests local dysregulation of adipogenesis possibly involving local external factors. We hypothesize that variants in TBL1R1 that result in Pierpont syndrome alone are not sufficient to result in a locally disturbed adipogenesis and that additional, yet unknown, external factors are essential for the development of fat pads.

Of note, the increased expression of both Fasn mRNA and Ucp1 mRNA was also observed in the mouse model of chronic β3-adrenergic receptor activation (29), which shows more browning and a leaner phenotype, similar to our mouse model. However, the link between Tbl1xr1 and adrenergic stimulation is unknown at present.

In summary, we have generated a mouse model for Pierpont syndrome using the CRISPR-Cas9 technology, which successfully mimics several but not all aspects of the human phenotype. The current mouse model can be of value for future studies on the pathogenesis of the syndrome and several of its main characteristics, especially the altered body composition and hearing loss.

Materials and Methods
The generation of targeted mice bearing the Tbl1xr1\*Y446C/Y446C mutation
Targeted mice bearing the Tbl1xr1\*Y446C/Y446C mutation, named TBL1XR1-Y446C-EM1-B6, were generated at MRC Harwell (Didcot, UK) using CRISPR-Cas9 technique. A sgRNA (sequence 5′-TCAAGAGCCGTGTACAGTGTGG-3′) targeting Tbl1xr1 exon 14 was synthesized in vitro (Fig. 1). Cas9 mRNA, sgRNAs and donor DNA (sequence ssODNs 5′-GCCAGATACCTGCCATCAGGACTAAAAGCGACACTGCCAGACACTGCA CACCGGCTTGTGTATGTTTGTTCAAGATGTGGTACCGAACACTGGC CCTCTGTCTACGTCCCATAACCTA-3′) were microinjected into fertilized one-cell embryos of C57BL/6J mice. The injected one-cell embryos were transferred into the pseudopregnant female mice. The presence of genomic modification in the F0 generation was determined by genomic DNA analysis via PCR and Sanger sequencing on ear clip samples [see Supplementary Material as described in (30)]. F0 generation mice were mated with WT C57BL/6J mice of the same age to obtain F1 generation mice. These mice underwent PCR and Sanger sequencing to enable definite characterization of the mutant allele. Animals that showed the desired base changes also underwent copy counting analysis via digital droplet PCR to check against additional donor integrations in the genome. Subsequently, mature F1 generation mice were backcrossed to obtain F2 generation Tbl1xr1\*Y446C/+ mice and F2 generation were intercrossed to obtain homozygous Tbl1xr1\*Y446C/Y446C mice. All F2 offsprings were genotyped by qPCR.

All procedures conducted were done in accordance with the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 (SI 4 2012/3039) at Harwell (Didcot, UK). Mice were housed in Tecniplast IVC cages (1284L and 1285L) with Aspen bedding (Datesand). Food and water were provided ad libitum. The lighting regime was 12 h light, 12 h dark with 30 min dusk to dawn, dawn to dusk period. Health checks were conducted daily and a maximum number of five animals were housed per cage. Male and female mice were used at the age of 10–12 weeks. For auditory testing, mice were maintained and analyzed at NIDDK, National Institutes of Health under approved protocols and following the guidelines for care at the National Institutes of Health.

Genotyping
F0 and F1 samples were genotyped by qPCR and sequencing. F2 offspring and subsequent generations were genotyped by qPCR. Mouse ear DNA was extracted using the DNA Extract All Reagents Kit (Thermo Fisher Scientific) and stored at −20°C until use. Samples were genotyped using qPCR with a FAM-labeled TaqMan assay (LGC, Biosearch Technologies, 5 μm probe, 15 μm each primer) designed to detect either the WT Tbl1xr1 allele or the mutant Tbl1xr1 allele, run in multiplex with a VIC-labeled internal control Dot1L (Thermo Fisher Scientific, 5 μm probe, 20 μm each primer), TaqMan GTxpress Master Mix (Thermo Fisher Scientific) and 1:10 dilution of DNA Extract All Reagents Kit preparation.

Body weight and body composition
Body weight was weekly recorded from week 4 to week 10. Body composition and BMD and BMC of anesthetized mice at the age of 11 weeks were assessed using the PIX-Imus Dual Energy X-Ray Absorption machine (GE Medical Systems, USA), the so-called DEXA system. The PIX-IMUS Densitometer provides BMD and body composition results from total body imaging in less than 5 min by taking high-energy X-ray images. Results include BMD in mg/cm², BMC in mg, bone area in cm², % fat, lean tissue in grams, and fat tissue in grams.

