SIRT1 is a Direct Coactivator of Thyroid Hormone Receptor β1 with Gene-Specific Actions

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Abstract
Sirtuin 1 (SIRT1) NAD+-dependent deacetylase regulates energy metabolism by modulating expression of genes involved in gluconeogenesis and other liver fasting responses. While many effects of SIRT1 on gene expression are mediated by deacetylation and activation of peroxisome proliferator activated receptor coactivator α (PGC-1α), SIRT1 also binds directly to DNA bound transcription factors, including nuclear receptors (NRs), to modulate their activity. Since thyroid hormone receptor β1 (TRβ1) regulates several SIRT1 target genes in liver and interacts with PGC-1α, we hypothesized that SIRT1 may influence TRβ1. Here, we confirm that SIRT1 cooperates with PGC-1α to enhance response to triiodothyronine, T3. We also find, however, that SIRT1 stimulates TRβ1 activity in a manner that is independent of PGC-1α but requires SIRT1 deacetylase activity. SIRT1 interacts with TRβ1 in vitro, promotes TRβ1 deacetylation in the presence of T3 and enhances ubiquitin-dependent TRβ1 turnover; a common response of NRs to activating ligands. More surprisingly, SIRT1 knockdown only strongly inhibits T3 response of a subset of TRβ1 target genes, including glucose 6 phosphatase (G-6-Pc), and this is associated with blockage of TRβ1 binding to the G-6-Pc promoter. Drugs that target the SIRT1 pathway, resveratrol and nicotinamide, modulate T3 response at dual TRβ1/SIRT1 target genes. We propose that SIRT1 is a gene-specific TRβ1 coregulator and TRβ1/SIRT1 interactions could play important roles in regulation of liver metabolic response. Our results open possibilities for modulation of subsets of TR target genes with drugs that influence the SIRT1 pathway.

Introduction
Thyroid hormone receptors (TRs) are members of the nuclear hormone receptor (NR) family [1]. Both TRs regulate gene transcription by binding to specific DNA sequences (thyroid hormone response elements, TREs) and nucleating formation of protein assemblies which, in turn, influence organization and post-translational modification of nearby chromatin and RNA polymerase II recruitment and processivity [2–4]. TRs, like other NRs, harbor a hormone-dependent docking surface that binds several general coregulators, including the steroid receptor coactivator (SRC) family, TR associated protein 220 (TRAP220) and others, which are required for target gene induction by active thyroid hormone, predominantly triiodothyronine (T3). Coregulators are recruited sequentially in a dynamic and ordered process and activated TRs are eventually ubiquitinated and channeled into proteasomal degradation pathways [5,6].

TR transcription complexes must also integrate responses to T3 signals with those of other signaling pathways. TRs cooperate with particular subsets of heterologous DNA bound transcription factors (TFs) in composite modules, including CTCF [7] and SREBP1 [8], and particular TR/TF combinations could be responsible for integration of different signaling pathways. TR coregulators could also play a role. For example, peroxisome proliferator activated receptor γ coactivator 1α (PGC-1α) is induced in different tissues in response to a variety of different environmental signals and strongly potentiates TR activity [9–15]. In liver, PGC-1α is induced under fasting conditions and is required for optimal T3 activation of genes involved in fatty acid β-oxidation, mitochondrial activity and other metabolic pathways [13–15].

Sirtuin 1 (SIRT1) is an NAD+-dependent deacetylase that is activated by resveratrol and regulates the expression of genes involved in fasting response and resistance to metabolic diseases [16,17]. SIRT1 regulates activity of several TFs that bind to PGC-1α via targeted deacetylation of multiple PGC-1α lysine residues and enhancement of PGC-1α activity [18]. For example, SIRT1 enhances the activity of the NR PPARα in liver through PGC-1α [19]. In addition, SIRT1 binds directly to DNA bound TFs, including NRs, to influence TF activity [20–22]. For example, SIRT1 enhances the response of liver X receptor (LXR) α to agonists and this is accompanied by LXR deacetylation and SIRT1-dependent channeling of active LXRα into ubiquitin-dependent degradation pathways [20].

There are superficial overlaps between the actions of TRs and SIRT1 in liver [16,23]; both factors exert similar effects upon genes involved in gluconeogenesis, fatty acid oxidation and mitochondrial function. Since TR binds PGC-1α [9], we tested the possibility that SIRT1 may enhance activity of TRβ1, the predominant TR subtype in liver, via effects upon PGC-1α...
activity. We confirm that SIRT1 synergizes with PGC-1α to potentiate T₃ response, in accordance with recent findings of another group [24], but also find that SIRT1 enhances TRβ₁ activity independently of PGC-1α. More surprisingly, requirements for SIRT1 in T₃ response are highly gene-specific and, in one case, associated with hormone and SIRT1-dependent transcription complex assembly on DNA. We propose that TRβ₁/SIRT1 complex formation may serve as a checkpoint for regulation of key genes with important roles in metabolic response and our results open possibilities for modulation of subsets of T₃ dependent genes with drugs that target the SIRT1 pathway.

