Thyroid hormone receptor regulates most genes independently of fibroblast growth factor 21 in liver

Aijun Zhang1, Douglas H Sieglaff1, Jean Philippe York1, Ji Ho Suh1, Stephen D Ayers1,∗, Glenn E Winnier1, Alexei Kharitonenkov2, Christopher Pin3,4, Pumin Zhang5, Paul Webb1 and Xuefeng Xia1,6

1Houston Methodist Research Institute, Genomic Medicine Program, 6670 Bertner Ave, Houston, Texas 77030, USA
2College of Arts and Sciences, Chemistry Department, Indiana University Bloomington, Bloomington, Indiana, USA,
3Departments of Paediatrics, Oncology, and Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada
4Children’s Health Research Institute, London, Ontario, Canada
5Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas, USA
6The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China
*(S D Ayers is now at Personalis Inc., Department of Genomics, Menlo Park, CA, USA)

Correspondence should be addressed to X Xia or P Webb
Emails
xxia2@tmhs.org or pwebb@tmhs.org

Abstract

Thyroid hormone (TH) acts through specific receptors (TRs), which are conditional transcription factors, to induce fibroblast growth factor 21 (FGF21), a peptide hormone that is usually induced by fasting and that influences lipid and carbohydrate metabolism via local hepatic and systemic endocrine effects. While TH and FGF21 display overlapping actions when administered, including reductions in serum lipids, according to the current models these hormones act independently in vivo. In this study, we examined mechanisms of regulation of FGF21 expression by TH and tested the possibility that FGF21 is required for induction of hepatic TH-responsive genes. We confirm that active TH (triiodothyronine (T3)) and the TRβ-selective thyromimetic GC1 increase FGF21 transcript and peptide levels in mouse liver and that this effect requires TRβ. T3 also induces FGF21 in cultured hepatocytes and this effect involves direct actions of TRβ1, which binds a TRE within intron 2 of FGF21. Gene expression profiles of WT and Fgf21-knockout mice are very similar, indicating that FGF21 is dispensable for the majority of hepatic T3 gene responses. A small subset of genes displays diminished T3 response in the absence of FGF21. However, most of these are not obviously directly involved in T3-dependent hepatic metabolic processes. Consistent with these results, T3-dependent effects on serum cholesterol are maintained in the Fgf21−/− background and we observe no effect of the Fgf21-knockout background on serum triglycerides and glucose. Our findings indicate that T3 regulates the genes involved in classical hepatic metabolic responses independently of FGF21.

Key Words
- nuclear receptor
- FGF
- gene expression
- metabolism

Introduction

Fibroblast growth factor 21 (FGF21) is one of three endocrine FGFs that influence metabolic responses via a combination of local and systemic actions. FGF21 is expressed in the liver, pancreas, and white and brown adipose tissue (Johnson et al. 2009, Fon Tacer et al. 2010, Kliwer & Mangelsdorf 2010). Hepatic Fgf21 expression is
induced by the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPARα) under fasting conditions, and secreted FGF21 peptide exerts autocrine/paracrine effects on liver, including regulation of genes involved in lipogenesis, lipolysis, and fatty acid oxidation, as well as additional endocrine effects on other tissues (Badman et al. 2007). Administration of FGF21 to overweight and diabetic mice results in decreases in the levels of plasma triglycerides and glucose, and increases in both insulin sensitivity and thermogenesis associated with decreased body weight (Coskun et al. 2008, Berglund et al. 2009, Xu et al. 2009). Conversely, mice that lack FGF21 exhibit late-onset obesity. Thus, there is intense interest in FGF21 as a therapeutic for metabolic disorders, weight loss, and diabetes. Potentially adverse effects of FGF21 have also come to light. FGF21 is involved in integration of metabolism and bone turnover, raising the possibility that FGF21 could promote bone loss, and elevated FGF21 levels are associated with abnormal lipid profiles in coronary heart disease, raising concerns about cardiovascular events (Iglesias et al. 2012, Wei et al. 2012). Moreover, FGF21 levels become elevated in obesity and nonalcoholic fatty liver disease, indicating that serum FGF21 levels do not always correlate with an improved metabolic profile. Given these concerns, it is important to understand how to manage local production of FGF21 in desired target tissues and the consequences of changes in FGF21 concentrations.

Thyroid hormones (THs) act as master regulators of metabolism and there is evidence for interdependency of TH and FGF21 signaling pathways. TH receptors (TRα and TRβ) are members of the nuclear hormone receptor family that regulate target genes in response to the main active form of TH, triiodothyronine (T3). TRs usually work by binding DNA-response elements (TREs) in the proximal promoters of target genes as heterodimers with retinoid X receptors (RXRs) but can also regulate target genes via protein–protein interactions with heterologous transcription factors (Baxter & Webb 2009, Cheng et al. 2010, Lin et al. 2013). T3 induces FGF21 in mouse liver and, in this study, TRβ selectively enhances FGF21 expression and promoter activity via interactions with PPARα bound to PPAR-α-response elements, but does not require PPARα to induce other T3-regulated genes (Adams et al. 2010). In addition, FGF21 administration to mice reduces serum levels of the parental form of TH, thyroxine (T4), and T3, implying mutual regulatory dependency of these signaling systems (Domouzoglou et al. 2014). In spite of these potential relationships, key actions of FGF21 and TH appear largely independent (Domouzoglou et al. 2014). Recent assessments of administration of TH to Fgf21-knockout mice have revealed no differences in serum lipids, the expression of selected T3-regulated genes in liver and brown adipose tissue, or overall energy expenditure. Conversely, FGF21 elicited similar alterations in serum lipids and gene expression in hypothyroid and euthyroid mice.

In this study, we set out to understand the mechanisms and downstream effects of TH-dependent changes in hepatic Fgf21 expression. We find that both T3 and the TRβ-selective thyromimetic GC1 act through TRβ to directly induce FGF21 expression and that the Fgf21 locus harbors a functional TRE within intron 2. This finding added another layer of complexity to previous studies of T3 regulation of FGF21 expression, which indicated that TRβ works primarily through PPARα bound to the FGF21 promoter (Adams et al. 2010). While patterns of hepatic T3 responses are similar in WT and Fgf21-knockout mice, similar to previous studies of a subset of hepatic genes involved in lipid metabolism (Domouzoglou & Maratos-Flier 2011, Domouzoglou et al. 2014), we also find that optimal response of a small subset of hepatic TH-target genes requires Fgf21. These genes, however, are not obviously related to metabolic regulation and we find no changes in serum metabolic parameters in Fgf21-knockout mice. Thus, our data support the idea that hepatic effects of TH on gene regulation and metabolism are largely independent of Fgf21.