Hearing and histology of the cochlea
Auditory function was measured in male and female mice using a Smart EP system (Intelligent Hearing System, Miami, FL) to test the ABR under avertin anesthesia (0.25 mg/g body weight), as described in (31). Two independent groups of mice at 3–5 months of age were tested. No differences in outcomes were observed.
between males and females. Cochleae were fixed in 2% paraformaldehyde/3% glutaraldehyde in phosphate-buffered saline (PBS) overnight, then decalcified in 0.1 M EDTA in PBS for 5 days at 4°C, using established methods (32). Groups included four mutant (8 cochleae) and three control (6 cochleae) mice at ~3 months of age. Glycol methacrylate plastic sections of 6 μm thickness were stained with hematoxylin.

**Locotronic® test**

To evaluate the effect of the Tkl1xr1 mutation on locomotor coordination and the balance of mice, the Locotronic® Locomotor Test (Intellibio, France) was used (33). Mice at the age of 10 weeks traverse a horizontal ladder with evenly spaced rungs, along a narrow corridor to reach the exit. The number of errors (when the mouse foot/tail slipped between rungs) was recorded automatically. Each animal was tested three times. The more errors, the worse the psychomotor development of mice (33,34).

**T3, T4 and TSH plasma concentrations**

Male and female mice at the age of 12 weeks were killed by exsanguination and trunk blood was taken at the same time. Plasma was separated by centrifugation and immediately frozen at −80°C. Serum T3 and T4 were measured with in-house radioimmunoassay (35). TSH levels were measured by a mouse pituitary magnetic bead panel for TSH (the Milliplex assay), following the manufacturer’s instructions (Merck-Millipore Corp., Darmstadt, Germany), and read on a BioPlex (BioRad). The obtained values were expressed in picograms per milliliter (pg/ml). All samples from one experiment were measured within the same assay.

**Fluorescence in situ hybridization**

Brains were removed, immediately frozen in 2-methylbutane on dry ice and stored at −80°C until further processing. Coronal cryo-sections (20 μm) containing the paraventricular nucleus (PVN) were pre-treated as described before (36). In brief, defrosted and air-dried sections were fixed for 1 h in 4% PFA in PBS (pH 7.4), permeabilized in PBS containing 0.4% Triton X-100, and acetylated in 0.1 M tri-ethanolamine (pH 8.0) as described before (37). The paraventricular nucleus (PVN) were pre-treated as described before (36). In brief, defrosted and air-dried sections were fixed for 1 h in 4% PFA in PBS (pH 7.4), permeabilized in PBS containing 0.4% Triton X-100, and acetylated in 0.1 M tri-ethanolamine (pH 8.0) containing 0.25% (v/v) acetic anhydride. Subsequently, sections were dehydrated in rising concentrations of ethanol and air-dried. Third-generation fluorescence in situ hybridization (FISH) experiments were carried out as described previously (37,38). A probe against TRH consisting of a set of 20 individual sequences for the target was commercially designed and generated (Molecular Instruments). All buffers and amplifiers were obtained from Molecular Instruments. Sections were incubated for 10 min with hybridization buffer at 37°C before probe in hybridization buffer (0.4 pmol per 100 μl) was applied. Hybridization was carried out at 37°C for 20 h. Following rinsing with probe wash buffer and 5× SSC + 0.1% Tween20 (SSCT), sections were incubated with amplification buffer for 30 min.