Materials and Methods

Plasmids and Reagents

Mammalian expression vectors for human TRβ₁ and PGC-1α and the bacterial expression vector for TRβ₁ (GST-TRβ₁) were described previously [9]. SIRT1 were cloned by PCR from a cDNA library of HepG2 cells in frame at BamHI and XbaI sites of pcDNA3Flag vector. The mammalian expression vectors for mouse SIRT1 wild type and SIRT1 mutant (H355Y) were provided as gifts from Dr. Tadahiro Kitamura (Gunma University). The DR-4-luc reporter plasmid was described previously [9].

Adenovirus Infection

Adenoviruses that express mouse wild type or mutant (H355Y) SIRT1 were kindly provided as gifts from Dr. Tadahiro Kitamura (Gunma University). Flag tagged TRβ₁ overexpressing HepG2 cells were infected with adenovirus expressing SIRT1 (AdSIRT1) or null adenovirus at multiplicity of infection = 50 and treated with T₃.

siRNA

SiRNAs for SIRT1 (ID no. HSS177403, HSS177404 and HSS177409) and PGC-1α (ID no. HSS16797, HSS16798, HSS16799) were purchased from Invitrogen Life Technologies. HepG2-TRβ₁ cells were transfected with siRNA (100 nM) using Lipofectamine RNAiMAX reagent (Invitrogen Life Technologies) according to manufacturer’s instructions.

qPCR

Real-time PCR was performed as described previously [26], using the Roche LightCycler 480 RT PCR machine and SYBR Green Mastermix (Roche) according to the manufacturer’s procedure. Sequences of primers used for Real-time PCR are available upon request. Relative mRNA levels were calculated by comparative the cycle threshold method using GAPDH as the internal control. GAPDH level was not affected by T₃.

Microarray Hybridization and Analysis

HumanHT-12 v4 whole genome expression arrays were purchased from Illumina. cRNA synthesis and labeling were

SIRT1/TRβ₁ Interactions

GST Pull-down

GST and GST-TRβ₁ fusions were expressed in Escherichia coli BL21 cells and isolated with glutathione-Sepharose-4B beads (GE Healthcare Life Sciences). Immobilized GST fusions were then incubated with SIRT1 protein produced by in vitro translation using the TNT-coupled transcription-translation system (Promega). Binding reactions were carried out in 250 μL of GST binding buffer (20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 % glycerol, 0.05 % NP-40, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol, and 1.5 % bovine serum albumin) for 4 h at 4°C. The beads were washed three times with 1 ml of GST binding buffer. Bound proteins were eluted by the addition of 20 μl of SDS loading buffer, and were analyzed by Western blot analysis using anti-SIRT1 antibody.
Figure 1. SIRT1 is a TRβ1 co-regulator. (A) Graph representing luciferase activity measured in extracts of HepG2 TRβ1 cells transfected with DR-4-luc and expression vectors for PGC-1α, SIRT1 or both and treated +/- T3. (B) Results of luciferase assay in HepG2 TRβ cells when siRNA against PGC-1α is used. Inset represents a western blot of cell extracts using PGC-1α antibody to confirm PGC-1α knockdown. (C) As in Fig. 1B, but using expression vectors for wild type SIRT1 and deacetylase defective mutant of SIRT1 (H355Y). (D) Luciferase activity measured in 293T cells transfected with expression vectors for TRβ1 or TRα +/- SIRT1 expression vector and treated +/- T3. The levels of luciferase activity were normalized to lacZ expression. All data are representative of at least three independent experiments with similar results. All values represent the mean ± SD of duplicate samples. **, P < 0.01; ***, P < 0.001.
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performed using Illumina® TotalPrep™-96 RNA Amplification Kit (Ambion). Labeling in vitro transcription reaction was performed at 37°C for 14 h. Biotinylated cRNA samples were hybridized to arrays at 58°C for 18 h according to manufacturer’s protocol. Arrays were scanned using BeadArray Reader. Unmodiﬁed microarray data obtained from GenomeStudio was background-subtracted and quantile-normalized using the limma package [35] and analyzed with the limma package [36] within R [37].

All analysis was corrected for multiple hypotheses testing [38], and effects determined to be signiﬁcant when 2-fold with an adjusted p-value 0.05. To facilitate comparisons among the various datasets, all data was uploaded into a SQLite3 database [39]. Heatmaps were produced and clustered using multiarray viewer [40].

To determine T3-responsive gene transcripts affected by SIRT1 KD, we compared the T3-response in the NC-siRNA against SIRT1 siRNA through pattern analysis as done previously (26). In brief, including the three affects (T3-response in NC-siRNA, T3-response in SIRT1 siRNA, and SIRT siRNA in the absence of T3) a 3-by-3 permutation of down-regulated (0.5) and up-regulated (2) and no change (1) results in 27 possible patterns (supplemental). The Euclidean distance between experimental data and the 27 patterns were calculated, with the matching pattern receiving the minimal Euclidean distance.