Materials and method

Mice

All animal experiments were approved by the Animal Care and Use Committee of Houston Methodist Hospital Research Institute. Mouse strains C57BL/6, ApoE−/−, Ldlr−/−, and TRβ−/− were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) at 9 weeks of age. Fgf21−/− mice on a C57/B6 background (Dutchak et al. 2012) were received from The University of Texas Southwestern Medical Center (Dallas, TX, USA). For short-term ligand treatments (1 and 3 days) T3, GC1, or vehicle (control) were administered to mice (n = 5) by oral gavage at a dose of 1 mg/kg body weight, after which, mice were killed and dissected for tissue harvesting. For long-term treatments (2 weeks), GC1 was admixed into diet at a dose of 0.8 mg/kg body weight, after which the mice were killed for collection of blood and tissue samples.

Quantitative RT-PCR

Total RNA was isolated from livers or cultured cells using the RNAeasy Kit (Qiagen) and reverse transcribed to cDNA using the ABI Reverse Transcription Kit according to the
manufacturer’s instructions. Gene transcript expression was measured by quantitative RT-PCR (qPCR) (Roche LightCycler 480 II), in 20 µl reactions that included cDNA (10 ng of initial RNA input), 900 nM of each primer, PCR-grade water, and 10 µl of 2× TaqMan Master Mix (Applied Biosystems). The transcript expression levels were normalized to peptidylprolyl isomerase A (PPIA) expression levels. Independent samples were prepared from five to six different mice per genotype or three separate cell culture seedings and analyzed in technical triplicates. Gene transcript abundance was assessed by the standard ΔΔCp method and results were expressed as relative fold change compared with respective control group. Primer catalog numbers are available on request.

Serum FGF21 analyses

Mouse serum FGF21 concentrations were determined by ELISA (Phoenix Secretomics (Burlingame, CA, USA), PS001-01-EK-M-1), according to the manufacturer’s protocol. Serum samples of six to seven mice from each treatment were analyzed.

Immunoblot analysis

One hundred micrograms of liver were homogenized in 1 ml homogenization buffer (10 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 30 mM sodium phosphate, 10% glycerol, 0.5% NP40) containing complete protease inhibitor cocktail (Roche) using a Polytron homogenizer. The homogenate was centrifuged at 14 000 g for 8 min at 4 °C and the supernatant collected for further analysis. The proteins were separated by SDS–PAGE, transferred to polyvinylidine difluoride membranes (Millipore, Billerica, MA, USA), and detected with primary FGF21 and β-actin (Sigma) antibodies at 1:1000 and 1:10 000 dilutions, respectively, and HRP-conjugated secondary antibody at 1:5000 dilution. Immunoreactive proteins were visualized using the ECL western blotting detection system (Amersham, RPN2106), and densitometry analysis was conducted with Scion Image v4.0.3.2 software.

Cell culture

HepG2 (ATCC, Bethesda, MD, USA) and HepG2-TRβ (Lin et al. 2013) cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin, 0.1 g/l of streptomycin, and 4 mmol/l glutamine, under 95% air and 5% CO2 at 37 °C. For cycloheximide (CHX) treatment, HepG2-TRβ cells were maintained within 10% resin-stripped FBS-DMEM media for 24 h before treatment. The cells were pretreated with CHX at 10 µg/ml for 30 min, followed by 10 nM T3 incubation for 6 h, after which RNA was collected using the RNAeasy Kit (Qiagen). For RNA interference assays, HepG2-TRβ cells were plated in 10% resin-stripped FBS-DMEM media and grown to 50% confluency. The cells were transfected with PPARα ON-TARGET plus SMART pool or negative control siRNA (Dharmacon, Waltham, MA, USA) at a 50 nM final concentration, and 48 h later treated with 10 nM T3 or bezofibrate for 6 h, after which RNA was collected using the RNAeasy Kit (Qiagen).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the EZ ChIP Kits (Millipore) according to the manufacturer’s instructions. Briefly, mice livers were homogenized with a hand-held homogenizer in PBS containing a broad-spectrum protease inhibitor cocktail and then cross-linked with 1% formaldehyde. The nuclei were collected by adding lysis buffer following cross-linkage, which was stopped through addition of glycine. The liver nuclear solution was sonicated with a Fisher 60 Sonic Dismembrator (Thermo Fisher Scientific, Waltham, MA, USA). The sonicated solution was incubated with anti-TRβ polyclonal antibody or control rabbit IgG (Millipore) overnight with agitation at 4 °C. The TRβ antibody used for ChIP has also been used for TRβ studies of mouse cerebellum and liver (Dong et al. 2009, Paquette et al. 2011) and was originally purchased from Affinity Bioreagents (Golden, CO, USA), but was later made available through Thermo Fisher Scientific and Pierce (Grand Island, NY, USA), as PAI-213A. We have used the same antibody for western blotting analysis of TRβ expression in cell lines and mouse liver with appropriate controls for TRβ specificity shown (Ayers et al. 2014, Lammel Lindemann et al. 2014). Antibody-bound chromatin was precipitated with protein G-conjugated agarose beads, washed with gradient stringent buffers, and eluted with the elution buffer provided with the kit. The isolated DNA was analyzed by qPCR analysis using oligonucleotides corresponding to the following regions of the FGF21 locus: Fg21+715/+902, forward: 5′-CAGACTGCCCCTCA-GAGAAG-3′, reverse: 5′-CTCTGGCTAGTTGCGATT-3′. Fg21+3727/+3880 forward: 5′-CATTTGATATTCTTGG-GATTGG-3′, reverse: 5′-GACCAGGCTGGACTCAAGTT-3′.

Transient transfection

For cloning of Fgf21-luciferase reporters, a 339-bp fragment of Fgf21 intron 2 was PCR amplified from C57BL/6 mouse
Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed by incubating 32P-labeled oligonucleotide probes with in vitro translated TRβ and RXRα. The results were visualized by autoradiography. Competition assays were performed by adding 100× molar excess of either unlabeled oligonucleotide probes (specific competitor) or unlabeled mutant oligonucleotide probe (nonspecific competitor, mutated bases are indicated in each figure).

