

RESEARCH ARTICLE

Regulation of type 1 iodothyronine deiodinase by LXR α

Yoriko Sakane^{1‡a}, Naotetsu Kanamoto^{1‡b*}, Ichiro Yamauchi¹, Tetsuya Tagami², Yusuke Morita³, Masako Miura^{1‡c}, Masakatsu Sone¹, Akihiro Yasoda¹, Takeshi Kimura³, Kazuwa Nakao⁴, Nobuya Inagaki¹

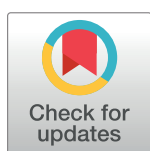
1 Department of Diabetes, Endocrinology and Nutrition, Kyoto University Graduate School of Medicine, Kyoto, Japan, **2** Division of Endocrinology and Metabolism, Clinical Research Institute, National Hospital Organization Kyoto Medical Center, Kyoto, Japan, **3** Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **4** Medical Innovation Center, Kyoto University Graduate School of Medicine, Kyoto, Japan

‡a Current address: Preemptive Medicine and Lifestyle Disease Research Center, Kyoto University Hospital, Kyoto, Japan

‡b Current address: Department of Endocrinology, Osaka City General Hospital, Osaka, Japan

‡c Current address: Department of Endocrinology, Rakuwakai Otowa Hospital, Kyoto, Japan

* kyotetsu@kuhp.kyoto-u.ac.jp



OPEN ACCESS

Citation: Sakane Y, Kanamoto N, Yamauchi I, Tagami T, Morita Y, Miura M, et al. (2017) Regulation of type 1 iodothyronine deiodinase by LXR α . PLoS ONE 12(6): e0179213. <https://doi.org/10.1371/journal.pone.0179213>

Editor: Makoto Makishima, Nihon University School of Medicine, JAPAN

Received: December 4, 2016

Accepted: May 25, 2017

Published: June 15, 2017

Copyright: © 2017 Sakane et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by JSPS KAKENHI (Grant Number 16H06902), MEXT KAKENHI (Grant Numbers 21229013, 20790659, 18790635, and 19591075; <https://www.jsps.go.jp/j-grantsinaid/>), and Takeda Science Foundation (<http://www.takeda-sci.or.jp>). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

The iodothyronine deiodinases are selenoenzymes that regulate the activity of thyroid hormone via specific inner- or outer-ring deiodination. In humans, type 1 deiodinase (D1) is highly expressed in the liver, but the mechanism by which its gene expression is regulated remains to be elucidated. Liver X receptor α (LXR α), a transcription factor of the nuclear receptor superfamily, is highly expressed in the liver, where it functions as a sensor for excess intracellular oxysterols. LXR α interacts with other nuclear receptors on promoters of genes that contain a binding core sequence for nuclear receptors. In addition, it is reported that the promoter of the gene encoding human D1 (*hDIO1*) contains the core sequence for one of nuclear receptors, thyroid hormone receptor (TR). We investigated the involvement of LXR α in the regulation of *hDIO1*, in the liver. We performed *hDIO1* promoter-reporter assays using a synthetic LXR agonist, T0901317, and compared promoter activity between a human liver carcinoma cell line, HepG2, and a clone of human embryonic kidney cells, TSA201. We defined the region between nucleotides –131 and –114, especially nucleotides –126 and –125, of the *hDIO1* promoter as critical for basal and LXR α -mediated specific transcriptional activation in HepG2 cells. An increase in *hDIO1* expression was observed in LXR α -stimulated cells, but absent in cycloheximide-treated cells, indicating that new protein synthesis is required for LXR α -mediated regulation of *hDIO1*. On the other hand, electrophoretic mobility shift assays revealed that LXR α and RXR α bound to the *hDIO1* promoter. We also demonstrated that LXR α and TR β compete with each other on this specific region of the promoter. In conclusion, our results indicated that LXR α plays a specific and important role in activation of TH by regulating D1, and that LXR α binds to and regulates the *hDIO1* promoter, competing with TR β on specific sequences within the promoter.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Iodothyronine deiodinases type 1, 2, and 3 (D1, D2, and D3, respectively) are selenoenzymes that regulate the activity of thyroid hormone (TH) via specific inner- or outer-ring deiodination [1]. Biologically active intracellular triiodothyronine (T3) is supplied from the serum and via activation of thyroxine (T4) by D1 and D2 [1]. In human, D1 is expressed at high levels in the liver, kidney, and thyroid, whereas D2 is expressed in the brain, pituitary, and brown adipose tissue. The local T3 concentration in hepatic cells depends on serum TH concentration, T3 generated from T4 by D1, and clearance of T3 from hepatic cells. The expression and activity of D1 are modulated by multiple factors including T3, cAMP, retinoic acid, and TSH [2–4]. In a previous report, we demonstrated that expression of the human D1 gene (*hDIO1*) is liver-specific and cooperatively regulated by forkhead box A1 (FOXA1), FOXA2, and upstream stimulatory factor (USF) via specific regions of the *hDIO1* promoter [5]. However, much remains unknown regarding the mechanisms of regulation of *hDIO1* and its physiological role in humans.

Liver X receptors (LXRs) are ligand-activated transcription factors of the nuclear receptor superfamily that bind oxidized cholesterol and function as sensors for excess intracellular oxysterols [6]. LXRs exist in two isoforms, LXR α and LXR β . LXR β is expressed ubiquitously, whereas LXR α is highly expressed in the liver, and at lower levels in adipose tissue, adrenal glands, intestines, lungs, kidneys, and cells of myeloid origin [7]. LXRs form heterodimers with the retinoid X receptor (RXR) and bind to the LXR-responsive element (LXRE) in promoters of target genes that contain direct repeats (DRs) of the core sequence AGGTCA separated by four nucleotides (DR4) [8].

LXRs bind to and compete with another subgroup of nuclear receptor, the thyroid hormone receptors (TRs), at the same sites in the 5'-region of promoters, e.g., those of the genes encoding acetyl-CoA carboxylase- α , cholesterol 7- α -hydroxylase, and ATP-binding cassette transporter A1 [9–11]. Toyoda *et al.* previously reported that the *hDIO1* promoter contains two functional TH response elements (TREs), each of which also consists of two DRs with the core sequence, AGGTCA [3]. Although LXRs and TRs belong to two distinct receptor subgroups with respect to ligand-binding affinity [12], the two receptor systems have similar molecular mechanisms, i.e., both form heterodimers with RXRs and to bind to DR4 with identical geometry and polarity [13,14]. To date, it remains unknown whether LXRs regulate the expression of *hDIO1*. Because LXR α is expressed at very high levels in the liver, and the *hDIO1* promoter contains TREs, we hypothesized that LXR α is involved in regulation of *hDIO1* in a liver-specific manner by unknown mechanisms.

Thus, in this study, we investigated the involvement of LXR α in the regulation of *hDIO1*, in the liver using a human liver carcinoma cell line HepG2.

Materials and methods

Ligand

A synthetic LXR agonist, T0901317 (TO), was purchased from Cayman Chemical (Ann Arbor, MN, USA), and a synthetic LXR antagonist, GSK2033 (GSK), was purchased from Axon Medchem BV (Groningen, The Netherlands). TO and GSK were dissolved in and diluted with dimethyl sulfoxide at various concentrations.

Cell culture

A human liver carcinoma cell line, HepG2 [5], and a clone of human embryonic kidney 293 cells, TSA201 [15], were cultured as described previously.

Plasmid construction

Deletion mutants of the 5'-flanking region of *hDIO1* (−2023, −803, −187, −131, −113, −103/−4; the transcription start site was defined as +1, nucleotide 53894211, NC_000001.11) were prepared and subcloned into pGL 4.10 (Promega, Madison, WI, USA) to create a fusion with the luciferase gene (−2023, −803, −187, −131, −113, −103/−4 *hDIO1*-Luc) as described previously [5]. Mutations were created in the −131/−4 *hDIO1*-Luc construct at nucleotides −124, −119/−118, −108/−106, −126/−125, or both −126/−125 and −108/−106 of the *hDIO1* promoter using −131/−4 *hDIO1*-Luc construct as a template and the QuikChange® Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Sequences of the 5'-flanking region of mutated constructs are provided in Table 1. These constructs were verified by sequencing. Each plasmid expressing cDNA for human TR β , human RXR α , human LXR α , and human farnesoid X receptor (FXR) was subcloned into pCMX as described previously [16]. A plasmid expressing cDNA for human pregnane X receptor (pFN21A-hPXR) was generated by Kazusa DNA Research Institute (Chiba, Japan) and purchased from Promega. We also prepared a plasmid expressing cDNA for human PAR-related orphan receptor alpha (ROR α). The open reading frame of ROR α was generated by PCR using cDNA from HepG2 cells as a template. We used primers containing *SgfI* or *PmeI* linker: 5'-GACCGCGATCGCCATGGAGTCAGCTCCGGCAGCCC-3' and 5'-ATTAGTTTAAACCCCATCAATTTGCATTGCATTGCTGGTCA-3'. The PCR product was digested with *SgfI* and *PmeI* and cloned into *SgfI*/*PmeI*-digested pFN21A CMV Flexi vector (Promega). The plasmid was verified by sequencing and synthesis of ROR α was confirmed by western blot.