### Table 1. List of primers used for qPCR

| Gene name                          | Symbol | Primer                  | Forward (5’–3’)                  | Reverse (5’–3’)                  | Products length (bp) |
|------------------------------------|--------|-------------------------|----------------------------------|----------------------------------|----------------------|
| Eukaryotic translation elongation  | Ef1a1  | mmEF1a1a                | AGTGCCTTTGGACGTTCTT              | ATTGTAGATCGAGGTGSCGG             | 174                  |
| factor 1 alpha 1                   |         |                         |                                  |                                  |                      |
| Ribosomal protein, large, P0       | Rplp0  | RPLP-1                  | GCGCCCTGACCTCTCGCTTTTCG         | TGGCAAGGACGGCTTTGT               | 124                  |
| Hypoxanthine guanine               | Hprt   | mmHprt                  | GCAGTAGAACCCCAAAATTGGGAAGATCTTGCTTAC              | TGGCAAGGACGGCTTTGT               | 84                   |
| phosphoribosyl transferase         |         |                         |                                  |                                  |                      |
| Solute carrier family 2 (facilitated glucose transporter), member 4 | Slc2a4 | mmGLUT4                 | TCCCTTCAGGTTGGATACCATGG         | AGCTGTGATGGTTACCTCTCTTACTCA      | 69                   |
| Malic enzyme 1, NADP(+)-dependent, cytosolic | Me1     | mmME                   | GAAAGAGGTTGTTGCCCTGAGATTGATCA   | AATTCGCAACTCTCTTACTTGGG           | 96                   |
| Phosphoenolpyruvate carboxykinase 1, cytosolic | Pck1    | mmPEPCK                 | ATGTCCCAGGCGGATGTAAG            | TACGGTTCAAGGCCTTTTCTCA           | 81                   |
| Peroxisome proliferative activated receptor Gamma, Coactivator 1 alpha | Ppargc1a | mmPGC1a                | CAATGAATGCAAGGCGTTCTTA          | GTGTTGAGAGGTTGTTTTTTCCAG         | 97                   |
| Thyroid hormone responsive         | Thrsp  | mmSpot14                | CATCCCATACCCACCTGAGCCCTGGAGT    | TGGCCAGTCTGGGTGTTATGTCGTCA        | 157                  |
| Peroxisome Proliferator Activated Receptor alpha | Ppara   | mmPPARA                | TCTCATGATGAGAGAGACGTTG          | ACTGCGCAGTTGGAAACCAGAT          | 113                  |
| Uncoupling protein 1               | Ucp1   | mmUCP1                  | CGACTCTCCCAAGAGCTCTTCT         | GCCCAGCTGAACTTCTTCTGTCTTCA        | 72                   |
| Uncoupling protein 3               | Ucp3   | mmUCP3b                 | GAGATGTTGAACCTAGCACCAGCAATCTTCTC | GCCGTCTGACTTCTTCTTCTTCTTCTADGCA   | 153                  |
| Fatty acid synthase                | Fasn   | mmFASN                  | GAGAGTGGTGTAGACCAGCCAG          | TGGTAATTCCATAGACCGGCCAG          | 139                  |
| Carnitine palmitoyltransferase 1C  | Cpt1c  | mmCPT1c-2               | GAGCATTGCAAACAAGAGGAGA          | GCAGCATGCTGATACGTTATGTCGCAAGTGCTCAITTCATTCGCAAGTCTGAGCTGACTG             | 197                  |
| Glucokinase                        | Gck    | mmGck                   | CACATTGCTGCAGCAGACT             | ACTTGTGACCGAGCGAGCAGCAT          | 107                  |
| Hexokinase 1                       | Hk1    | mmHK1                   | GAGGCATCTCGAGACCAAG             | TCTCAGGATCTTCTTCCACACAAGAGCAC   | 205                  |

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Table 2. List of significant gene sets related to adipogenesis

| Name               | Gene set                                    | Description                                                                                                                                  | Nb  | Direction | Effect          | P-value | FDR   |
|--------------------|---------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------|-----|------------|-----------------|---------|-------|
| M2432              | VERNOCHE_T ADIPOGENESIS                    | Genes upregulated during adipogenic differentiation of 3T3-L1 cells and downregulated by troglitazone                                       | 17  | ↓          | Less adipogenesis | 0.00    | 0.00004 |
| M2271              | STEGER_ADIPOGENESIS_DN                     | Genes downregulated during adipogenesis of 3T3-L1 cells                                                                                  | 25  | ↓          | More adipogenesis | 0.00    | 0.00028 |
| M2182              | WANG_CLASSIC_ADIPOGENIC_TARGETS_OF_PPAR    | Classic adipogenic genes that are induced by PPARγ during adipogenesis in 3T3-L1 preadipocytes.                                            | 25  | ↓          | Less adipogenesis | 0.00    | 0.00063 |
| M2270              | STEGER_ADIPOGENESIS_UP                     | Genes upregulated during adipogenesis of 3T3-L1 cells.                                                                                     | 21  | ↓          | Less adipogenesis | 0.00    | 0.00141 |
| M1579              | BURTON_ADIPOGENESIS_8                      | Progressively downregulated during differentiation of 3T3-L1 cells into adipocytes.                                                          | 77  | ↓          | More adipogenesis | 0.00    | 0.00154 |
| M1645              | LI_ADIPOGENESIS_BY_ACTIVATED_PPAR         | Adipocyte genes induced in 3T3-L1 cells by constitutively active PPARγ or its agonist TZD.                                                   | 17  | ↓          | Less adipogenesis1| 0.00    | 0.00263 |
| M1577              | BURTON_ADIPOGENESIS_3                      | Strongly upregulated during differentiation of 3T3-L1 cells into adipocytes.                                                                 | 99  | ↑          | More adipogenesis | 0.00    | 0.00807 |
| M1626              | BURTON_ADIPOGENESIS_PEAK_AT_0HR           | Downregulated during differentiation of 3T3-L1 into adipocytes in response to adipogenic hormones.                                          | 60  | ↓          | More adipogenesis | 0.00    | 0.00945 |
| M2420              | WAKABAYASHI_ADIPOGENESIS_PPAR_RXRA_BOUND_WITH_H4K20ME1_MARK | Genes upregulated during adipocyte differentiation of 3T3-L1 cells and newly modified by H4K20me1.                                      | 146 | ↓          | Less adipogenesis | 0.00    | 0.02537 |
| M1675              | GERHOLD_ADIPOGENESIS_UP                    | Selected genes upregulated during differentiation of 3T3-L1 cells into adipocytes in response to adipogenic hormones.                   | 47  | ↓          | Less adipogenesis | 0.00    | 0.02838 |

*aSystematic name in MSigDB. bNumber of genes in set. cTwo-tailed P-value. dBenjamini and Hochberg FDR adjusted P-value.