Chromatin Immunoprecipitation (ChIP) Assay

Flag tagged TRβ1 overexpressed HepG2 cells were transfected with SIRT1 siRNA or control siRNA and treated +/− 10 nM T3 for 24 h following transfection. ChIP assays were performed by SimpleChip Enzymatic Chromatin IP Kit (Cell Signaling) according to manufacturer’s instructions. 10% (v/v) of the supernatant was saved as ‘input’ chromatin prior to immunoprecipitation. Anti-Flag antibody conjugated agarose bead slurry (Sigma Aldrich A2220) and anti-SIRT1 (Cell Signaling #2496) were used for immunoprecipitation. Immuneoprecipitated DNA and input-sheared DNA were subjected to PCR using a primer pair for G-6-Pc (forward, 5'-GAGGGCTTCTCAGAAACACAGG-3', and reverse, 5'-GCAGTGACCTCTGAGATGAG-3') or for PCK1 (forward, 5'-AGTTTCTCCTCTCCTCTCAGAAC-3', and reverse, 5'-AGGACCTGACGAGATCGAGAAC-3'), which amplify regions spanning the thyroid response element (TRE). IgG was used as an immunoprecipitation control.

Microarray

Flag tagged TRβ1 overexpressed HepG2 cells were transfected with control siRNA (100 nM) or SIRT1 siRNA (100 nM) and treated +/− T3. Hybridizations and microarrays were performed as described previously.

Statistical Analysis

All results are the means ± SD. Statistical analysis was performed using GraphPad Prism software (GraphPad Inc., San Diego, CA). Comparisons of groups were performed using a Student’s t test.

P < 0.05 was considered statistically significant. All experiments were performed at least three times.

Results

SIRT1 Enhances TRβ1 Activity in PGC-1α Dependent and Independent Manners

To evaluate the possibility that SIRT1 modulates TRβ1 activity, we examined SIRT1 effects on T3 response at a standard T3 inducible reporter driven by a TRE composed of a direct repeat of the consensus TR binding half-site AGGTCA (DR-4) [27]. We transfected the DR-4 reporter into HepG2 liver cells that stably express TRβ1 +/− expression vectors for PGC-1α and SIRT1 and measured T3 effects on luciferase activity (Fig. 1A). As expected, T3 response was enhanced approximately 20-fold by PGC-1α whereas SIRT1 increased T3 response 2.5 fold. Cotransfection of SIRT1 and PGC-1α resulted in synergistic effects determined to be significant when 2-fold with an adjusted p-value 0.05. To facilitate comparisons among the various datasets, all data was uploaded into a SQLite3 database [39]. Heatmaps were produced and clustered using multiarray viewer [40].

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increases in TRβ1 activity; together, SIRT1 and PGC-1α potentiated T3 response by greater than 200-fold, consistent with the notion that SIRT1 cooperates with PGC-1α to stimulate TRβ1 activity [24]. Interestingly, SIRT1 overexpression had no obvious influence on acetylation of PGC-1α in these conditions, suggesting that SIRT1 must exert additional effects upon activity of the TRβ1/PGC-1α complex (Fig. S1).

We next examined effects of knockdown of endogenous PGC-1α expression in the HepG2-TRβ cells (Fig. 1B). PGC-1α protein levels were greatly diminished after transfection of PGC-1α siRNA, as determined by western analysis of cell extracts (Fig. 1B, inset). Under these conditions, however, SIRT1 coactivation of TRβ1 was unaffected, and even, partially enhanced. Thus, SIRT1-dependent enhancement of T3 response is independent of PGC-1α in this assay. SIRT1 enzymatic activity was required for TRβ1 coactivation; a deacetylase defective mutant (H355Y) of SIRT1 did not enhance T3 response (Fig. 1C). We also verified that SIRT1 enhanced activity of both TR subtypes in transfections into 293T cells, which do not express endogenous TRs, albeit with a modest preference for TRβ1 versus TRα1 (Fig. 1D).

SIRT1 Interacts with TRβ1

To investigate whether SIRT1 interacts with TRβ1, we performed co-immunoprecipitations from extracts of 293 cells transfected with affinity tagged versions of SIRT1 (flag) and TRβ1 (myc) to qualitatively assess SIRT1/TRβ1 interactions in different conditions. SIRT1 precipitation with anti-flag antibody resulted in co-precipitation of myc-tagged TRβ1 (Fig. 2A). Conversely, TRβ1 precipitation with anti-myc antibody resulted in co-precipitation of SIRT1 (Fig. 2B). Within this context, SIRT1/TRβ1 interactions appeared unaffected by T3. We also performed co-immunoprecipitation assays in the HepG2 stably expressing TRβ1 and showed that endogenous SIRT1 co-precipitated with TRβ1 and, again, this effect was ligand-independent (Fig. 2C).
To test whether SIRT1 interacts with TRβ1 in vivo, we performed glutathione-S-transferase (GST) pull-downs with purified bacterially expressed TRβ1. As shown in Fig. 2D, [35]S-Methionine-labeled SIRT1 protein was retained by GST-TRβ1, but not by GST protein, in the absence and presence of T3. Thus, SIRT1 directly interacts with TRβ1 in a ligand-independent manner.

**Figure 5. SIRT1 induces proteasome-dependent TRβ1 degradation and ubiquitination.** (A) Western analysis of 293T cells transfected with myc-TRβ1 and SIRT1 expression vectors and treated with T3 for 24 hours and 20 μM MG-132 for 6 hours. Note the MG132-dependent increase in TRβ1 levels observed in SIRT1 and T3. The lower panel represents GAPDH loading control. (B) SIRT1 leads to ubiquitination of TRβ1 protein. The panel represents western analysis of extracts of 293T cells transfected with TRβ1 and SIRT1 expression vectors and treated with T3 for 24 hours and 20 μM MG-132 for 6 hour, immunoprecipitated with anti-myc antibody and blotted with anti-ubiquitin antibody.