Transient transfection and luciferase assay

Transient transfections were performed in 24-well tissue culture plates using the Lipofectamine™ LTX reagent (Thermo Fisher Scientific, Waltham, MA, USA) for HepG2 cells, and the Lipofectamine™ 2000 reagent (Thermo Fisher Scientific) for TSA201 cells, as described previously [5]. Briefly, cells were seeded in 24-well plates 1 day before transfection and maintained in 0.5 ml of antibiotic-free medium supplemented with 10% charcoal-stripped bovine serum (Thermo Fisher Scientific). For transfection of HepG2 cells, we used 500 ng of experimental reporter constructs, 50 ng of each expression vector, and 25 ng of pGL 4.74, which contains the cDNA encoding *Renilla* luciferase (Promega) as an internal control for transfection efficacy. For transfection of TSA201 cells, we used 100 ng of experimental reporter constructs, 10 ng of each expression vector, and 5 ng of pGL 4.74. In both cell types, culture media were replaced with antibiotic-free medium supplemented with 10% charcoal-stripped bovine serum with vehicle or 10^{−7} M TO 6–8 h after transfection. The cells were then cultured for an additional 48 h and harvested. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega), and luminescence was measured by a 2030 ARVOX

Table 1. Wild-type and mutated sequence of constructs used in mutational analysis.

Mutated construct and control	Sequence of 5'-flanking region of the construct between the nucleotides −131 and −104
−126/−125, −108/−106 Mut −131/−4 <i>hDIO1</i> -Luc	5'-TCTGA AA TGACTCCTTCCCCTGA AAA GG
−126/−125 Mut −131/−4 <i>hDIO1</i> -Luc	5'-TCTGA AA TGACTCCTTCCCCTGACCCGG
−124 Mut −131/−4 <i>hDIO1</i> -Luc	5'-TCTGAC CC GACTCCTTCCCCTGACCCGG
−119/−118 Mut −131/−4 <i>hDIO1</i> -Luc	5'-TCTGACCTGACT TG TCCCCTGACCCGG
−108/−106 Mut −131/−4 <i>hDIO1</i> -Luc	5'-TCTGAAATGACTCCTTCCCCTGA AAA GG
−131/−4 <i>hDIO1</i> -Luc	5'-TCTGACCTGACTCCTTCCCCTGACCCGG

The mutated base pairs between nucleotides −131 and −104 of the *hDIO1* promoter are indicated by bold italic letters with underlines.

<https://doi.org/10.1371/journal.pone.0179213.t001>

multilabel reader (PerkinElmer, Waltham, MA, USA). Firefly luciferase activity was normalized against *Renilla* luciferase activity in each well to control for transfection efficacy.

RNA isolation, reverse transcription, and quantitative PCR

HepG2 cells were cultured in antibiotic-free medium supplemented with 10% charcoal-stripped bovine serum (Thermo Fisher Scientific) and treated with TO at various concentrations and 10^{-6} M GSK, or pre-treated with cycloheximide for 30 min before treatment with 10^{-7} M TO for 24 h. Then, total RNA was extracted from HepG2 cells using the RNeasy® Plus Mini Kit (QIAGEN, Valencia, CA, USA). One microgram of total RNA was reverse-transcribed with random hexamers using First-strand cDNA Synthesis Kit (GE Healthcare UK Ltd, Buckinghamshire, UK). The resultant cDNA was diluted 1:10 by addition of TE buffer.

Quantitative PCR was performed, recorded, and analyzed using TaqMan® Gene Expression Assays with the StepOnePlus™ Real-time PCR System (Thermo Fisher Scientific). The probe/primer sets were Hs00174944_m1 (*hDIO1*), Hs00230861_m1 (*THRB*), and Human *PPIA* (Cyclophilin A) Endogenous Control, purchased from Thermo Fisher Scientific. Diluted cDNA was amplified as described previously [5]. Expression levels of each gene were normalized against the corresponding mRNA levels of cyclophilin A to compensate for variations in input RNA.

Preparation of electrophoresis mobility shift assay (EMSA)

Nuclear extracts were prepared from HepG2 cells and TSA201 cells using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). HepG2 cells were treated with vehicle or TO, with or without co-transfection of expression vectors for LXRα and RXRα (LXRα/ RXRα). One microgram of each expression vector was transfected into HepG2 cells in 60-mm dishes using the Lipofectamine™ LTX reagent (Thermo Fisher Scientific). Vehicle or TO was added to the culture media 24 h after transfection. The cells were then cultured for an additional 24 h and harvested. EMSAs and supershift assays were conducted using the LightShift™ Chemiluminescent EMSA kit (Thermo Fisher Scientific) as directed by the manufacturer with slight modifications, as described previously [5]. The antibodies used in the supershift assays were as follows: anti-human LXRα mouse monoclonal antibody (PP-PPZ0412-00, Perseus Proteomics, Tokyo, Japan), anti-human RXRα mouse monoclonal antibody (PP-K8508-00, Perseus Proteomics), anti-human TRβ1 mouse monoclonal antibody (sc-738X, Santa Cruz Biotechnology, Dallas, TX, USA), and normal mouse IgG (sc-2025, Santa Cruz Biotechnology). The sequences of oligonucleotides used for EMSA are provided in Table 2.

Table 2. Sequences of double-stranded oligonucleotides used in EMSA.

Oligonucleotides	Sequences
Wt1	5' -GCAAACATCT TCTGACCTGACTCCTT CCCC-3'
-124 mut1	5' -GCAAACATCT TCTGACCCG ACTCCTTCCCC-3'
-126/-125 mut1	5' -GCAAACATCT TCTGAAATGACTCCTT CCCC-3'
Wt2	5' - TCTGACCTGACTCCTT CCCCTGACCCGG-3'
-126/-125 mut2	5' - TCTGAAATGACTCCTT CCCCTGACCCGG-3'
LXRE	5' -GATCTTAGTTCACTCAAGTTCAAGGATC-3'

Wt1, oligonucleotide containing the wild-type sequence of the region between nucleotides -141 and -112 of the *hDIO1* promoter; -124 mut1, oligonucleotide containing a mutation at nucleotide -124 of the Wt1 oligonucleotide; -126/-125 mut1, oligonucleotide containing mutations at nucleotides -126 and -125 of the Wt1 oligonucleotide; Wt2, oligonucleotide containing the sequence of the region between nucleotides -131 and -104 of the *hDIO1* promoter [3]; -126/-125 mut2, oligonucleotide containing mutations at nucleotides -126 and -125 of the Wt2 oligonucleotide; LXRE, oligonucleotide containing the consensus sequence of the LXR response element [17]. The oligonucleotide sequence between nucleotides -131 and -114 of the *hDIO1* promoter is indicated by bold letters, and mutated base pairs are indicated by bold italic letters with underlines.

<https://doi.org/10.1371/journal.pone.0179213.t002>

Transfection of short interfering RNA (siRNA)

An aliquot of 2.5 pmol siRNA specific for *THRB* (s14121, Silencer Select[®] siRNA, Thermo Fisher Scientific) or a negative control siRNA (Negative Control #2, Silencer Select[®] siRNA, Thermo Fisher Scientific) was introduced into HepG2 cells using 24-well plates and the Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) by reverse transfection. Transfections of siRNA were performed 24 h before transfections of expression vectors for the luciferase assay. To determine the knockdown efficacy of siRNA, mRNA was extracted 72 h after siRNA transfection and analyzed by quantitative PCR as described above. Changes in levels of TR β protein (coded by *THRB*) following siRNA transfections were verified by western blot. See [S1 File](#) for detailed information.

Statistical analysis

Data are expressed as means $\pm$ standard error of the mean (SEM) obtained from at least three separate experiments. Significance of differences was evaluated using analysis of variance (ANOVA) followed by the Tukey–Kramer method, unless otherwise specified. *P* values < 0.05 were considered to be statistically significant.

Results

Identification of the specific region of the *hDIO1* promoter required for its basal activity and LXR α -mediated in HepG2 cells

To identify the specific region of the *hDIO1* promoter important for its activation by the synthetic LXR agonist, TO, we transiently transfected a series of 5′-deletion constructs into HepG2 and TSA201 cells, along with or without expression vectors for LXR α and RXR α (LXR α /RXR α). The cells were then cultured with or without TO ([Fig 1](#) and [S2 File](#)). In both cell lines, basal luciferase activity was significantly decreased by deletion of nucleotides –131 to –114 (*P* < 0.01) ([Fig 1A](#) and Fig A in [S2 File](#)). Luciferase activity following addition of TO was significantly increased (1.48-fold) without expression vectors for LXR α /RXR α (Fig B in [S2 File](#)) and further increased (1.93-fold) with those vectors in HepG2 cells transfected with the –131/–4 *hDIO1*-Luc construct (*P* < 0.01) ([Fig 1B](#)). These increases in response to TO were abolished by deletion of nucleotides –131 to –114. On the other hand, in TSA201 cells transfected with the –131/–4 *hDIO1*-Luc construct, no significant increase in luciferase activity was observed in response to TO ([Fig 1B](#)). These results indicated the importance of the specific region between nucleotides –131 and –114 for both basal activity and LXR α -mediated activation of the *hDIO1* promoter in HepG2 cells.

Regulation of *hDIO1* expression by LXR α

To confirm the change in *hDIO1* mRNA levels, we performed quantitative PCR analysis. The *hDIO1* mRNA level was increased by TO in a dose-dependent manner ([Fig 2A](#)). Furthermore, the increase in *hDIO1* mRNA following addition of TO was diminished by co-treatment with a LXR antagonist GSK ([Fig 2B](#)).

TO is a ligand for FXR, PXR, and ROR α as well as LXR α [18,19]. To verify that the effect of TO on the *hDIO1* promoter depends on LXR α , we performed luciferase assays using expression vectors for these receptors in HepG2 cells ([S3 File](#)). Exclusively in cells co-transfected with expression vectors for LXR α /RXR α , luciferase activity in response to TO was significantly higher than in cells transfected with empty vectors. Overexpression of FXR and ROR α did not alter luciferase activity, and PXR instead decreased, in comparison with the results of each empty vector. These results indicated that LXR α specifically activates the *hDIO1* promoter.