Microarray analysis

Mouse WG-6 v2 whole-genome expression arrays were purchased from Illumina (San Diego, CA, USA), and cRNA synthesis and labeling were performed using Illumina TotalPrep-96 RNA Amplification Kit (Ambion, Austin, TX, USA), with the labeling in vitro transcription reaction performed at 37 °C for 14 h. Biotinylated cRNA samples were hybridized to arrays at 58 °C for 18 h according to the manufacturer’s protocol. Arrays were scanned using BeadArray Reader (Illumina). Unmodified microarray data obtained from GenomeStudio were background-subtracted and quantile-normalized using the lumi package (Du et al. 2008) and analyzed with the limma package (Smyth 2005) within R (R Development Core Team 2010). All analysis was corrected for multiple hypotheses testing (Benjamini & Hochberg 1995) and effects were determined to be significant when there was a greater than or equal to twofold increase/decrease relative to the control and they had an adjusted P value <0.05. Raw microarray data sets have been deposited in the NCBI Gene Expression Omnibus (GEO).

gDNA and cloned into the BglII and HindIII sites of pGL4-23. Positive clones were confirmed through sequencing. For luciferase assays, HepG2-TRβ1 cells (2 × 10⁵ cells/well) were seeded into six-well plates 12 h before transfection of 1 μg/well of Fgf21-luciferase reporter constructs and 0.1 μg/well of Renilla-luciferase control plasmid using Fugene 6 transfection reagent. Twenty-four hours post transfection, cells were incubated with 10 nM T3 for another 24 h, after which luciferase activity was determined using the Promega Dual Luciferase Assay Kit following the manufacturer’s instructions.

Figure 1
Fgf21 is upregulated in livers of T3-treated mice. (A) Results of qRT-PCR analysis of hepatic FGF21 transcript levels in WT (C57BL/6) mice treated with T3 or GC1 for 24 h (n = 6/group) along with other secreted hepatic factors, as follows: angiopoietin-like factors (ANGPTL) 3, 4, and 6; angiotensinogen (AGT), hepatocyte growth factor (HGF), insulin-like growth factor 1 (IGF1). (B) Hepatic Fgf21 mRNA levels in LDL receptor knockout (Ldlr−/−) and apolipoprotein E-knockout (ApoE−/−) mice treated with T3 or GC1 for 24 h (n = 6/group). (C) Circulating levels of FGF21 in Ldlr−/− mice treated with T3 or GC1 for 24 h (n = 6/group). (D) Hepatic Fgf21 mRNA levels in Ldlr−/− mice 28 days following GC1 treatment (n = 6/group). Statistical significance of the observed changes is denoted by single or double asterisks, which indicate and refers to P < 0.05 or P < 0.01 respectively.

Figure 2
Fgf21 induction requires TRβ1. (A) qRT-PCR analysis of hepatic Fgf21 transcript levels in WT (C57BL/6) mice treated with T3 for 24 h or TRβ1−/− knockout mice (n = 6/group). (B) Western blotting analysis of hepatic FGF21 protein levels in WT and TRβ1−/− mice with representative gel images at the top and quantitation below. The statistical significance of the observed changes is denoted by double asterisks, which indicate P < 0.01.
Thyroid hormone/FGF21 interactions

**Research**

A ZHANG and others

**Figure 3**

TRβ-dependent induction of FGF21 in cultured cells. (A) FGF21 mRNA levels in HepG2 cells treated with GC1 (10 nM), T3 (10 nM) or vehicle for 24 h (n=4). (B) Expression levels of FGF21 mRNA in HepG2 cells that express exogenous TRβ1 or in parental HepG2 cells examined by RT-qPCR at different time points after T3 induction (n=4). (C) T3 concentration dependence of induction of FGF21 mRNA in HepG2 cells that expressed exogenous TRβ1 was examined by RT-qPCR for various concentrations of T3 for 24 h (n=4). (D) T3 induction of FGF21 mRNA is insensitive to CHX. Cells were pretreated with CHX for 30 min, followed by T3 for 6 h. (E) PPARα knockdown does not affect T3 response of FGF21 in HepG2 cells. qRT-PCR analysis of FGF21 transcript levels after treatment of HepG2-TRβ cells with bezafibrate or T3 for 2 h. Results are shown in the presence of control scrambled siRNA or PPARα-specific siRNA. Knockdown levels of PPARα transcripts are shown in the inset. The statistical significance of the observed changes is denoted by single or double asterisks, which indicate P<0.05 and P<0.01 respectively. Thyromimetic GC1 also elicited increases in hepatic Fgf21 expression, which persisted for at least 2 weeks with continuous treatment (Fig. 1D). Optimal hepatic T3 induction of FGF21 required TRβ, the predominant TR subtype in liver (Fig. 2A and B). Thus, T3 acts primarily through TRβ to induce hepatic Fgf21 expression.

**Data analysis and statistics**

All values are expressed as mean ± S.E.M. Statistical analyses were performed using GraphPad Prism Version 5.0 Software (GraphPad, Inc., San Diego, CA, USA). Data were analyzed by Student’s t-test, Mann–Whitney U test, or one-way ANOVA, where P values <0.05 were considered to be statistically significant.

**Serum metabolites**

Serum aliquots were sent to the comparative pathology laboratory of Baylor College of Medicine (Houston, TX, USA) for serum chemistry analysis.

**Results**

**T3 and GC1 induce Fgf21 in liver in a TRβ-dependent manner**

We examined the effects of acute (24 h) treatment of male C57BL/6 mice with T3 or the thyromimetic GC1 on hepatic Fgf21 expression. Fgf21 mRNA was induced by both ligands, an effect not observed for genes that encode other secreted hepatic factors (Fig. 1A). Similar effects of TR ligands on Fgf21 expression were seen in two mouse models of dyslipidemia (ApoE<sup>−/−</sup> and Ldlr<sup>−/−</sup>) (Fig. 1B) and FGF21 induction was accompanied by increases in circulating FGF21 peptide in serum (Fig. 1C). The selective

**Figure 4**

A TRβ-binding site in the FGF21 second intron. (A) Results of ChIP-Seq analysis with HepG2-TRβ cells showing binding events in the FGF21 second intron. (B) Representative gel showing results of ChIP analysis with an anti-TRβ antibody to verify binding within FGF21 intron 2 along with an IgG control. (C) qRT-PCR analysis of ChIP experiments showing specific binding of TRβ to the FGF21 intron 2 in native mouse liver. Immunoprecipitations were carried out with either TRβ antibodies or control IgG, and immunopurified DNA was analyzed with primers flanking intron 2 of FGF21 (+713 to 902) or a control region (+3727 to +3880) (n=4). The statistical significance of the observed changes is denoted by double asterisks, which indicate P<0.01.
results not shown). A modest T₃ response was also observed in parental HepG2 cells after extended T₃ treatment (Fig. 3B); this delayed effect resembles the kinetics of T₃ induction of other bona fide hepatic TR-target genes in this cell type and is probably a consequence of low levels of TRβ that are present in these cells (Yuan et al. 2012). T₃-dependent FGF21 induction exhibited classical dose dependence with maximal activation with 10 nM hormone (Fig. 3C), implying that this T₃ effect was TR-dependent, and was maintained after pretreatment with the protein synthesis inhibitor CHX, implying a direct transcriptional effect of TR (Fig. 3D).