Probe initiator-specific hairpins h1 and h2 labeled with AxF647 (6 pmol per 100 μl amplification buffer) were separately heat-shocked for 90 s at 95°C and cooled down at room temperature for 30 min. Hairpins were mixed in amplification buffer and applied onto the sections. Signal amplification was performed for 20 h, sections were rinsed in SSCT, incubated for 5 min with Hoechst33258 (1:2000) and cover-slipped using Fluoromount (Sigma-Aldrich). Pictures were taken on a confocal microscope (Leica SP8) as z-stacks. For TRH quantification, those optical sections from the z-stacks with the most intense TRH signals were used. TRH signal integrated density in the PVN was measured in ImageJ and background determined in a signal-free area was subtracted. Four to eight sections from 5 to 6 animals per group were analyzed.

RNA isolation and qPCR

Total RNA from liver, WAT, BAT and gastrocnemius muscle was isolated using TriReagent (Sigma) and the ISOLATE II RNA Mini Kit (BIOLINE). RNA yield was determined using the Nanodrop (Nanodrop) and cDNA was synthesized with equal RNA input with the First-Strand cDNA synthesis kit (AMV) for qPCR with 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 LightCycler 480 (Roche Molecular Biochemicals) and LightCycler 480 SYBR Green 1 Master mix (Roche Molecular Biochemicals). 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 Eef1a1, Rplp0 and Hprt, all showing stable expression under the experimental conditions.

RNA library preparation

Total RNA was extracted from WAT of 12 mice (six Tβ1xγr1Y446C/Y446C mice and six WT mice; three males and three females of each genotype). To create cDNA libraries, samples were prepared using the Kapa mRNA Hyperprep kit (Roche, Basel, Switzerland). Concentration of the library samples was measured using the
Quant-it DNA HS assay (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were equimolar pooled and the pool was checked on Tapestation using DNA1000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). The libraries were sequenced on the NovaSeq6000 PE150 sequencer (Illumina, San Diego, CA, USA), producing at least 40 M 150-bp paired-end reads per library.

RNA sequencing of WAT and data analysis

RNA sequencing data analyses were carried out with Bioconductor (v3.13) packages in R (v4.1.0). Raw sequencing data were subjected to quality control using FastQC (v0.11.15) and dupRadar (v1.0.0) (39,40). All samples were of sufficient quality and kept for analysis. Reads were trimmed for adapter sequences with Trimomatic (v0.36) (41). Trimmed reads were aligned to the Mus musculus genome (Ensembl GRCm38v93) using HISAT2 (v2.1.0) (42). Gene level counts were obtained using HTSeq (v0.11) (43) with default parameters except stranded = reverse and the mouse GTF from Ensembl (release 93). Additional gene annotation was retrieved from Ensembl (release 104) using the biomaRt R/Bioconductor (v2.48.1) (44). Genes with more than 2 counter-per-million reads (CPM) in three or more of the samples were kept. Counts were normalized using the trimmed mean of M values normalization method of edgeR (3.4.0) (45). Then, count data were transformed to log2-counts per million (logCPM) using voom, estimating the mean–variance relationship (46). Differential expression was assessed with an empirical Bayes moderated t-test using the linear model framework from the limma package (47). The Benjamini–Hochberg FDR adjustment was used to correct for multiple testing of resulting P-values.

GSEA was performed using CAMERA (limma package) with a preset value of 0.01 for the intergene correlation (48) using the Hallmark, C1, C2, C3, C5, C6, C7 and C8 gene set collections retrieved from the Molecular Signatures Database (v7.4; Entrez Gene ID version) (49). Entrez gene IDs were mapped from mouse to human with HomoloGene (v68). P-values were calculated for each gene set for two alternative hypotheses (‘up’ or ‘down’) and adjusted using the Benjamini–Hochberg FDR. Gene sets with an FDR adjusted P-value of <0.05 were considered significant. The RNA sequencing data have been deposited in NCBI Gene Expression Omnibus (GEO) and are accessible under GEO Series accession number GSE198787.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Variations between Tbl1xr1Y446C/Y446C and WT mice were evaluated by two-way ANOVA using Graphpad Prism 9.0 software with two grouping factors (gender and strain) followed by Tukey post hoc analysis. If there was no difference between genders, data of male and female mice were pooled. Statistical significance was defined at a level of P < 0.05.
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