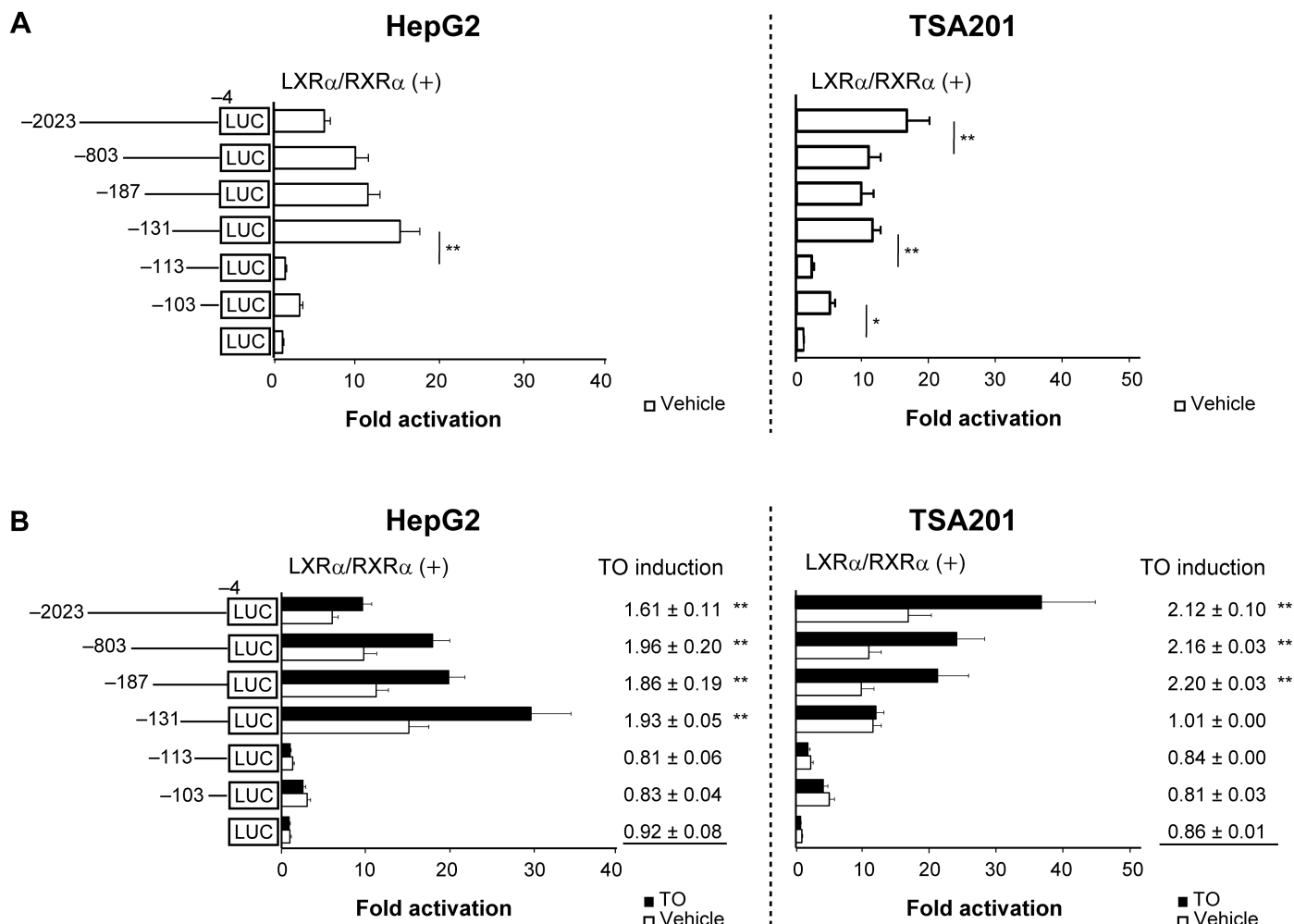


Fig 1. HepG2-specific regulation of the *hDIO1* promoter by T0901317 (TO). A series of 5'-deletion constructs of the *hDIO1* promoter were transiently transfected into HepG2 and TSA201 cells along with expression vectors for human LXR α and human RXR α (LXR α /RXR α) with and without 10^{-7} M TO. Promoter activity was normalized against *Renilla* luciferase activity, and the normalized value is expressed relative to that of promoterless pGL 4.10 in the absence of TO. Results are expressed as means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$. A. Basal luciferase activity of each construct. Statistical analysis was performed on pairwise comparisons of constructs, and significant pairs are presented. B. Luciferase activities of each construct with and without 10^{-7} M TO. TO induction indicates ratio of promoter activity with TO to the activity without TO. Statistical analysis was performed to compare TO induction of each construct with that of promoterless pGL 4.10, and significant differences are presented.

<https://doi.org/10.1371/journal.pone.0179213.g001>

To determine whether LXR α needs *de novo* protein synthesis to stimulate *hDIO1* transcription, we performed the TO stimulation experiment using HepG2 cells pre-treated with cycloheximide. In this case, the mRNA level of *hDIO1* was not increased by TO (Fig 2C).

Investigation of the key nucleotides within the region between nucleotides -131 and -114 of the *hDIO1* promoter

To narrow down the specific site required for the basal activity and LXR α -mediated activation of the *hDIO1* promoter, we performed luciferase assays using HepG2 cells transfected with wild-type or mutant *hDIO1* promoter-reporter constructs. From the previous reports of Toyoda et al. [3] and a computational analysis [20], the region between nucleotides -131 and -114 of the *hDIO1* promoter contains a half-site of TRE, whose binding site consists of two octamer half-site motifs (YYRGGTCA) separated by 10 bp [3], as well as an activator protein 1

(AP-1) site, whose consensus sequence contains TGA(C/G) TCA [21–23], as shown in Fig 3A. Thus, mutations were introduced to disrupt the sequences constituting the TRE [3] and putative AP-1 site (Fig 3A). Basal luciferase activity was significantly reduced when nucleotides –126/–125 or nucleotide –124 was mutated, in comparison to the construct lacking any mutations (Fig 3B); in the former case, the reduction was larger, reaching the baseline level (i.e., the level in cells transfected with promoterless pGL 4.10). On the other hand, basal luciferase activity increased when nucleotides –108/–106 were mutated, but did not change when nucleotides –119/–118 were mutated (Fig 3B). The increase in luciferase activity of the *hDIO1* promoter by TO was abolished when nucleotides –126/–125 or a nucleotide –124 was mutated, but not when nucleotides –108/–106 or –119/–118 were mutated (Fig 3C).

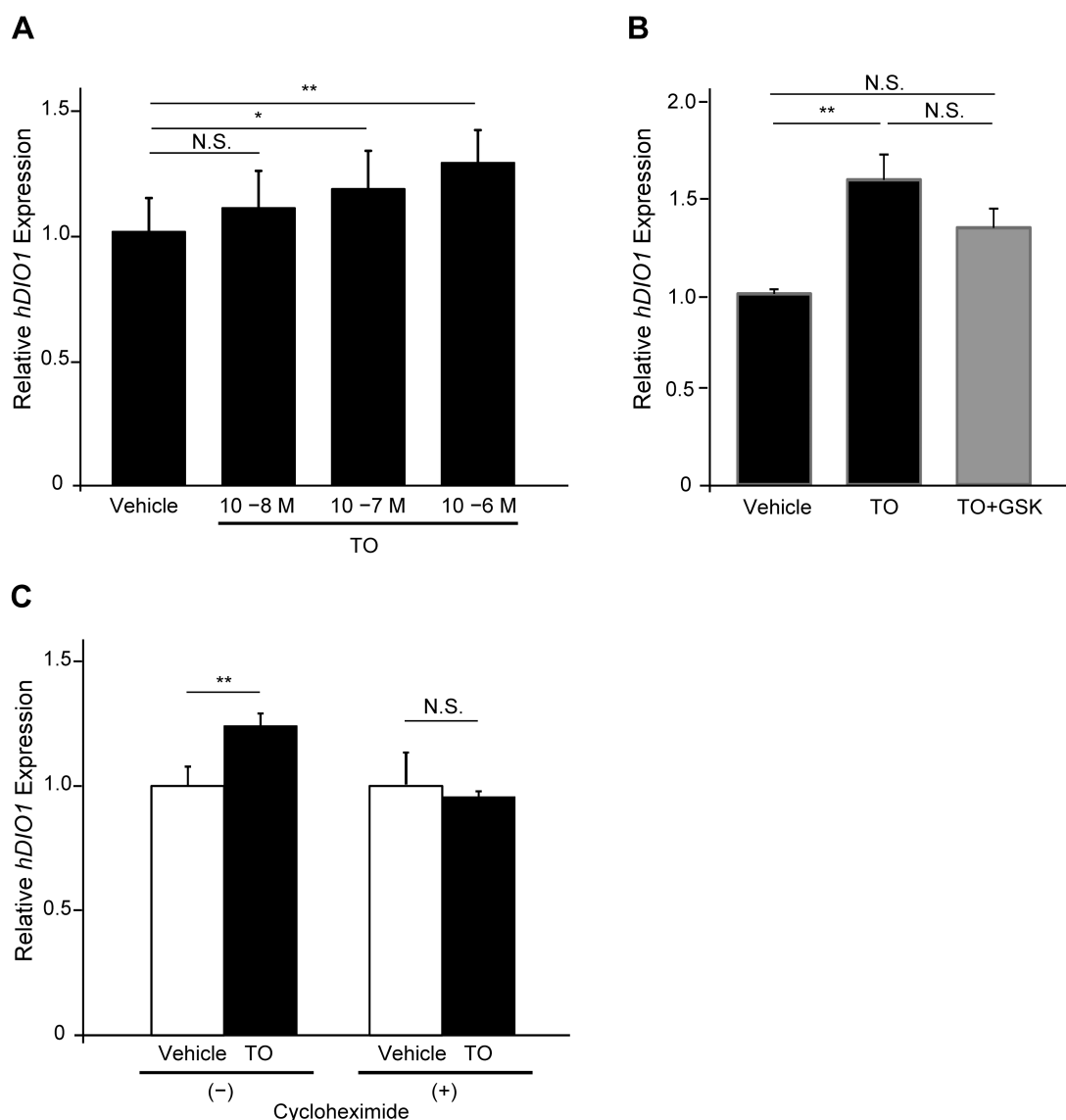


Fig 2. Analysis of relative *hDIO1* mRNA levels in HepG2 cells. *hDIO1* mRNA levels were normalized against the corresponding levels of cyclophilin A mRNA, and the value in HepG2/vehicle cells was defined as 1. Values are expressed as means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; N.S., not significant. A. HepG2 cells were treated at different concentrations of TO for 24 h. B. HepG2 cells were treated with vehicle, 10⁻⁶ M TO, or 10⁻⁶ M TO and 10⁻⁶ M GSK2033 (GSK) for 24 h. C. HepG2 cells were exposed to cycloheximide for 30 min before being treated with TO (10⁻⁷ M) for 24 h.