Interestingly, the effects of TRβ on FGF21 expression were not absolutely dependent on PPARα in HepG2 (Fig. 3E). Although induction of Fgf21 mRNA by the PPARα ligand bezofibrate was abrogated by a PPARα-specific siRNA that reduced PPARα transcript levels by 80% (inset), the T₃ response was unaffected.

**A functional TRE in the Fgf21 second intron**

To determine whether TRβ might bind the Fgf21 locus independently of PPARα, we consulted a published ChIP-chip dataset and our own preliminary ChIP-Seq results. Results of recent studies have indicated the

---

**Figure 5**

An intronic TRE in the FGF21 locus. (A) Reporter assays were performed in HepG2-TRβ1 cells co-transfected with a reporter construct harboring intron 2 of FGF21 in the presence of PPARα or TRβ expression plasmid. The cells were treated with either 100 nM WY14643 or 10 nM T₃ (n=4). (B) As shown in Fig. 5A, results of luciferase assays performed with a variety of reporter genes bearing intron 2 truncations are shown in the schematic diagram. (C) Diagrammatic representation of the FGF21 promoter within the reporter vector (the region that displayed enrichment within the ChIP-assay). The FGF21 promoter used in our studies contains binding sites for TRβ. The TRE determined to mediate the TRβ-response is also shown, as indicated by truncation and mutant analysis. (D) As shown in Fig. 5A, HepG2-TRβ1 cells co-transfected with a reporter construct harboring intron 2 of FGF21 or TRE-mutant reporter constructs or control vector pGL4 in the presence of TRβ expression plasmid. The cells were treated with either vehicle or 10 nM T₃ (n=4). (E) Electrophoretic mobility shift assay (EMSA) depicting TRβ binding to the discovered TRE. The response elements were measured by mixing 100 ng of purified TRβ (DBD–LBD) with 1 pM of biotin-labeled double-stranded oligonucleotide corresponding to C₇₁₅ to C₁₀₅₄ of FGF21. Binding specificity of the regulatory region was verified with competition experiments in which 50-fold molar excess of unlabeled +715 to +1054 of FGF21. Binding specificity of the regulatory region was verified with competition experiments in which 50-fold molar excess of unlabeled +715 to +1054 of FGF21. Binding specificity of the regulatory region was verified with competition experiments in which 50-fold molar excess of unlabeled +715 to +1054 oligomer or mutant-TRE +715 to +1054 oligomer (n=4). The statistical significance of the observed changes is denoted by double asterisks, which indicate P<0.01.
presence of an intronic TRβ-binding peak within the Fgf21 locus in mouse cerebellum (Dong et al. 2009) and we later confirmed the existence of this peak in the data derived from our own ChIP-Seq analysis of TRβ-binding events in HepG2 cells (Fig. 4A) (Ayers et al. 2014). We verified TRβ binding at this location with conventional ChIP-PCR performed on human HepG2-TRβ cells (Fig. 4B) and on native mouse liver (Fig. 4C).

Cloned sequences corresponding to the second intron of human FGF21 conferred T3 responsiveness upon a luciferase reporter (Fig. 5A). There was no response to PPARα ligand (WY14643). Deletion mapping indicated that important T3-responsive elements lay between the intronic sequences +771 and +842 downstream of the Fgf21 transcriptional start site (Fig. 5B). This region of the mouse gene harbored a degenerate nonconsensus TRE (DR-5) with one well conserved half site (AGGTCA; reverse strand TCCAGT) (Fig. 5C). Mutation of the putative TRE bound bacterially expressed TRβ with conventional ChIP-PCR (Fig. 5D). Furthermore, the putative TRE bound bacterially expressed TRβ in EMSA and this interaction was abolished by mutation of the AGGTCA sequence or an excess of WT oligonucleotide (Fig. 5E).

Altered hepatic T3-responses in Fgf21–/– mice

As T3 induces FGF21 in liver, and secreted FGF21 peptide may exert autocrine/paracrine effects upon gene expression in hepatocytes, we determined whether T3 regulated the same genes in the livers of WT and euthyroid Fgf21–/– mice using Illumina BeadArray analysis. More than 500 gene transcripts displayed significant response (greater than or equal to twofold with and adjusted P value <0.05) to a 3-day T3 treatment in WT mice, in accordance with previous studies. In general, T3-dependent gene regulation patterns were similar in both WT and Fgf21-knockout mice and the majority of T3 responses differed by not more than 50% (black points located within the dotted lines that delimit a twofold difference between WT and Fgf21–/– mice) (Fig. 6A).

We detected two clusters of genes with altered T3 response in the Fgf21–/– background (red points, outside of dotted delimiting lines). One group (Table 1, 31 genes) exhibited diminished T3-dependent activation in the Fgf21–/– background, and the other altered group (Table 2, 23 genes) displayed diminished T3-dependent repression. These altered responses to T3 were readily reproduced in independent high-throughput qRT-PCR experiments (Fig. 6B). Examples of such genes with diminished responses to T3 in the Fgf21-knockout mice include Dio1, Insl2, Cdkn1, and Glut1 (Fig. 6C, full gene names in Table 1). Genes that displayed similar levels of T3 response in both strains include Cpne2, Rab34, Crel2d, and Wfdc6, as identified in our previous studies (Lin et al. 2013) (Fig. 6D). Thus, targeted ablation of Fgf21 in the mouse germ line did not result in the global inhibition of a large percentage of hepatic T3 responses, but FGF21 was required for induction of a small subset of T3-regulated genes.