To determine which acetyl lysine specific antibody (Fig. 5) confirmed that TRβ1 is acetylated [28,29] and also reveals that acetylation levels were specifically decreased in cells which overexpress SIRT1 and were treated with T3 (Fig. 3).

To determine how SIRT1 affects TRβ1 protein levels, we decreased SIRT1 levels using a specific SIRT1 siRNA in HepG2-TRβ1 cells. This treatment reduced SIRT1 mRNA and protein levels by more than 90% with no effect upon TRβ1 mRNA levels (Fig. 4A-C). TRβ1 protein levels were unaffected by SIRT1 knockdown in the absence of T3, however, there were T3-dependent reductions of TRβ1 steady state levels and these were partially reversed in the presence of the SIRT1 siRNA (Fig. 4C). Hormone activation usually results in diminished steady state levels of nuclear receptors and this phenomenon reflects increased ubiquitin-dependent receptor turnover; an essential step for renewal of NR transcription complexes [5]. Over the course of the study, T3 consistently reduced steady state TRβ1 levels, although the extent of this effect varied from modest (20%) to large (>90%). In this case, effects were large and we also observed that this effect was partly dependent upon SIRT1 enzymatic activity; wild type SIRT1 potentiates T3-dependent decreases in TRβ1 levels in transfected 293T cells but no decrease was observed in the presence of the deacetylase-defective mutant of SIRT1 (Fig. 4D). Nicotinamide, a SIRT1 inhibitor, also reversed T3-dependent reductions in TRβ1 levels in this cell type (Fig. 4E).

This is consistent with the idea that SIRT1 activity is required for hormone-dependent reductions in TRβ1 steady state levels in these conditions, although nicotinamide may also slightly reduce SIRT1 levels and this could also contribute to this effect. We conclude that SIRT1 enhances T3-dependent reductions in TRβ1 steady state levels.

Since previous studies suggested that SIRT1 dependent deacetylation of LXRα is associated with enhanced ubiquitin-dependent receptor turnover [20], we examined effects of inhibition of proteasome activity upon TRβ1 steady state levels and ubiquitination. In the absence of T3, TRβ1 protein levels were not changed by SIRT1 overexpression, treatment of proteasome inhibitor (MG132), or both in transfected 293T cells (Fig. 5A). In the presence of T3, however, treatment with MG132 led to increases in TRβ1 levels relative to that seen with overexpressed SIRT1 and T3 (Fig. 5A). Overexpression of SIRT1 was also associated with accumulation of higher molecular weight ubiquitinated forms of TRβ1, similar to that seen in the presence of proteasome inhibitor MG132 (Fig. 5B). Thus, SIRT1 overexpression results in T3-dependent deacetylation of TRβ1 and enhanced proteasome-mediated degradation and ubiquitination of TRβ1.

**SIRT1 Influences Expression of a Subset of TRβ1 Target Genes**

To understand how knockdown of SIRT1 would influence expression of TRβ1 target genes involved in fasting responses, we examined effects of transfected SIRT1 siRNA on T3 response in HepG2-TRβ1 cells using qPCR analysis of selected TRβ1 targets [26]. SIRT1 siRNA treatment led to complete loss of T3 induction of the glucose 6 phosphatase (G-6-Pc) gene, which encodes an enzyme that catalyzes a key rate limiting step in gluconeogenesis (Fig. 6A). In fact, T3 suppressed G-6-Pc mRNA levels in the presence of SIRT1 siRNA relative to basal levels seen with SIRT1 siRNA alone. SIRT1 also modestly inhibited T3 induction of two other fasting response genes, phosphoenol pyruvate carboxykinase 1 (PCK1) and fibroblast growth factor 1 (FGF21) (Fig. 6B, C). However, T3 induction of other target genes, including Hairless (HR; Fig. 6D), was completely unaffected by SIRT1 knockdown. PGC-1α knockdown only slightly decreased mRNA levels of these target genes, G-6-Pc and PCK1 (Fig. S3). This finding strengthens the suggestion that SIRT1-dependent regulation of these genes is independent of PGC-1α in these assay conditions.

To explore the influences of SIRT1 knockdown upon T3 response more fully, we examined T3 induction +/- SIRT1 siRNA in HepG2-TRβ1 cells using an array based assay. As we documented previously [26], hundreds of genes displayed significant T3 response in this cell type, with most induced by T3 and a minority repressed (Fig. S2). However, SIRT1 siRNA treatment only inhibited T3 induction of a small subset of these TRβ1 target genes (Fig. 7) with the vast majority unaffected by SIRT1 (Fig. S2).
There is no obvious defined functional association among the affected genes (i.e., functional ontology; not shown). Thus, SIRT1 is absolutely required for T3-induction of a very limited number of genes.