<https://doi.org/10.1371/journal.pone.0179213.g002>

Identification of nuclear proteins that bind to nucleotides –131 to –114 of the *hDIO1* promoter in HepG2 and TSA201 cells

To confirm the interaction between DNA and proteins within nucleotides –131 to –114 of the *hDIO1* promoter, we performed EMSA using oligonucleotides containing the wild-type sequence of the region between nucleotides –141 and –112 of the *hDIO1* promoter (Wt1) (Fig 4A). Incubation of nuclear proteins from HepG2 cells or TSA201 cells with biotin-labeled Wt1 oligonucleotides led to the formation of several DNA/protein complexes, as shown in lanes 2 and 4, respectively. In both cell lines, specific formation of these complexes was inhibited by incubation with excess unlabeled Wt1 oligonucleotides (lanes 3 and 5).

Furthermore, we performed EMSA using nuclear extracts from HepG2 cells and mutated unlabeled nucleotides (–124 mut1 or –126/–125 mut1) (Fig 4B). The same DNA/protein complexes observed in previous experiments (lane 2) were abolished when the nuclear proteins were incubated with excess unlabeled Wt1 oligonucleotides (lane 3), but not with excess unlabeled –126/–125 mut1 oligonucleotides (lane 5), supporting the idea that the specific DNA/protein complexes formed within the region between nucleotides –131 and –114 of the *hDIO1* promoter. However, both DNA/protein complexes were abolished when nuclear extracts were incubated with excess unlabeled –124 mut1 oligonucleotides (lane 4).

Binding of LXR α and RXR α on the *hDIO1* promoter

The results of EMSA using nuclear extracts from vehicle-treated, TO-treated, and TO-treated and LXR α /RXR α -overexpressing HepG2 cells were shown in Fig 5A. The DNA/protein complexes were observed at the same positions and appeared slightly stronger using nuclear extracts from TO-treated cells (lane 3) and much stronger using nuclear extracts from TO-treated and LXR α /RXR α -overexpressing cells (lane 4), in comparison with those using nuclear extracts from vehicle-treated cells (lane 2).

To identify the protein that binds this region, we performed supershift assays using antibodies against LXR α , TR β , and RXR α with nuclear extracts from TO-treated and LXR α /RXR α -overexpressing HepG2 cells (Fig 5A and 5B). The results of validation of the antibodies used in supershift assays are shown in S4 File. The supershift of the complexes was not observed with antibodies against LXR α (Fig 5A, lane 7) or TR β (Fig 5B, lane 5), but was observed with an antibody against RXR α (Fig 5B, lane 6). These results indicated that the DNA/protein complexes formed within nucleotides –131 to –114 of the *hDIO1* promoter contained RXR α .

Considering that RXR α forms a heterodimer with other nuclear receptors on the 3' side of RXR α binding position, such as LXR α and TR β [24], and that nucleotides –131 to –114 contain a half-site of TRE while nucleotides –113 to –104 contain another half-site, we performed EMSA using an oligonucleotide containing the sequence of the region between nucleotides –131 and –104 (Wt2) [3] with nuclear extracts from TO-treated and LXR α /RXR α -overexpressing HepG2 cells (Fig 5C). As a result, a DNA/protein complex was formed (lane 2), but this complex abolished when the nuclear extracts were incubated with excess unlabeled Wt2 oligonucleotides (lane 3) or excess unlabeled –126/–125 mut2 oligonucleotides (lane 4). In a supershift assay, the formed complex was supershifted with antibodies against both LXR α and RXR α (lanes 6 and 8), but not TR β (lane 7). These results indicated that LXR α , as well as RXR α , can bind to the region between nucleotides –131 and –104 of the *hDIO1* promoter. In addition, unlike RXR α binding, LXR α binding also required the region between nucleotides –113 and –104.

Furthermore, to determine whether LXR α binds to the *hDIO1* promoter under physiological conditions, we performed ChIP assays using an antibody against LXR α . We described materials and methods in S1 File. Using both primer sets, approximately 6-fold to 8-fold

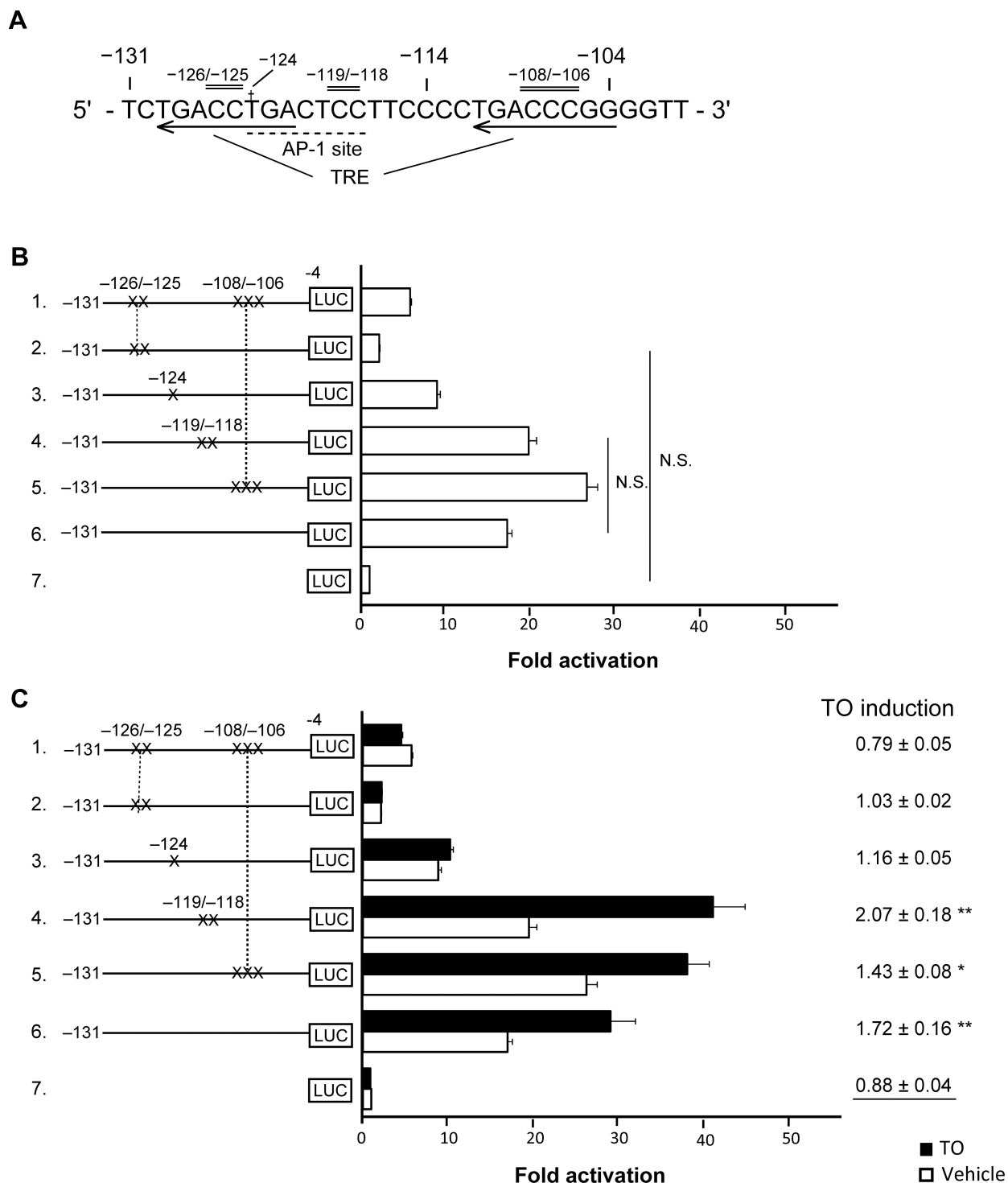


Fig 3. Mutational analysis of activation of the *hDIO1* promoter by T0901317 (TO). A. The nucleotide sequence of the 5'-flanking region of the *hDIO1* promoter used in the analysis, along with the positional relationship among mutated oligonucleotides, thyroid hormone response element (TRE), and the putative activator protein 1 (AP-1) site. † and black horizontal bars represent the site-specific mutation. B and C. A series of mutated *hDIO1* promoter constructs was transiently transfected into HepG2 cells along with expression vectors for LXRα/RXRα, with or without 10^{-7} M TO. Schematic diagram to the left of the figure representing mutant (No. 1–5) and wild-type (No. 6) *hDIO1* promoters, which were introduced upstream of the luciferase gene. No. 7 represents the promoterless pGL 4.10 construct. Promoter activity was normalized against *Renilla* luciferase activity, and is expressed relative to that of promoterless pGL 4.10 in the absence of TO. Values are expressed as means ± SEM. *, $P < 0.05$; **, $P < 0.01$; N.S., not significant. B. Basal

luciferase activity of each construct. Statistical analysis was performed on comparisons between all constructs. Because most pairs exhibited significance, only non-significant pairs are presented. C. Luciferase activities of each construct with and without 10^{-7} M TO. TO induction indicates ratios of promoter activity with TO to the activity without TO. Statistical analysis was performed on comparisons of TO induction of each construct with that of promoterless pGL 4.10, and significant differences are presented.