We could not perceive many obvious relationships between genes that display altered responses to T3 in the Fgf21-knockout mice and canonical hepatic metabolic process regulated by TH (Tables 1, 2, and 3). Results of ingenuity pathway analysis (IPA) indicated that the gene subsets that showed altered T3 response in the Fgf21-knockout background might affect the action of vitamin C,
the complement pathway, and other processes (Table 3 and results not shown). Similar biological functions were predicted through Genecodis analysis (Tabas-Madrid et al. 2012) (not shown). More directed queries in which we utilized IPA function pathway analysis to probe for the association of defined hepatic metabolic pathways with the T3/FGF21-dependent gene subset revealed the connections of several members of this gene set with different aspects of lipid metabolism, amino acid metabolism, and carbohydrate metabolism (Table 4). Inspection of these relationships did not allow us to generate many obvious predictions about the influences of these genes upon local hepatic and systemic metabolism (see ‘Discussion’).

Given that there were weak associations of the T3/FGF21-dependent gene set with classical T3-dependent hepatic metabolic pathways, we determined whether Fgf21-knockout influenced the levels of serum metabolites under the conditions of our experiment (Fig. 7). Previously, treatment with T3 and a thyromimetic has been shown to result in rapid reductions in serum cholesterol levels in WT mice (Lin et al. 2012, Lammel Lindemann et al. 2014). While we were able to reproduce this effect with short T3

<table>
<thead>
<tr>
<th>Probe_ID</th>
<th>Symbol</th>
<th>Entrez gene name</th>
<th>Fold change</th>
<th>Adjusted P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILMN_2874352</td>
<td>Cyp17A1</td>
<td>Cytochrome P450, family 17, subfamily A</td>
<td>8.861</td>
<td>3.110</td>
</tr>
<tr>
<td>ILMN_2647234</td>
<td>Dio1</td>
<td>Deiodinase, iodothyronine, type I</td>
<td>8.268</td>
<td>3.229</td>
</tr>
<tr>
<td>ILMN_2813830</td>
<td>Nt5e</td>
<td>5’-nucleotidase, ecto (CD73)</td>
<td>7.634</td>
<td>2.870</td>
</tr>
<tr>
<td>ILMN_3009501</td>
<td>Kcnk1</td>
<td>Potassium channel, subfamily K, member 1</td>
<td>7.393</td>
<td>3.370</td>
</tr>
<tr>
<td>ILMN_1241350</td>
<td>Vsig4</td>
<td>V-set and immunoglobulin domain containing 4</td>
<td>6.151</td>
<td>1.941</td>
</tr>
<tr>
<td>ILMN_1239959</td>
<td>E020018b13rik</td>
<td>RIKEN CDNA E020018b13 gene</td>
<td>6.088</td>
<td>-1.005</td>
</tr>
<tr>
<td>ILMN_2619620</td>
<td>C1qb</td>
<td>Complement component 1, q subcomponent, B chain</td>
<td>6.041</td>
<td>1.988</td>
</tr>
<tr>
<td>ILMN_12715840</td>
<td>C1qc</td>
<td>Complement component 1, q subcomponent, C chain</td>
<td>5.785</td>
<td>1.596</td>
</tr>
<tr>
<td>ILMN_2775030</td>
<td>Folr2</td>
<td>Folate receptor 2 (fetal)</td>
<td>5.643</td>
<td>2.751</td>
</tr>
<tr>
<td>ILMN_2528155</td>
<td>Loc227995</td>
<td>Predicted gene 13607, actin-like 6A pseudogene</td>
<td>5.618</td>
<td>2.070</td>
</tr>
<tr>
<td>ILMN_2878071</td>
<td>Lyz1/Lyz2</td>
<td>Lysozyme 2</td>
<td>5.506</td>
<td>2.081</td>
</tr>
<tr>
<td>ILMN_1234858</td>
<td>Insig2</td>
<td>Insulin-induced gene 2</td>
<td>5.022</td>
<td>1.302</td>
</tr>
<tr>
<td>ILMN_2671923</td>
<td>Ly86</td>
<td>Lymphocyte antigen 86</td>
<td>4.760</td>
<td>1.954</td>
</tr>
<tr>
<td>ILMN_1252636</td>
<td>Mmd2</td>
<td>Monocyte to macrophage differentiation-associated 2</td>
<td>4.749</td>
<td>1.231</td>
</tr>
<tr>
<td>ILMN_2925653</td>
<td>Ear2</td>
<td>Eosinophil-associated, ribonuclease A family, # 2</td>
<td>4.691</td>
<td>1.715</td>
</tr>
<tr>
<td>ILMN_2634083</td>
<td>Cdkn1a</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, CIP1)</td>
<td>4.595</td>
<td>1.083</td>
</tr>
<tr>
<td>ILMN_1216880</td>
<td>Emr1</td>
<td>EGF-like module containing, mucin-like, hormone receptor-like 1</td>
<td>4.462</td>
<td>1.711</td>
</tr>
<tr>
<td>ILMN_2640346</td>
<td>P2ry13</td>
<td>Purinergic receptor P2Y, G-protein-coupled, 13</td>
<td>4.249</td>
<td>2.000</td>
</tr>
<tr>
<td>ILMN_2785648</td>
<td>Cd207</td>
<td>CD207 molecule, langerin</td>
<td>4.242</td>
<td>1.781</td>
</tr>
<tr>
<td>ILMN_2533376</td>
<td>Mmp27</td>
<td>Matrix metalloepptidase 27</td>
<td>4.132</td>
<td>1.960</td>
</tr>
<tr>
<td>ILMN_2752524</td>
<td>Paqr9</td>
<td>Progestin and adipoQ receptor family member IX</td>
<td>3.988</td>
<td>1.724</td>
</tr>
<tr>
<td>ILMN_2524986</td>
<td>Ear3</td>
<td>Eosinophil-associated, ribonuclease A family, #3</td>
<td>3.869</td>
<td>1.493</td>
</tr>
<tr>
<td>ILMN_2948296</td>
<td>Wfdc12</td>
<td>WAP four-disulfide core domain 12</td>
<td>3.706</td>
<td>1.438</td>
</tr>
<tr>
<td>ILMN_2819319</td>
<td>Rrm2</td>
<td>Ribonucleotide reductase M2</td>
<td>3.687</td>
<td>1.627</td>
</tr>
<tr>
<td>ILMN_1241695</td>
<td>Ms46a</td>
<td>Membrane-spanning 4-domains, subfamily A, member 6A</td>
<td>3.376</td>
<td>1.641</td>
</tr>
<tr>
<td>ILMN_2896170</td>
<td>Apcs</td>
<td>Amyloid P component, serum</td>
<td>3.291</td>
<td>1.301</td>
</tr>
<tr>
<td>ILMN_3118707</td>
<td>Sla</td>
<td>Src-like-adaptor</td>
<td>3.113</td>
<td>1.191</td>
</tr>
<tr>
<td>ILMN_2548010</td>
<td>Hopx</td>
<td>HOP homeobox</td>
<td>2.970</td>
<td>1.422</td>
</tr>
<tr>
<td>ILMN_2788073</td>
<td>Hmx1</td>
<td>Heme oxygenase (decycling) 1</td>
<td>2.882</td>
<td>1.411</td>
</tr>
<tr>
<td>ILMN_2761585</td>
<td>2400009b08Rik</td>
<td>RIKEN CDNA 2400009b08 gene</td>
<td>2.409</td>
<td>1.109</td>
</tr>
<tr>
<td>ILMN_2728300</td>
<td>Mdm1</td>
<td>Mdm1 nuclear protein homolog (mice)</td>
<td>2.386</td>
<td>1.185</td>
</tr>
<tr>
<td>ILMN_1258159</td>
<td>Glut1</td>
<td>Glucose transporter 1</td>
<td>1.580</td>
<td>1.087</td>
</tr>
</tbody>
</table>