SIRT1 Potentiates TRβ1 Activity at Native Regulatory Elements

To examine effects of SIRT1 on TRβ1 activity at native TREs in the G-6-Pc and PCK1 genes, we used computer aided analysis to localize potential regulatory elements in both loci. For the G-6-Pc gene, we detected a hitherto unknown DR-4 site approximately 2.3 kb upstream of the transcriptional initiation site (Fig. 8A). While previous studies suggested that the rodent PCK1 proximal promoter harbors a variant TRE [30–32], we were unable to locate functional TREs within the human PCK1 proximal promoter or demonstrate TRβ1 interaction with this region of DNA by transfection analysis or gel shift (not shown). However, we did localize a previously unknown non-canonical DR-4 site around 13KB downstream of the gene (Fig. 8A).

As expected, TRβ1 conferred T3 response upon reporters driven by the native G-6-Pc promoter and the PCK1 downstream TRE (Fig. 8B, C). This effect was enhanced by SIRT1 cotransfection (Fig. 8B, C) and, conversely, SIRT1 knockdown inhibited T3 response in both contexts (Fig. 8D, E). However, effects of SIRT1 overexpression and knockdown were more prominent at the G-6-Pc promoter versus the PCK1 downstream
TRE (Fig. 8) and other native TREs (not shown), in parallel with strong SIRT1 requirements for T₃ induction of the native G-6-Pc gene. T₃ treatment and SIRT1 overexpression did not alter activity of the parental pGL4.23 reporter, indicating that effects were specific to G-6-Pc and PCK1 sequences (Fig. S4B).

To directly compare SIRT1 effects upon T₃ response at TREs that localized to proximal promoters, analogous to G-6-Pc, and to rule out the possibility that the relative lack of a SIRT1 effect upon PCK1 was a consequence of the unusual source and composition of this element, we also assessed SIRT1 effects upon proximal promoters of other TRβ1 target genes (Fig. S4C, D). While SIRT1 potentiated T₃ response at the SLC16A6 and MYH6 promoters, the extent of SIRT1 potentiation was promoter-specific with relatively modest effects at SLC16A6 and stronger effects upon MYH6 (Fig. S4C, D). Thus, SIRT1 enhances TRβ1 dependent T₃ response in a promoter-specific action.

To verify that TRβ1 and SIRT1 co-localized to the G-6-Pc and PCK1 TREs in cultured HepG2-TRβ1 cells, ChIP analysis was performed (Fig. 9A, B). We observed that TRβ1 (using an antibody to the Flag-tag at the N-terminus of TRβ1 expressed in these cells) and SIRT1 were present at both elements in the absence of hormone and SIRT1 siRNA knockdown reduced the
amount of detectable SIRT1 protein at both TREs. Unlike many previously documented cases of hormone-independent interactions of TRs with TREs [1], T3 enhanced TRβ1 binding to the G-6-Pc promoter. SIRT1 recruitment to the G-6-Pc TRE also appeared hormone-dependent, even though TRβ1/SIRT1 interactions are unaffected by T3. More surprisingly, SIRT1 knockdown inhibited T3-dependent TRβ1 interactions with the G-6-Pc promoter. T3 weakly enhanced TRβ1 and SIRT1 binding to the PCK1 TRE and SIRT1 knockdown reversed the hormone-dependent component of this interaction. Thus, SIRT1 is recruited to TRE region of TRβ1 target genes and is required for T3-dependent association of TRβ1 with the G-6-Pc promoter and, to a lesser extent, the PCK1 downstream TRE.

Drugs that Target SIRT1 Modulate TRβ1 Activity

Finally, we determined whether TRβ1 action at dual TRβ1/ SIRT1 target genes was influenced by drugs that target the SIRT1 pathway. Treatment of HepG2-TRβ1 cells with resveratrol, an indirect activator of SIRT1, did not affect basal mRNA levels of G-6-Pc, but did enhance T3 response (Fig. 10A). In parallel, resveratrol enhanced basal PCK1 and FGF21 mRNA levels and also potentiated T3 response at both genes (Fig. 10B, C). Treatment of HepG2-TRβ1 cells with the SIRT1 inhibitor nicotinamide potently inhibited T3 induction of G-6-Pc (Fig. 10D), similar to effects of SIRT1 siRNA treatment. In parallel, nicotinamide modestly inhibited T3 response at PCK1 and FGF21 (Fig. 10E, F). Thus, chemical manipulation of SIRT1
influences T₃ response and these SIRT1-dependent effects display similar gene context-specificity to that seen with SIRT1 siRNA.