<https://doi.org/10.1371/journal.pone.0179213.g003>

enrichment of ChIP-DNA following LXRα immunoprecipitation was observed in comparison with the negative control (S5 File), indicating that LXRα could physiologically bind to the *hDIO1* promoter.

Interaction of LXRα/RXRα and TRβ on a specific region of the *hDIO1* promoter

TRs bind to or compete with LXRs on the same regions in the promoters of some genes [9–11], and basal suppression of gene promoters by un-liganded TRs has been reported [25,26]. Therefore, we examined the interaction of LXRα, RXRα, and TRβ on a specific region of the *hDIO1* promoter using the –131/–4 *hDIO1*-Luc construct (Fig 6). Basal luciferase activity, which was significantly decreased by overexpression of TRβ, was not abolished when expression vector for either LXRα or RXRα was transfected alone (Fig 6A and 6B), but was abolished when expression vectors for LXRα/RXRα were co-transfected (Fig 6C). Similarly, overexpression of TRβ decreased TO induction, except when expression vectors for LXRα/RXRα were co-transfected (Fig 6C).

Next, we examined TO induction in TRβ-knockdown conditions (Fig 6D). The siRNA specific for TRβ efficiently knocked down the *THRB* mRNA and the encoded protein (S6 File). Basal luciferase activity was significantly increased in TRβ-knockdown cells co-transfected with expression vectors for LXRα/RXRα. Furthermore, in TRβ-knockdown cells, TO

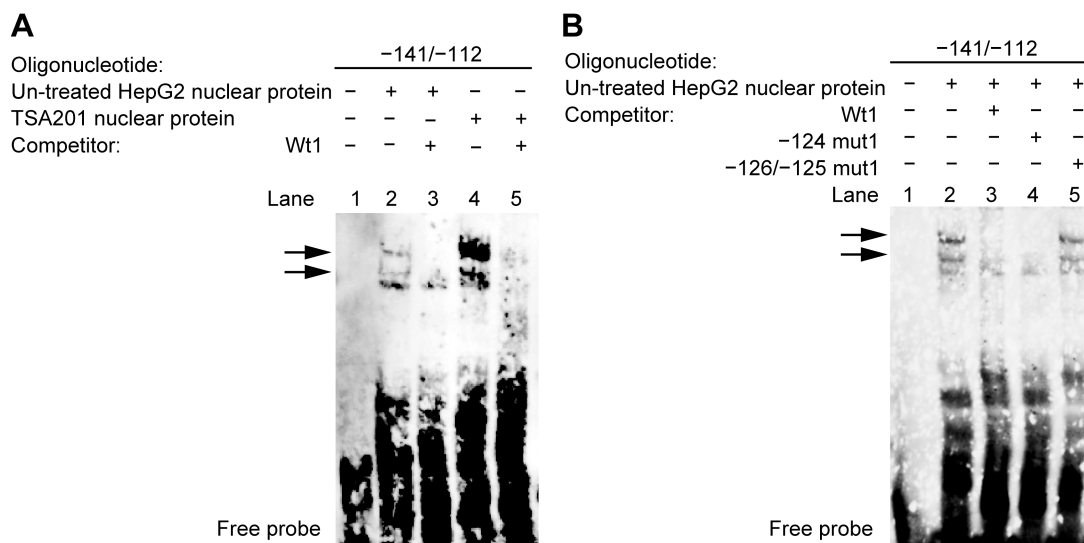


Fig 4. Specific binding of transcription factors to the region between nucleotides –131 and –114 of the *hDIO1* promoter. A. EMSA with oligonucleotide containing the wild-type sequence of the region between nucleotides –141 and –112 of the *hDIO1* promoter (Wt1) and nuclear extracts from un-treated HepG2 cells and TSA201 cells. In lane 1, as a control, only the biotin-labeled Wt1 oligonucleotide was present. Biotin-labeled Wt1 oligonucleotide was incubated with nuclear extracts from HepG2 or TSA201 cells without competitors in lanes 2 and 4, respectively, and with 25-fold molar excesses of unlabeled Wt1 oligonucleotides in lanes 3 and 5, respectively. The specific DNA/protein complexes formed are indicated by arrows. B. EMSA with mutant oligonucleotides and nuclear extracts from HepG2 cells. Biotin-labeled Wt1 oligonucleotide was incubated with nuclear extracts from HepG2 cells without competitors in lane 2, and with 25-fold molar excesses of unlabeled Wt1, –124mut1, and –126/–125mut1 oligonucleotides in lanes 3, 4, and 5, respectively. The specific DNA/protein complexes formed are indicated by arrows.

<https://doi.org/10.1371/journal.pone.0179213.g004>

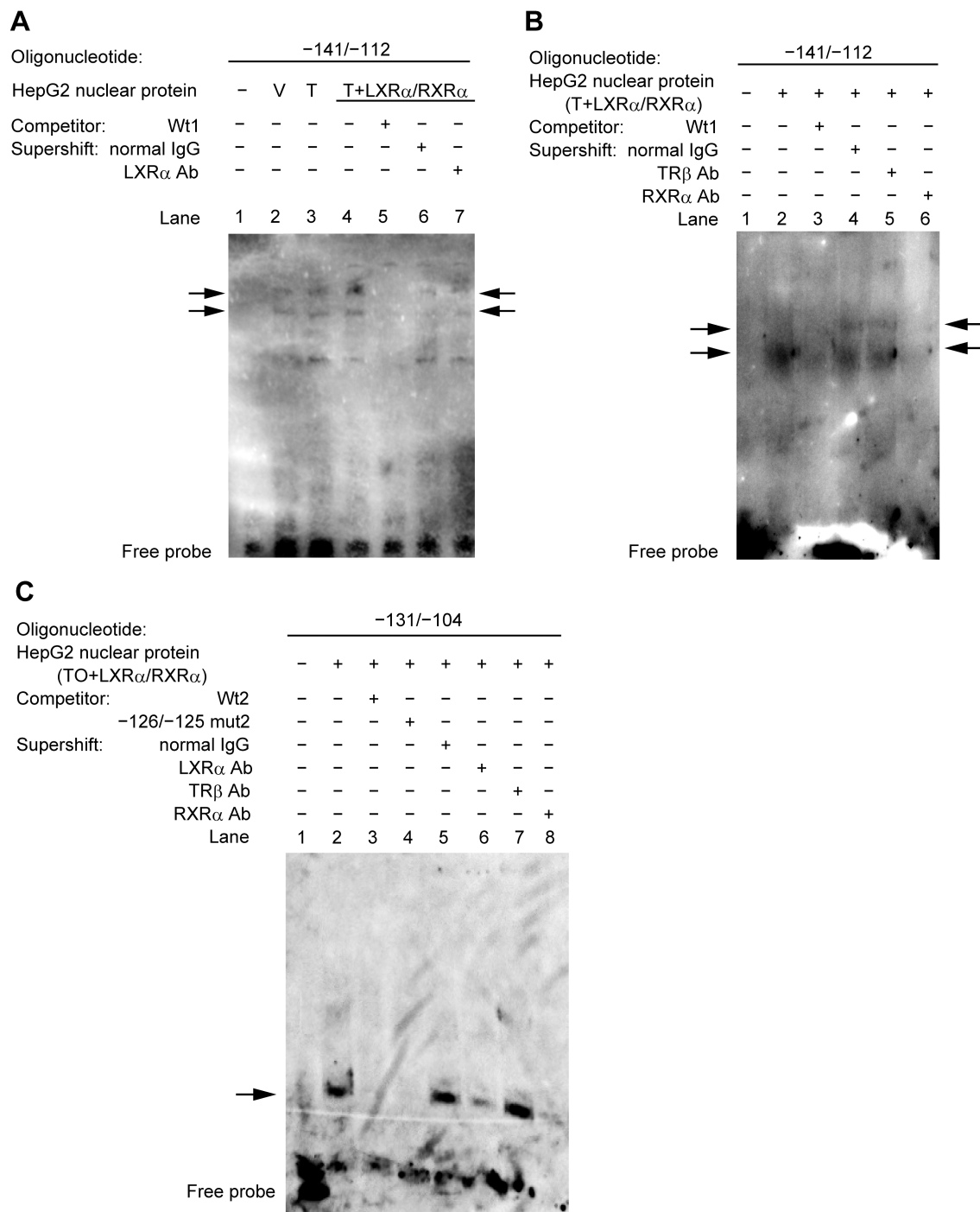


Fig 5. Binding of LXRα on the *hDIO1* promoter. A. EMSA with oligonucleotide containing the wild-type sequence of the region between nucleotides -141 and -112 of the *hDIO1* promoter (Wt1) with nuclear extracts from vehicle-treated (V), T0901317 (TO)-treated (T), or TO-treated and LXRα/RXRα-overexpressing (T+LXRα/RXRα) HepG2 cells; also shown is a supershift assay with an antibody against LXRα. Specific DNA/protein complexes are indicated by arrows. In lane 1, as a control, only biotin-labeled Wt1 oligonucleotide was present. In lanes 2, 3, and 4, biotin-labeled Wt1 oligonucleotide was incubated with nuclear extracts from V, T, and T+LXRα/RXRα HepG2 cells without competitor, respectively. In lane 5, biotin-labeled Wt1 oligonucleotide was incubated with nuclear extracts from T+LXRα/RXRα HepG2 cells with excess unlabeled Wt1 oligonucleotides as competitor. The results of the supershift assay are shown with normal mouse IgG as a control in lane 6 and with an antibody against LXRα in lane 7. B. EMSA with Wt1 oligonucleotides with nuclear extracts from T+LXRα/RXRα HepG2 cells and a supershift assay with antibodies against TRβ and RXRα. Specific DNA/protein complexes are indicated by arrows. In lane 1, as a control, only biotin-labeled Wt1 oligonucleotide was present. Biotin-