The table shows positively T3-regulated genes influenced by Fgf21 knockout with their respective fold change and adjusted P value.
Table 2 Negatively T3-regulated genes influenced by Fgf21 knockout

<table>
<thead>
<tr>
<th>Probe_ID</th>
<th>Symbol</th>
<th>Entrez gene name</th>
<th>Fold change WT</th>
<th>Fold change Fgf21KO</th>
<th>Adjusted P value WT</th>
<th>Adjusted P value Fgf21KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILMN_2852624</td>
<td>Hmapi/Hamp2</td>
<td>Hemicidin antimicrobial peptide</td>
<td>11.73</td>
<td>5.69</td>
<td>0.006</td>
<td>0.049</td>
</tr>
<tr>
<td>ILMN_1253233</td>
<td>Dsg1c</td>
<td>Desmoglein 1 gamma</td>
<td>8.159</td>
<td>3.932</td>
<td>0.002</td>
<td>0.029</td>
</tr>
<tr>
<td>ILMN_2976211</td>
<td>Cyp2b23</td>
<td>Cytochrome P450, family 2, subfamily b, polypeptide 23</td>
<td>7.294</td>
<td>1.075</td>
<td>0.022</td>
<td>0.970</td>
</tr>
<tr>
<td>ILMN_2796472</td>
<td>Vldlr</td>
<td>Very-LDL receptor</td>
<td>7.191</td>
<td>2.436</td>
<td>0.011</td>
<td>0.253</td>
</tr>
<tr>
<td>ILMN_1232758</td>
<td>Gm3065</td>
<td>Predicted gene 3065</td>
<td>4.219</td>
<td>1.550</td>
<td>0.010</td>
<td>0.480</td>
</tr>
<tr>
<td>ILMN_1214219</td>
<td>Col27a1</td>
<td>Collagen, type XXVII, alpha 1</td>
<td>3.491</td>
<td>1.564</td>
<td>0.001</td>
<td>0.189</td>
</tr>
<tr>
<td>ILMN_1230145</td>
<td>Acvr2b</td>
<td>Activin A receptor, type IIB</td>
<td>3.352</td>
<td>1.575</td>
<td>0.032</td>
<td>0.488</td>
</tr>
<tr>
<td>ILMN_2565942</td>
<td>Fnib</td>
<td>Filamin B, beta</td>
<td>3.277</td>
<td>1.451</td>
<td>0.038</td>
<td>0.605</td>
</tr>
<tr>
<td>ILMN_1256423</td>
<td>Ranpb3</td>
<td>RAN-binding protein 3-like</td>
<td>3.274</td>
<td>1.440</td>
<td>0.009</td>
<td>0.452</td>
</tr>
<tr>
<td>ILMN_1218441</td>
<td>Slc23a3</td>
<td>Solute carrier family 23, member 3</td>
<td>3.073</td>
<td>1.479</td>
<td>0.030</td>
<td>0.519</td>
</tr>
<tr>
<td>ILMN_2875737</td>
<td>Cyp2d6</td>
<td>Cytochrome P450, family 2, subfamily D, polypeptide 6</td>
<td>2.945</td>
<td>1.313</td>
<td>0.022</td>
<td>0.655</td>
</tr>
<tr>
<td>ILMN_2523012</td>
<td>Meg3</td>
<td>Maternally expressed 3</td>
<td>2.936</td>
<td>1.143</td>
<td>0.033</td>
<td>0.879</td>
</tr>
<tr>
<td>ILMN_2625233</td>
<td>9130230123r</td>
<td>RIKEN cDNA 9130230L23 gene</td>
<td>2.890</td>
<td>1.395</td>
<td>0.026</td>
<td>0.559</td>
</tr>
<tr>
<td>ILMN_1213804</td>
<td>Lrp4</td>
<td>LDL receptor-related protein 4</td>
<td>2.767</td>
<td>1.030</td>
<td>0.020</td>
<td>0.975</td>
</tr>
<tr>
<td>ILMN_2624238</td>
<td>Adams7</td>
<td>ADAM metallopeptidase with thrombospondin type 1 motif, 7</td>
<td>2.635</td>
<td>1.154</td>
<td>0.002</td>
<td>0.713</td>
</tr>
<tr>
<td>ILMN_1239872</td>
<td>5830473c10rik</td>
<td>RIKEN cDNA 5830473C10 gene</td>
<td>2.597</td>
<td>1.026</td>
<td>0.011</td>
<td>0.975</td>
</tr>
<tr>
<td>ILMN_1227319</td>
<td>Vwce</td>
<td>Von Willebrand factor C and EGF domains</td>
<td>2.440</td>
<td>1.212</td>
<td>0.011</td>
<td>0.662</td>
</tr>
<tr>
<td>ILMN_1247135</td>
<td>Znf276</td>
<td>Zinc finger protein 276</td>
<td>2.209</td>
<td>1.095</td>
<td>0.007</td>
<td>0.838</td>
</tr>
<tr>
<td>ILMN_2764325</td>
<td>Brics5</td>
<td>BRICOS domain containing 5</td>
<td>2.202</td>
<td>1.089</td>
<td>0.036</td>
<td>0.902</td>
</tr>
<tr>
<td>ILMN_2557871</td>
<td>Kit1b</td>
<td>Kinesin family member 1B</td>
<td>2.135</td>
<td>1.021</td>
<td>0.033</td>
<td>0.980</td>
</tr>
<tr>
<td>ILMN_2954098</td>
<td>Lgs16</td>
<td>Lecin, galactose-binding, soluble 6</td>
<td>2.065</td>
<td>1.024</td>
<td>0.011</td>
<td>0.969</td>
</tr>
<tr>
<td>ILMN_2718600</td>
<td>Nfyb</td>
<td>Nuclear transcription factor Y, beta</td>
<td>2.061</td>
<td>1.027</td>
<td>0.005</td>
<td>0.956</td>
</tr>
<tr>
<td>ILMN_2622891</td>
<td>C12orf43</td>
<td>Chromosome 12 open reading frame 43</td>
<td>2.059</td>
<td>1.028</td>
<td>0.044</td>
<td>0.973</td>
</tr>
</tbody>
</table>