**Discussion**

In this study, we have investigated interactions of SIRT1 and TR signaling pathways. We hypothesized that PGC-1α and SIRT1 would cooperate to enhance TRβ1 activity in liver cells and, accordingly, we find that PGC-1α and SIRT1 synergize to potentiate T₃ response at a TRE-dependent reporter, a very large enhancement of T₃ activation in response to coregulator transfection compared with previous studies [9,33]. Similar findings were recently reported by another investigator [24], who also demonstrated that SIRT1 promotes T₃-dependent deacetylation of PGC-1α and that SIRT1 is required for optimal T₃ response of endogenous TR-regulated genes in cultured liver cells, liver primary cultures and native rat liver. We have also obtained other evidence which suggests, however, that effects of SIRT1 are not completely dependent upon PGC-1α and that SIRT1 is also a direct TRβ1 coactivator. SIRT1 binds to TRβ1 in co-immunoprecipitation experiments and in vitro pulldowns and ChIP studies demonstrate colocalization of TRβ1 and SIRT1 at TREs located near target genes and similar results were also seen by Thakran and colleagues [24]. Furthermore, SIRT1 potentiates T₃ response at a transfected reporter in the absence of exogenous PGC-1α and this effect is actually potentiated by knockdown of endogenous PGC-1α. Additionally, knockdown of endogenous PGC-1α does not diminish T₃ response at two endogenous genes, opposite to effects of knockdown of SIRT1, implying that T₃ response is PGC-1α independent in these conditions. Finally, SIRT1 overexpression triggers TRβ1 deacetylation and enhanced T₃- and ubiquitin-dependent turnover of TRβ1. We acknowledge the possibility that SIRT1 could influence TRβ1 indirectly in the absence of PGC-1α, through actions upon another TR cofactor such as PGC-1β or other TR interacting proteins. However, correlation between SIRT1 enhancement of TRβ1 activity and SIRT1/TRβ1 interactions leads us to suggest that SIRT1 can modulate in two ways; indirectly, via potentiation of PGC-1α activity, and directly through TRβ1 contact.

While SIRT1 is a direct TR cofactor, requirements for SIRT1 in T₃ response appear different from general TR coregulators such as the SRCs and TRAP220, which are needed for T₃ activation of most TRβ1 target genes [3]. Instead, SIRT1 enhances TRβ1 activity in a strongly gene-specific manner. We found that SIRT1 knockdown abolished T₃ induction of G-6-Pc, but only modestly inhibited T₃ induction of the PCK1 and FGF21 genes, and left T₃ response at HR and other genes completely unaffected. These results were internally consistent with experiments that utilized a small molecule activator (resveratrol) or an inhibitor (nicotinamide) of the SIRT1 pathway. Array-based analysis of effects of SIRT1 knockdown in HepG2-TRβ cells confirmed that most T₃ responses were independent of SIRT1, but also revealed that a small subset of TRβ1 target genes were strongly inhibited by SIRT1 knockdown. Studies of Thakran et al. are also indicative of gene specificity in TRβ1/SIRT1 cooperation [24]. Whereas T₃ and resveratrol synergized to activate the pyruvate dehydrogenase kinase 4 (PDK4) gene in HepG2 cells, they only displayed modest additive effects at carnitine palmitoyl transferase 1α (CPT1α), and resveratrol did not enhance T₃ response at other genes.

How does SIRT1 modulate TRβ1 activity? SIRT1 binds TRβ1 in a hormone-independent fashion. This is different from general coactivators such as the SRCs which interact with a ligand-dependent activation function (AF-2) in the receptor ligand binding domain [33,33] and could imply that SIRT1 plays a distinct role from other TR coregulators. SIRT1 nevertheless largely influences T₃ response, with only modest effects upon unliganded TRs in some conditions, implying that it must cooperate with factors that act upon T₃-ligated TRs. There are also close parallels between effects of SIRT1 upon TRβ1 activity, acetylation state and turnover (this study) and previously reported effects of SIRT1 on LXRα [20]. In the latter study, the authors proposed that LXRα first induce the target gene in response to activating ligands and that SIRT1 subsequently deacetylates LXRα to trigger its ubiquitination and turnover, thereby allowing novel transcription complex formation. We have not investigated kinetics of changes in TRβ1 acetylation state through the transcription cycle, but it seems reasonable to suggest that SIRT1 could fulfill a similar function for TRs. TR acetylation is mediated by histone acetyl transferases (HATs) such as CBP/p300, among the first factors recruited to target genes after T₃ binding [23]. We therefore suggest that SIRT1 must act at an essential step of TRβ1 activation that occurs after CBP/p300 dependent acetylation and that separate acetylation and deacetylation steps may be important components of the transcription cycle. To fully investigate this idea, it will be important to understand kinetics of recruitment of different cofactors that, respectively, acetylate and deacetylate TRs, the role of different TR acetylation sites in T₃ response and the correlation of these events with TR acetylation status and transcriptional activity.

Data mentioned above describe general effects of SIRT1 upon TRβ activity, modification state and turnover, but it is hard to completely reconcile these effects with occasional strong gene-specific requirements for SIRT1 in T₃ response. We instead propose that gene-specific effects of SIRT1 upon T₃ response may be related to additional effects upon TRβ1 transcription complex formation. Three lines of evidence support this idea. T₃ enhances SIRT1 recruitment to the G-6-Pc TRE. This is unexpected; TRβ1/SIRT1 interactions appear independent of hormone (Fig. 2). Further, T₃ strongly enhances TRβ1 binding to the G-6-Pc promoter in cultured cells. This is also unusual; previous ChIP studies indicated that TRβ1/TRE interactions are usually unaffected by T₃. Finally, T₃-dependent TRβ1 binding to the G-6-Pc promoter is potently inhibited by SIRT1 knockdown. We do not know of any other case in which TRβ1/TRE interactions in cultured cells are dependent upon a cofactor. Together, these observations suggest that T₃ must trigger steps involved in TRβ1/SIRT1 complex assembly on the G-6-Pc TRE. Interestingly, absolute requirements for SIRT1 in T₃ response of G-6-Pc were recapitulated in transfections with a G-6-Pc promoter dependent reporter. Thus, information required for strong SIRT1 dependence of T₃ response is located within this segment of DNA. It may be interesting to consider contributions of other TFs that bind the G-6-Pc promoter and cooperate with TRβ1 and/or SIRT1 in these effects and the possibility that TRβ1 acetylation inhibits TRβ binding in this context.