labeled Wt1 oligonucleotide was incubated with the nuclear extracts in lane 2 and with the nuclear extracts and excess unlabeled Wt1 oligonucleotides as competitor in lane 3. The results of the supershift assay are shown with normal mouse IgG as a control in lane 4, with an antibody against TR β in lane 5, and with an antibody against RXR α in lane 6. C. EMSA was performed with oligonucleotides containing the wild-type sequence of the region between nucleotides -131 and -104 of the *hDIO1* promoter (Wt2) with nuclear extracts from T+LXR α /RXR α HepG2 cells; also shown is a supershift assay with antibodies against LXR α , TR β , and RXR α . A specific DNA/protein complex is indicated by an arrow. In lane 1, as a control, only biotin-labeled Wt2 was present. Biotin-labeled Wt2 was incubated with nuclear extracts in lane 2, with nuclear extracts and excess unlabeled Wt2 oligonucleotides as competitor in lane 3, and with excess unlabeled -126/-125 mut2 as competitor in lane 4. The results of the supershift assay are shown with normal mouse IgG as a control in lane 5, with an antibody against LXR α in lane 6, with an antibody against TR β in lane 7, and with an antibody against RXR α in lane 8.

<https://doi.org/10.1371/journal.pone.0179213.g005>

induction was increased (from 1.32-fold to 1.79-fold) in the absence of co-transfection of expression vectors for LXR α /RXR α , and further increased (from 1.55-fold to 2.13-fold) in the presence of LXR α /RXR α . These results indicated that LXR α /RXR α compete with TR β in TO induction, as well as basal activity, in this specific region of the *hDIO1* promoter.

Discussion

In this study, we investigated the involvement and mechanism of regulation of *hDIO1* mediated by LXR α using the synthetic LXR agonist TO. We identified a specific region between nucleotides -131 and -114 of the *hDIO1* promoter that was important for basal activity and LXR α -mediated activation of *hDIO1* specifically in HepG2 cells. The results of mutational analysis showed that basal and TO-induced luciferase activities of the *hDIO1* promoter were abolished when nucleotides -126/-125 or a nucleotide -124 was mutated. EMSA using nuclear extracts from HepG2 and TSA201 cells revealed that some DNA/protein complexes were formed on oligonucleotides containing the sequence of the region between nucleotides -131 and -114 of the *hDIO1* promoter. Supershifts of DNA/protein complexes were observed with an antibody against RXR α using oligonucleotides containing the sequence of the region between nucleotides -131 and -114, as well as with antibodies against LXR α and RXR α using the oligonucleotides containing the sequence of the region between nucleotides -131 and -104. In addition, luciferase assays revealed that LXR α /RXR α and TR β compete with each other on this specific region of the *hDIO1* promoter when TR β is either overexpressed or knocked down.

The specific region we identified between nucleotides -131 and -114 of the *hDIO1* promoter contains a TRE, as reported by Toyoda et al. [3]. Within this region, nucleotides -126/-125 were critical for both basal and LXR α -mediated activation and protein binding on the *hDIO1* promoter. This result is consistent with reports that the CC residues within TRE are the most important determinants for TR β binding [27,28]. Luciferase assays revealed that basal and LXR α -mediated activation was also abolished when a nucleotide -124 was mutated. However, based on the results of EMSA using excess mutated unlabeled oligonucleotide, a nucleotide -124 (unlike nucleotides -126/-125) did not seem to be essential for formation of DNA/protein complexes.

We used a synthetic LXR ligand, TO, in this study. Some synthetic ligands for LXRs, such as TO and GW3965, were developed because oxysterols, the natural ligands for LXRs, have relatively low affinities for LXRs [29,30]. We selected TO as a ligand for LXRs for our *in vitro* study because it is a stronger activator of LXRs than GW3965 in HepG2 cells [31]. TO is a potential activator of other nuclear receptors including FXR, PXR, and ROR α [18,19]. To confirm that TO specifically stimulates LXR α to regulate *hDIO1*, we used two strategies. First, we confirmed that the activation of luciferase activity in response to TO was not augmented by overexpression of FXR, PXR, or ROR α , but was augmented by LXR α /RXR α . Second, the response to TO in *hDIO1* mRNA was antagonized by a LXR antagonist GSK.

The results of EMSA and supershift assay suggested that DNA/protein complexes, including LXR α and RXR α were formed with oligonucleotides containing the sequences of the

region between the nucleotides -131 and -104 of the *hDIO1* promoter. The supershift with an antibody against RXR α was observed not only with an oligonucleotide containing the sequences of the region between nucleotides -141 and -112 of the *hDIO1* promoter (containing the sequences of the 5' single half-site of TRE), but also with an oligonucleotide containing

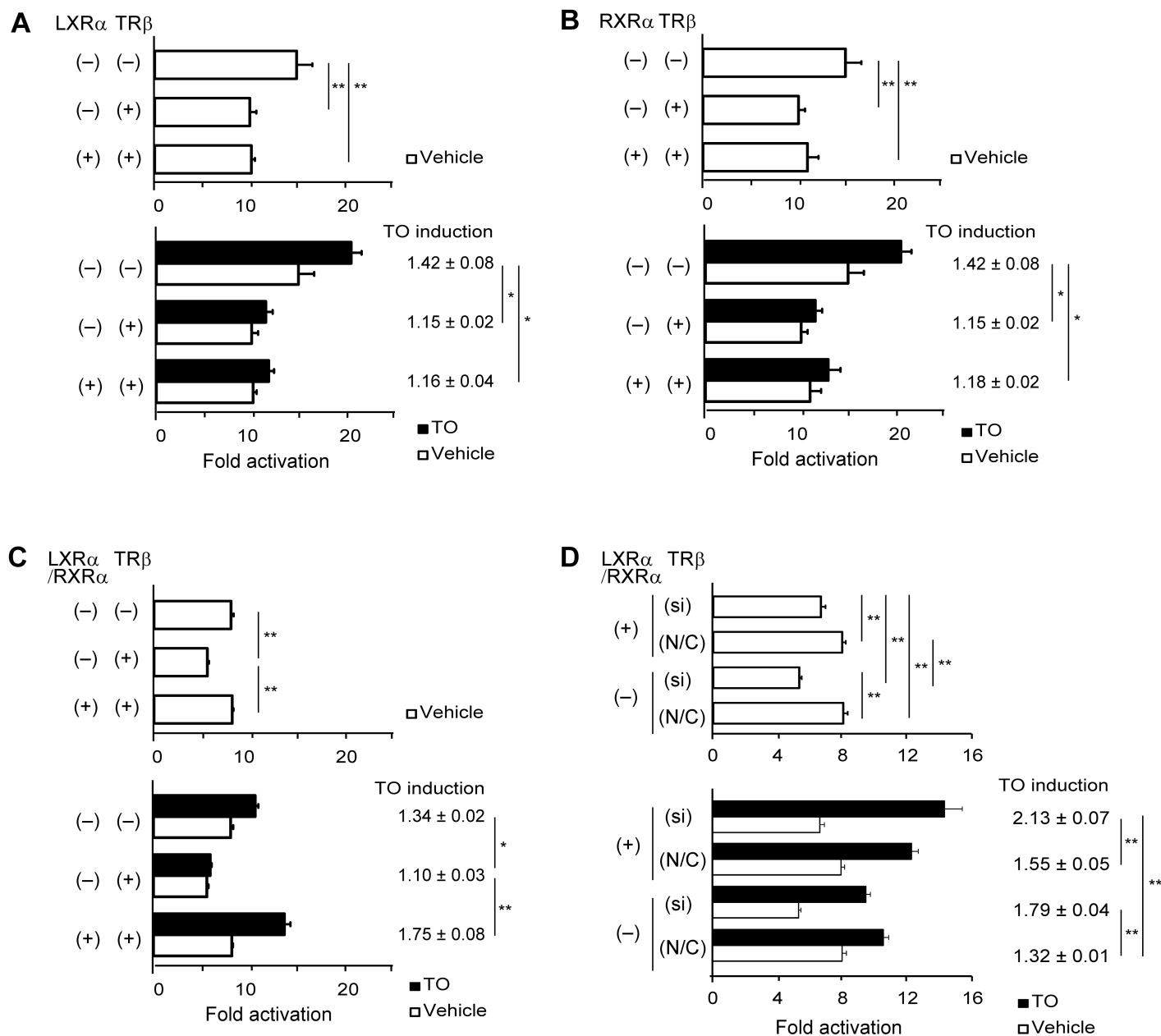


Fig 6. Interaction of LXR α /RXR α and TR β on the activity of the *hDIO1* promoter. HepG2 cells were transfected with or without expression vectors for LXR α , RXR α , or LXR α /RXR α , with or without TR β or siRNA specific for *THRB*. We performed luciferase assay using a -131/-4 *hDIO1*-Luc construct. All upper figures show only basal luciferase activities, whereas lower figures show both basal activities and activities with or without 10^{-7} M TO. TO induction indicates ratios of promoter activity with TO to the activity without TO. Promoter activity was normalized against *Renilla* luciferase activity, and the normalized value is expressed relative to that of promoterless pGL 4.10 in the absence of TO. Results are expressed as means $\pm$ SEM. Statistical analysis was performed on comparisons among all conditions; the results of basal activity are shown in the upper figure, and those of TO induction are shown in the lower figure; *, $P < 0.05$; **, $P < 0.01$. A. Comparison with and without overexpression of LXR α or TR β . B. Comparison with and without overexpression of RXR α or TR β . C. Comparison with and without overexpression of LXR α /RXR α or TR β . D. Comparison with and without overexpression of LXR α /RXR α and TR β knockdown using siRNA. si, siRNA specific for *THRB*; N/C, negative control siRNA.