Discussion

PPARα-dependent induction of FGF21 is required for changes in expression of genes that are involved in the fasting response in liver, and secreted hepatic FGF21 influences local hepatocyte metabolism and communicates information from liver to other tissues to coordinate fasting responses. Thus, FGF21 has attracted attention as a possible therapeutic to modulate metabolic disease. In this study, we verified previous observations which indicate that T3 induces hepatic FGF21 production (Adams et al. 2010, Domouzoglou et al. 2014) to show that sustained TR-dependent induction of FGF21 is elicited by a selective TRβ modulator and is dependent on TRβ. Furthermore, similar effects were also observed in a human liver cell line (HepG2), in which FGF21 induction was amplified in the presence of exogenous TRβ, and T3-dependent induction of FGF21 was also detectable in the presence of low endogenous levels of TRβ in this cell type (Yuan et al. 2012). Thus, the capacity for T3 regulation of hepatic FGF21 production is conserved in humans and in cultured cells of hepatic origin. Finally, TR regulation of FGF21

Table 3 IPA canonical pathway analysis of T3/FGF21-dependent genes

<table>
<thead>
<tr>
<th>Ingenuity canonical pathways</th>
<th>−log (P value)</th>
<th>Ratio</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antioxidant action of vitamin C</td>
<td>2.89</td>
<td>0.027</td>
<td>HMOX1, SLC23A3, PLA2G7</td>
</tr>
<tr>
<td>Complement system Phospholipases</td>
<td>2.61</td>
<td>0.057</td>
<td>C1QC, C1QB, HMOX1, PLA2G7, MMP27, CDKN1A</td>
</tr>
<tr>
<td>Bladder cancer signaling Pancreatic adenocarcinoma signaling</td>
<td>1.62</td>
<td>0.016</td>
<td>HMOX1, CDKN1A</td>
</tr>
</tbody>
</table>
FGF21 activate the (results not shown). In the light of this study (Adams luciferase reporter activity in transient transfections FGF21 to elicit additive increases in b proximal promoter activity (Adams Col27a1, Ly86, Lyz1/Lyz2, Ms4a6a, Nt5e, P2ry13 Insulin-dependent diabetes mellitus 6 0.0012 Carbohydrate metabolism 3 0.0037 Cdkn1a, Dio1 Amino acid metabolism 2 0.0030 Cdkn1a, Cyp17a1, Dio1, P2ry13, Vldlr Conversion of lipid 3 0.0049 Conversion of lipid 3 0.0094 Cyp17a1, Cyp2d6, Hmox1 Insulin-dependent diabetes mellitus 6 0.0012 Cdkn1a, Dio1, P2ry13, Vldlr Efflux of cholesterol 2 0.0192 Apcs, Vldlr Insulin-dependent diabetes mellitus 6 0.0012 Cdkn1a, Dio1, P2ry13, Vldlr Quantity of steroid 5 0.0067 Cdkn1a, Cyp17a1, Dio1, P2ry13, Vldlr Insulin-dependent diabetes mellitus 6 0.0012 Cdkn1a, Dio1, P2ry13, Vldlr Lipid Metabolism

While TRs do not bind to the Fgf21 proximal promoter, the results of studies by our own group and others indicate that TREs are often located within introns or the 3’ downstream region of target genes (Chatonnet et al. 2013, Gagne et al. 2013, Ayers et al. 2014, Ramadoss et al. 2014). We therefore searched published databases of TRE localization and noted that the mouse Fgf21 locus harbors a TR-binding site in intron 2. This binding site was first detected in a study of TR-binding events near of T3-target genes in cerebellum (Dong et al. 2009), which we later confirmed by our own genome wide assessment of TRβ1-binding events that utilized an engineered human HepG2 cell line that expresses exogenous TRβ1 (Ayers et al. 2014). We verified TR localization with human and mouse intron 2 using traditional ChIP-PCR approaches in HepG2 and native mouse liver and identified a functional TRE in this region of the mouse gene. We therefore suggest that TRs act through this intronic TRE to activate FGF21 expression in HepG2 cells and that this effect is independent of PPARα signaling.

While our results indicate that TR can bind to a functional TRE in FGF21 intron 2, we emphasize that they do not contradict the results of previous studies which indicate that TRs cooperate with PPARα to enhance FGF21 proximal promoter activity (Adams et al. 2010). Unlike the FGF21 proximal promoter, the putative FGF21 intronic TRE does not respond to PPARα ligands. Furthermore, bezofibrate activation of the human FGF21 gene is abolished by PPARα knockdown in HepG2 cells, with the T3 response unaffected by PPARα knockdown. Thus, T3 can activate the FGF21 gene independently of PPARα. However, we also verified that TRβ and PPARα cooperate to elicit additive increases in FGF21-promoter-dependent luciferase reporter activity in transient transfections (results not shown). In the light of this study (Adams et al. 2010) and other suggestions that TRs cooperate with PPARs to regulate gene expression (Lu & Cheng 2010), we suspect that TRs bound to the downstream intronic TRE can communicate with PPARα to regulate FGF21 expression. This idea leads to the prediction that T3 regulation of FGF21 could be influenced by changes in PPARα activity and it will be interesting to see whether dietary conditions or administration of PPARα ligands could alter the patterns of hepatic T3 regulation of FGF21 or hepatic gene response in general.