The possible physiologic significance of TRβ1/SIRT1 interactions is not completely clear. Given that PGC-1α is induced in fasting and that SIRT1 mediates beneficial effects of calorie restriction, it is reasonable to suggest that a TRβ1/PGC-1α/SIRT1 complex may be required for acute T₃ response of genes involved in gluconeogenesis and fatty acid β-oxidation in liver [24]. Results of our experiments add another angle, strong SIRT1 requirements for responses of particular T₃ regulated genes, including those seen at the G-6-Pc locus in this study and the PDK4 locus in another investigation [24] also raises the possibility that TRβ1/SIRT1 complex formation is an essential checkpoint for acute T₃ regulation of particular subsets of genes in vivo. Another recent study reveals inverse correlation between thyroid
hormone status and SIRT1 protein levels (but not mRNA levels) and SIRT1 activity in liver [41]. Given that physiological adaptation to fasting involves suppression of thyroid hormone actions, the authors suggest that increases in SIRT1 protein may be a specific response to reduced thyroid hormone status during caloric restriction. Our studies show that SIRT1 can exert PGC-1α independent effects on TRβ1, but we do not determine whether similar SIRT1 dependent effects on TRβ1 also occur in the presence of PGC-1α, whether these effects occur in mouse liver or precise physiologic conditions in which SIRT1 directly influences TRβ1. Thus, it is not obvious how molecular mechanisms described here may be involved in physiologic responses to fasting or hyperthyroidism. One interesting possibility, however, is that gene-specific SIRT1/TRβ1 interactions could preserve subsets of T3 regulated responses in conditions in which thyroid hormone levels and signaling pathways are broadly suppressed by starvation or fasting. We also note that thyroid hormone regulation of liver metabolic genes differs in different conditions; for example, thyroid hormone induction of gluconeogenic genes seen here in cell culture models can also be observed in mouse liver in some conditions but not others [42,43]. It will be interesting to define the role of TRβ1/SIRT1 interactions in these phenomena. Given the general roles of SIRT1 in TRβ activity, acetylation and turnover that resemble those seen with LXRα and PPARγ [20,44] and the additional gene-specific roles of TRβ1/SIRT1 interactions, it will also be important to understand what types of effects are active in different types of physiological response to changes in TRβ1 or SIRT1 levels or activity.

Regardless of the precise function of TRβ1/SIRT1 interactions, the fact that we can modulate T3 response at several endogenous genes with ligands that alter SIRT1 activity, resveratrol and nicotinamide, suggests that it may be possible to selectively manipulate subsets of key T3 responsive genes in vivo with combinations of thymrimetics and SIRT1 ligands. For example, SIRT1 activators could be used to enhance T3-dependent fatty acid oxidation in liver and a SIRT1 inhibitor could inhibit excessive gluconeogenesis associated with thyroid hormone excess states. These possibilities should be tested in animal models and could form the basis for novel therapeutic approaches to metabolic disease that employ SIRT1 modulators in combination with several thymrimetics that exhibit improved safety profiles relative to native thyroid hormones [34].

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Supporting Information

Figure S1 SIRT1 does not influence the acetylation of PGC-1α in transfected HepG2 cells. Immunoprecipitation analysis of 293T cells transfected with expression vectors for PGC-1α, SIRT1 or both and treated +/− T3. PGC-1α was immunoprecipitated with anti- PGC-1α antibodies and precipitates were blotted with anti-acetyl-lysine, PGC-1α or SIRT1 antibodies. Acetylated PGC-1α levels relative to total TRβ1 were quantified by Phosphor Imager (right panel). (TIF)

Figure S2 Heatmap representation of TRβ1 target genes that are inhibited by SIRT1 knockdown. T3-response was determined in the presence of Negative-control siRNA (NC-siRNA, column 1) and SIRT1 siRNA (Knock-down = KD, column 2) treatments through comparison against their respective vehicle control treatments. The specific effect of SIRT1 KD was determined through the comparison of effects of both SiRNA treatments in the absence of ligand (SIRT1-siRNA vs. NC-siRNA, lane 3), see Methods. Note that, in most instances, T3 responses are unaffected by SIRT1 knockdown but that a subset of T3 responsive genes exhibit significant changes in response to SIRT1 knockdown. Further, while SIRT1 knockdown does influence target gene expression in the absence of T3, many effects of SIRT1 knockdown are specific to T3. The SIRT1/T3 dependent cluster shown in the main text is marked at right of the heatmap. (TIF)

Figure S3 The effect of PGC-1α knockdown upon expression of TRβ1 target genes. qPCR analysis of HepG2/TRβ1 cells extracts treated +/− T3 and PGC-1α siRNA. G6-Pc (A) and PCK1 (B). All values represent the mean ± SD of duplicate samples. **, P < 0.01; *, P < 0.05. (TIF)