<https://doi.org/10.1371/journal.pone.0179213.g006>

the sequences of the region between nucleotides –131 and –104 (containing the sequences of complete TRE). On the other hand, the supershift with an antibody against LXR α was observed only with the oligonucleotide containing the sequences of the region between nucleotides –131 and –104. These results suggest that LXR α has effects on the region between nucleotides –111 and –104 of the *hDIO1* promoter, where the 3' half-site of TRE is located.

RXR forms heterodimers with numerous other nuclear receptors, including retinoic acid receptor, vitamin D receptor, TRs, and LXRs [8,32,33]. The consensus sequence of RXR, 5'–AGGTCA–3' [34], is located between nucleotides –129 and –124 of the *hDIO1* promoter, supporting our EMSA results showing that supershift with an antibody against RXR α was observed with oligonucleotides containing the sequences of the regions between nucleotides –131 and –114 and nucleotides –131 and –104. In addition, interactions mediated by dimerization of RXR α with other receptors occur only when RXR binds to the 5' half-site of their binding positions [35]. The importance of the polarity of RXR α was consistent with the results of the supershift assay with an antibody against LXR α . We observed that the DNA/protein complexes were more prominent when nuclear extracts from TO-treated (and especially TO-treated and LXR α /RXR α -overexpressing) HepG2 cells were used. This suggests that the DNA/protein complexes might contain some other proteins induced by TO or overexpression of LXR α /RXR α . Cycloheximide treatment abolished the increase in mRNA levels of *hDIO1* by TO, suggesting that some newly synthesized proteins are involved in stimulation of *hDIO1* transcription by LXR α .

Interactions between TR and LXR have been reported on promoters of some genes [9–11,36]. We confirmed that TR and LXR interact on the *hDIO1* promoter; TO induction was significantly decreased by overexpression of TR β , but this effect was abolished by co-transfection of expression vectors for LXR α /RXR α . Interestingly, the change in TO induction by TR β was not affected by transfection of expression vector for either LXR α or RXR α alone. This highlights the relevance of the EMSA/supershift results showing that complexes including both LXR α and RXR α bound to the specific region between nucleotides –131 and –104 of the *hDIO1* promoter. By contrast, in TR β -knockdown cells, TO induction was significantly increased with or without overexpression of LXR α /RXR α , indicating that deterioration of TR β function augments the effects of LXR α /RXR α on the *hDIO1* promoter.

Our observation that stimulation of LXR α by TO increased the expression of *hDIO1* in HepG2 cells was inconsistent with previous *in vivo* observations that TO decreases hepatic expression of *Dio1* in rat and mouse models [37,38]. Importantly, there is a large difference in the sequences of this gene's promoter regions between human and rodent; TREs have not been identified in the promoter regions of rat or mouse *Dio1*, whereas two TREs are present in the promoter region of human *DIO1* [3]. Therefore, we performed *in vitro* examinations using human cell lines, and obtained meaningful data on human *DIO1* gene.

In conclusion, we demonstrated that a specific region of the *hDIO1* promoter, which contains a TRE, was important for the basal activity and LXR α -mediated activation of this gene. Our results provide the additional insights that LXR α plays a specific and important role in activation of TH by regulating D1. Furthermore, LXR α binds to and regulates the *hDIO1* promoter, and competes with TR β on a specific region of the *hDIO1* promoter.

Supporting information

S1 File. Supplemental materials and methods.
(DOCX)

S2 File. A series of 5'-deletion analysis of the *hDIO1* promoter without LXR α /RXR α overexpression. A. A series of 5'-deletion constructs of the *hDIO1* promoter were transiently

transfected into HepG2 and TSA201 cells in the absence of expression vectors for human LXR α and human RXR α (LXR α /RXR α), with or without 10^{-7} M TO. Promoter activity was normalized against *Renilla* luciferase activity, and the normalized value is expressed relative to that of promoterless pGL 4.10 in the absence of TO. Results are expressed as means $\pm$ SEM. **, $P < 0.01$. A. Basal luciferase activity of each construct. Statistical analysis was performed for pairwise combinations of constructs, and significant pairs are presented. B. Luciferase activities of each construct with and without 10^{-7} M TO. TO induction indicates ratios of promoter activity with TO vs. without TO. Statistical analysis was performed between TO induction of each construct and that of promoterless pGL 4.10, and significant differences are presented. (TIF)

S3 File. Specificity of T0901317 (TO) in the regulation of *hDIO1* by LXR α . HepG2 cells were treated with and without 10^{-7} M TO and transfected using expression vectors as follows. pCMX-LXR α , co-transfection with pCMX-LXR α and pCMX-RXR α ; pCMX-FXR, co-transfection with pCMX-FXR and pCMX-RXR α ; pCMX-empty, transfection of pCMX-empty vector alone; pFN21A-ROR α , co-transfection of pFN21A-ROR α and pCMX-empty vector; pFN21A-PXR, co-transfection of pFN21A-PXR and pCMX-RXR α ; and pFN21A-empty, co-transfection of pCMX-empty vector and pFN21A-empty vector. Promoter activity was normalized against *Renilla* luciferase activity, and the normalized value is expressed relative to that of promoterless pGL 4.10 with pCMX-empty or pFN21A-empty in the absence of TO. TO induction indicates ratios of promoter activity with TO vs. without TO. Statistical analysis was performed to compare each group with the respective empty vector group. Results are expressed as means $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; N.S., not significant. (TIF)

S4 File. Positive control for antibodies against TR β , RXR α , and LXR α . A. EMSA with oligonucleotide containing the wild-type sequence of the region between nucleotides -131 and -104 of the *hDIO1* promoter (Wt2) with nuclear extracts from HepG2 cells overexpressing TR β and RXR α , and supershift assays with antibodies against TR β and RXR α . Specific DNA/protein complexes are indicated by black arrows and supershifted bands are by a gray arrow. In lane 1, as a control, only biotin-labeled Wt2 oligonucleotide was present. Biotin-labeled Wt2 oligonucleotide was incubated with nuclear extracts in lane 2 and with nuclear extracts and excess unlabeled Wt2 oligonucleotides as competitor in lane 3. The results of supershift assays are shown with normal mouse IgG as a control in lane 4, with an antibody against TR β in lane 5, and with an antibody against RXR α in lane 6. B. EMSA with oligonucleotide containing consensus sequence of LXR response element (LXRE) with nuclear extracts from HepG2 cells overexpressing LXR α and RXR α , and supershift assays with an antibody against LXR α . Specific DNA/protein complexes are indicated by black arrows and a supershifted band is by a gray arrow. In lane 1, as a control, only biotin-labeled LXRE oligonucleotide was present. Biotin-labeled LXRE oligonucleotide was incubated with nuclear extracts in lane 2 and with nuclear extracts and excess unlabeled LXRE oligonucleotides as competitor in lane 3. The results of supershift assays are shown with normal mouse IgG as a control in lane 4 and with an antibody against LXR α in lane 5. (TIF)

S5 File. Binding of LXR α on the *hDIO1* promoter. We purified chromatin fragments following immunoprecipitation from HepG2 cells using an antibody against LXR α . Normal mouse IgG was used as a negative control for an antibody against LXR α . The presence of specific DNA fragments binding to LXR α was determined by quantitative PCR using primer set 1 and 2, which covers nucleotides -128/+41 and -188/-25 of the genomic *hDIO1* promoter,

respectively. Enrichment of LXR α -bound DNA and that of normal mouse IgG-bound DNA were normalized against 10% of the corresponding input DNA, and the results of enrichment of LXR α -bound DNA were expressed as the fold change relative to normal mouse IgG-bound DNA. Data were compared by unpaired Student's t test. Values are expressed as means $\pm$ SEM. **, $P < 0.01$.

(TIF)

S6 File. Efficiency of siRNA-mediated knockdown of TR β . A. The results of quantitative PCR to detect *THRB* mRNA. We compared HepG2 cells transfected with negative control siRNA (N/C) or siRNA against *THRB* (si-TR β). *THRB* mRNA levels were normalized against the corresponding levels of cyclophilin A mRNA, and the value in N/C was defined as 1. Values are expressed as means $\pm$ SEM. **, $P < 0.01$. B. Protein levels of TR β after siRNA transfection were evaluated by western blot analysis. β -actin was used as a standard.

(TIF)

Acknowledgments

This work was supported by JSPS KAKENHI (Grant Number 16H06902), MEXT KAKENHI (Grant Numbers 21229013, 20790659, 18790635, and 19591075), and the Takeda Science Foundation. We gratefully acknowledge the work of past and present members of our laboratory and our discussions with them, which have helped to shape many of the ideas presented here.

Author Contributions

Conceptualization: NK.

Data curation: YS.

Formal analysis: YS.

Funding acquisition: NK AY KN NI.

Investigation: YS IY YM.

Methodology: NK AY.

Project administration: NK.

Resources: NK TT MM AY.