As FGF21 acts in an autocrine/paracrine fashion to regulate hepatic target genes involved in metabolic responses (Inagaki et al. 2007), we also tested the hypothesis that T3-dependent regulation of hepatic gene expression programs would require FGF21. Specifically, we used an unbiased approach to determine whether responses to T3 of any hepatic genes was diminished or abrogated in a mouse strain with a targeted deletion of the Fgf21 gene locus. While T3 regulates hundreds of genes in the livers of euthyroid mice, many with important roles in T3-regulation of metabolic responses, there were limited differences in T3 response patterns in WT and Fgf21−/− mice. During our studies, a group led by Maratos-Flier et al. examined T3

<table>
<thead>
<tr>
<th>Function and pathway</th>
<th>Number of molecules</th>
<th>P value</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration of cholesterol</td>
<td>4</td>
<td>0.0051</td>
<td>Cdkn1a, Dio1, P2ry13, Vldlr</td>
</tr>
<tr>
<td>Efflux of cholesterol</td>
<td>2</td>
<td>0.0192</td>
<td>Apcs, Vldlr</td>
</tr>
<tr>
<td>Accumulation of triacylglycerol</td>
<td>2</td>
<td>0.0139</td>
<td>Cdkn1a, Dio1</td>
</tr>
<tr>
<td>Conversion of lipid</td>
<td>3</td>
<td>0.0053</td>
<td>Cyp17a1, Cyp2d6, Hmox1</td>
</tr>
<tr>
<td>Quantity of steroid</td>
<td>5</td>
<td>0.0067</td>
<td>Cdkn1a, Cyp17a1, Dio1, P2ry13, Vldlr</td>
</tr>
<tr>
<td>Amino acid metabolism</td>
<td>2</td>
<td>0.0030</td>
<td>Cdkn1a, Dio1</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>3</td>
<td>0.0037</td>
<td>Lyz1/Lyz2, Nt5e, P1a2g7</td>
</tr>
<tr>
<td>Insulin-dependent diabetes mellitus</td>
<td>6</td>
<td>0.0012</td>
<td>Col12a1, Ly86, Lyz1/Lyz2, Ms4a6a, Nt5e, P2ry13</td>
</tr>
</tbody>
</table>

Figure 7 Analysis of serum metabolites in WT and Fgf21-knockout mice. (A) total serum cholesterol, (B) triglycerides, (C) glucose in WT and Fgf21−/− mice after 3-day T3 treatment. The statistical significance of the observed changes is denoted by double asterisks, which indicate P<0.01.
regulation of metabolic parameters in Fgf21-knockout mice and the effects of administered FGF21 in hypothyroid and euthyroid mouse backgrounds (Domouzoglou et al. 2014). Results of this study revealed that T3 and FGF21-dependent effects upon serum and hepatic lipid levels, thermogenesis and energy metabolism, and selected T3 and FGF21 target genes are almost completely independent of the other hormone signaling system. Our transcriptome-wide gene expression studies extend and lend further support to this idea, and moreover, our own investigation of the effects of FGF21 and T3 upon the levels of serum metabolites confirms that T3-dependent reductions in total serum cholesterol are completely independent of FGF21.

We detected a small subset of hepatic genes that displayed diminished responses to T3 in the Fgf21−/− background. In principle, such genes could be indirect targets that lie downstream of the FGF21 peptide, or FGF21 could be a permissive factor for induction of direct TR targets, analogous to the hepatic PPARz/FGF21 feed-forward loop established under fasting conditions in which FGF21 activation prolongs PPARz-dependent responses (Badman et al. 2007, Inagaki et al. 2007). While we have not determined the mechanisms of these effects, we suspect that the latter mechanism is at play at least in regulation of Dio1, which contains bona fide TREs within its proximal promoter (Zhang et al. 1998). Perhaps more surprising, however, was that T3/FGF21-dependent genes were associated with complement activation and the cell cycle and that many classical T3-regulated genes involved in cholesterol metabolism (Lin et al. 2012, Lindemann et al. 2014), fatty acid synthesis, β-oxidation, and gluconeogenesis (Singh et al. 2013, Suh et al. 2013, Thakran et al. 2013) did not appear in this list. The significance of the dual T3/FGF21 dependency displayed by this gene subset is not clear and requires further investigation.

We recognize that the notion that T3 and FGF21 regulate hepatic metabolic response independently of each other must remain qualified for several reasons. We analyzed T3-dependent changes in gene expression and serum lipid parameters after 72 h treatment. While this is long enough to obtain significant elevations of hepatic and serum FGF21 levels, it is possible that interactions between TR and FGF21-dependent pathways could emerge over longer treatment times. Furthermore, our studies were performed in euthyroid mice treated with T3 and not hypothyroid mice. Thus, it is possible that some T3- and FGF21-responsive genes with key roles in metabolic response could be fully induced at T3 concentrations observed in euthyroid mice (data shown in Lin et al. (2013)). Arguing against these possibilities, however, is the failure of the Maratos-Flier group also to identify defects in TR-dependent changes in metabolic parameters and gene expression in FGF21 knockouts over time courses of more than 1 week and in hypothyroid mice with varying levels of T3 (Domouzoglou et al. 2014).

It also remains possible that some members of the T3/FGF21-responsive gene subset could exert hard to predict effects upon metabolic responses. We attempted a more exhaustive effort to define the association of the T3/FGF21-responsive gene subset with classical metabolic pathways and discovered possible association of genes with various aspects of lipid, amino acid, and carbohydrate metabolic pathways and insulin-dependent diabetes mellitus (Table 4). For example, Dio1 encodes a product that catalyzes the conversion of T4 to T3 and therefore will alter local TH levels and, potentially, TR actions upon key genes involved in cholesterol metabolism. Insig2 is an inhibitor of sterol response element-binding protein (SREBP) activation and changes in levels of this transcription factor could result in widespread secondary alterations in the expression patterns of genes that regulate lipid metabolism. While it is conceivable that altered cooperation of TH and FGF21 at these, and other, genes could ultimately result in changes in systemic cholesterol and lipid levels, such effects were not obvious over the course of our study (Fig. 7). At present our results are most consistent with the notion that T3-dependent hepatic gene regulation patterns are largely independent of FGF21 signaling.

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.

Funding
This study was supported by NIH RC4 DK090849 (P W) and National Natural Science Foundation of China No. 81270868 (X X).
References


Dong H, Yauk CL, Rowan-Carroll A, You SH, Zoeller RT, Lambert I & Wade MG 2009 Identification of thyroid hormone receptor binding sites and target genes using ChIP-on-chip in developing mouse cerebellum. PLoS ONE 4 e4610. (doi:10.1371/journal.pone.0004610)


factor 21 promotes bone loss by potentiating the effects of peroxisome proliferator-activated receptor $\gamma$. PNAS 109 3143–3148. (doi:10.1073/pnas.1200797109)


Received in final form 8 December 2014
Accepted 11 December 2014
Accepted Preprint published online 11 December 2014