Software: NK.

Supervision: NK TT MM MS AY TK KN NI.

Validation: YS IY.

Visualization: YS.

Writing – original draft: YS.

Writing – review & editing: NK AY NI.

References

1. Bianco AC, Salvatore D, Gereben B, Berry MJ, Larsen PR (2002) Biochemistry, cellular and molecular biology, and physiological roles of the iodothyronine selenodeiodinases. *Endocr Rev* 23: 38–89. <https://doi.org/10.1210/edrv.23.1.0455> PMID: 11844744

2. Koenig RJ (2005) Regulation of type 1 iodothyronine deiodinase in health and disease. *Thyroid* 15: 835–840. <https://doi.org/10.1089/thy.2005.15.835> PMID: 16131326
3. Toyoda N, Zavacki AM, Maia AL, Harney JW, Larsen PR (1995) A novel retinoid X receptor-independent thyroid hormone response element is present in the human type 1 deiodinase gene. *Mol Cell Biol* 15: 5100–5112. PMID: 7651427
4. Schreck R, Schnieders F, Schmutzler C, Kohrle J (1994) Retinoids stimulate type I iodothyronine 5'-deiodinase activity in human follicular thyroid carcinoma cell lines. *J Clin Endocrinol Metab* 79: 791–798. <https://doi.org/10.1210/jcem.79.3.8077363> PMID: 8077363
5. Kanamoto N, Tagami T, Ueda-Sakane Y, Sone M, Miura M, Yasoda A, et al. (2012) Forkhead box A1 (FOXA1) and A2 (FOXA2) oppositely regulate human type 1 iodothyronine deiodinase gene in liver. *Endocrinology* 153: 492–500. <https://doi.org/10.1210/en.2011-1310> PMID: 22067325
6. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 383: 728–731. <https://doi.org/10.1038/383728a0> PMID: 8878485
7. Repa JJ, Mangelsdorf DJ (2000) The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu Rev Cell Dev Biol* 16: 459–481. <https://doi.org/10.1146/annurev.cellbio.16.1.459> PMID: 11031244
8. Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science* 294: 1866–1870. <https://doi.org/10.1126/science.294.5548.1866> PMID: 11729302
9. Zhang Y, Yin L, Hillgartner FB (2001) Thyroid hormone stimulates acetyl-coA carboxylase-α transcription in hepatocytes by modulating the composition of nuclear receptor complexes bound to a thyroid hormone response element. *J Biol Chem* 276: 974–983. <https://doi.org/10.1074/jbc.M005894200> PMID: 11027684
10. Hashimoto K, Cohen RN, Yamada M, Markan KR, Monden T, Satoh T, et al. (2006) Cross-talk between thyroid hormone receptor and liver X receptor regulatory pathways is revealed in a thyroid hormone resistance mouse model. *J Biol Chem* 281: 295–302. <https://doi.org/10.1074/jbc.M507877200> PMID: 16260782
11. Huuskonen J, Vishnu M, Pullinger CR, Fielding PE, Fielding CJ (2004) Regulation of ATP-binding cassette transporter A1 transcription by thyroid hormone receptor. *Biochemistry* 43: 1626–1632. <https://doi.org/10.1021/bi0301643> PMID: 14769039
12. Enmark E, Gustafsson JA (2001) Comparing nuclear receptors in worms, flies and humans. *Trends Pharmacol Sci* 22: 611–615. PMID: 11730970
13. Apfel R, Benbrook D, Lernhardt E, Ortiz MA, Salbert G, Pfahl M (1994) A novel orphan receptor specific for a subset of thyroid hormone-responsive elements and its interaction with the retinoid/thyroid hormone receptor subfamily. *Mol Cell Biol* 14: 7025–7035. PMID: 7935418
14. Berkenstam A, Farnegardh M, Gustafsson JA (2004) Convergence of lipid homeostasis through liver X and thyroid hormone receptors. *Mech Ageing Dev* 125: 707–717. <https://doi.org/10.1016/j.mad.2004.05.005> PMID: 15541766
15. Moriyama K, Tagami T, Akamizu T, Usui T, Saijo M, Kanamoto N, et al. (2002) Thyroid hormone action is disrupted by bisphenol A as an antagonist. *J Clin Endocrinol Metab* 87: 5185–5190. <https://doi.org/10.1210/jc.2002-020209> PMID: 12414890
16. Umesono K, Murakami KK, Thompson CC, Evans RM (1991) Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* 65: 1255–1266. PMID: 1648450
17. Zhao LF, Iwasaki Y, Nishiyama M, Taguchi T, Tsugita M, Okazaki M, et al. (2012) Liver X receptor alpha is involved in the transcriptional regulation of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene. *Diabetes* 61: 1062–1071. <https://doi.org/10.2337/db11-1255> PMID: 22415873
18. Mitro N, Vargas L, Romeo R, Koder A, Saez E (2007) T0901317 is a potent PXR ligand: implications for the biology ascribed to LXR. *FEBS Lett* 581: 1721–1726. <https://doi.org/10.1016/j.febslet.2007.03.047> PMID: 17418145
19. Houck KA, Borchert KM, Hepler CD, Thomas JS, Bramlett KS, Michael LF, et al. (2004) T0901317 is a dual LXR/FXR agonist. *Mol Genet Metab* 83: 184–187. <https://doi.org/10.1016/j.ymgme.2004.07.007> PMID: 15464433
20. Heinemeyer T, Wingender E, Reuter I, Hermjakob H, Kel AE, Kel OV, et al. (1998) Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res* 26: 362–367. PMID: 9399875
21. Ahmed AH, Gordon RD, Sukor N, Pimenta E, Stowasser M (2011) Quality of life in patients with bilateral primary aldosteronism before and during treatment with spironolactone and/or amiloride, including a

- comparison with our previously published results in those with unilateral disease treated surgically. *J Clin Endocrinol Metab* 96: 2904–2911. <https://doi.org/10.1210/jc.2011-0138> PMID: 21778218
22. Lee W, Mitchell P, Tjian R (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* 49: 741–752. PMID: 3034433
23. Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, et al. (1987) Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* 49: 729–739. PMID: 3034432
24. Orlov I, Rochel N, Moras D, Klaholz BP (2012) Structure of the full human RXR/VDR nuclear receptor heterodimer complex with its DR3 target DNA. *EMBO J* 31: 291–300. <https://doi.org/10.1038/emboj.2011.445> PMID: 22179700
25. Damm K, Thompson CC, Evans RM (1989) Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. *Nature* 339: 593–597. <https://doi.org/10.1038/339593a0> PMID: 2733791
26. Graupner G, Wills KN, Tzukerman M, Zhang XK, Pfahl M (1989) Dual regulatory role for thyroid-hormone receptors allows control of retinoic-acid receptor activity. *Nature* 340: 653–656. <https://doi.org/10.1038/340653a0> PMID: 2549424
27. Ayers S, Switnicki MP, Angajala A, Lammel J, Arumanayagam AS, Webb P (2014) Genome-wide binding patterns of thyroid hormone receptor beta. *PLoS One* 9: e81186. <https://doi.org/10.1371/journal.pone.0081186> PMID: 24558356
28. Katz RW, Koenig RJ (1994) Specificity and mechanism of thyroid hormone induction from an octamer response element. *J Biol Chem* 269: 18915–18920. PMID: 8034648
29. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, et al. (2000) Role of LXRs in control of lipogenesis. *Genes Dev* 14: 2831–2838. PMID: 11090131
30. Collins JL, Fivush AM, Watson MA, Galardi CM, Lewis MC, Moore LB, et al. (2002) Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. *J Med Chem* 45: 1963–1966. PMID: 11985463
31. Albers M, Blume B, Schlueter T, Wright MB, Kober I, Kremoser C, et al. (2006) A novel principle for partial agonism of liver X receptor ligands. Competitive recruitment of activators and repressors. *J Biol Chem* 281: 4920–4930. <https://doi.org/10.1074/jbc.M510101200> PMID: 16354658
32. Bugge TH, Pohl J, Lonnoy O, Stunnenberg HG (1992) RXR alpha, a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J* 11: 1409–1418. PMID: 1314167
33. Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Naar AM, et al. (1991) RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 67: 1251–1266. PMID: 1662118
34. Lee MS, Kliewer SA, Provencal J, Wright PE, Evans RM (1993) Structure of the retinoid X receptor alpha DNA binding domain: a helix required for homodimeric DNA binding. *Science* 260: 1117–1121. PMID: 8388124
35. Mader S, Chen JY, Chen Z, White J, Chambon P, Gronemeyer H (1993) The patterns of binding of RAR, RXR and TR homo- and heterodimers to direct repeats are dictated by the binding specificities of the DNA binding domains. *EMBO J* 12: 5029–5041. PMID: 8262045
36. Ishida E, Hashimoto K, Okada S, Satoh T, Yamada M, Mori M (2013) Crosstalk between thyroid hormone receptor and liver X receptor in the regulation of selective Alzheimer's disease indicator-1 gene expression. *PLoS One* 8: e54901. <https://doi.org/10.1371/journal.pone.0054901> PMID: 23359226
37. Davies JS, Kotokorpi P, Lindahl U, Oscarsson J, Wells T, Mode A (2008) Effects of the synthetic liver X receptor agonist T0901317 on the growth hormone and thyroid hormone axes in male rats. *Endocrine* 33: 196–204. <https://doi.org/10.1007/s12020-008-9067-9> PMID: 18473193
38. Stulnig TM, Steffensen KR, Gao H, Reimers M, Dahlgren-Wright K, Schuster GU, et al. (2002) Novel roles of liver X receptors exposed by gene expression profiling in liver and adipose tissue. *Mol Pharmacol* 62: 1299–1305. PMID: 12435796