Figure S4 SIRT1 differentially regulates the activity of alternate TRβ1 target gene promoters. (A) Schematic representation of TREs of TRβ1 target genes with sequences and positions of DR-4 site (~309 ~ −294) for SLC16A6 gene and DR-4 site (~148 ~ −133) for MYH6 gene. (B–D) Luciferase assays performed on extracts of 293T cells that were cotransfected with indicated reporters along with TRβ1 and SIRT1 expression vectors and treated +/− T3. The levels of luciferase activity were normalized to the lacZ expression. All values represent mean ± SD of duplicate samples. **, P < 0.01; *, P < 0.05. (TIF)

Author Contributions

Conceived and designed the experiments: JHS DHS XX PW. Performed the experiments: JHS DHS AZ AC. Analyzed the data: JHS DHS AZ AC. Contributed reagents/materials/analysis tools: XX. Wrote the paper: JHS GEW PW.

References

1. Brent GA (2012) Mechanisms of thyroid hormone action. J Clin Invest. 122: 3033–3043.
2. Weiss RE, Ramos HE (2004) Thyroid hormone receptor subtypes and their interaction with steroid receptor coactivators. Vitam Horm. 68: 183–207.
3. Fouladi JD (2013) The Mediator complex in thyroid hormone receptor action. Biochim Biophys Acta. 1830: 3867–3875.
4. Astapova I, Hollenberg AN (2013) The in vivo role of nuclear receptor corepressors in thyroid hormone action. Biochim Biophys Acta. 1830: 3876–3881.
5. Lonard DM, O’Malley BW (2009) Emerging roles of the ubiquitin proteasome system in nuclear hormone receptor signaling. Prog Mol Biol Transl Sci. 87: 117–135.
6. Brunelle M, Fayad T, Langlois MF (2011) Degradation of thyroid hormone receptor beta 1: existence of stable and unstable forms. Thyroid. 21: 311–318.
7. Wehl O, Wehl C, Barkulun M, Leers J, Uhle F, et al. (2010) Modular insulators: genome wide search for composite CTCF/thyroid hormone receptor binding sites. PLoS One. 5: e10119.
8. Weinhofer I, Kunze M, Ramlper H, Forss-Petter S, Samarut J, et al. (2008) Distinct modulatory roles for thyroid hormone receptors TRα and TRβ. J Steroid Biochem Mol Biol. 133: 58–65.
9. Yuan C, Nguyen P, Baxter JD, Webb P (2013) Distinct ligand-dependent and independent modes of thyroid hormone receptor (TR/PGC-1α) interaction. J Steroid Biochem Mol Biol. 133: 58–65.
10. Frige JN, Anwers J (2007) Transcriptional coregulators in the control of energy homeostasis. Trends Cell Biol. 17: 292–301.
11. Weitzel JM, Hamann S, Joek M, Lacey M, Filley A, et al. (2003) Hepatic gene expression patterns in thyroid hormone-treated hypothyroid rats. J Mol Endocrinol. 31: 291–303.

12. Weitzel JM, Radke C, Seitz HJ (2001) Two thyroid hormone-mediated gene expression patterns in vivo identified by cDNA expression arrays in rat. Nucleic Acids Res. 29: 5148–5155.

13. Zhang Y, Ma K, Song S, Elam MB, Cook GA, et al. (2004) Peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1 alpha) enhances the thyroid hormone induction of carnitine palmitoyltransferase I (CPT-I alpha). J Biol Chem. 279: 53963–53971.

14. Sadana P, Zhang Y, Song S, Cook GA, Elam MB, et al. (2007) Regulation of carnitine palmitoyltransferase I (CPT-I alpha) gene expression by the peroxisome proliferator activated receptor gamma coactivator (PGC-1) isoforms. Mol Cell Endocrinol. 267: 6–16.

15. Attila RR, Connaughton S, Boone LR, Wang F, Elam MB, et al. (2010) Regulation of pyruvate dehydrogenase kinase 4 (PDK4) by thyroid hormone: role of the peroxisome proliferator-activated receptor gamma coactivator (PGC-1 alpha). J Biol Chem. 285: 23725–23835.

16. Schug TT, Li X (2011) Sirtuin 1 in lipid metabolism and obesity. Ann Med. 43: 200–211.

17. Lagouge M, Argmann C, Gerhart-Hines Z, Meziane H, Lerin C, et al. (2006) Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell. 127: 1109–22.

18. Dominy JE Jr, Lee Y, Gerhart-Hines Z, Fuqerwer P (2010) Nutrient-dependent regulation of PGC-1alpha's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. Biochim Biophys Acta. 1804: 1674–1683.

19. Parsanezhad A, Schug TT, Xu Q, Surapureddi S, Guo X, et al. (2009) Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. Cell Metab. 9: 327–338.

20. Li X, Zhang S, Blander G, Tse JG, Krieger M, et al. (2007) SIRT1 deacetylates and positively regulates the nuclear receptor LXR. Mol Cell. 29: 91–106.

21. Kemper JK, Xiao Z, Ponugoti B, Miao J, Fang S, et al. (2009) FXR acetylation and regulation of PGC-1alpha's acetylation state and metabolic function through the enzymatic activities of Sirt1/GCN5. Biochim Biophys Acta. 1804: 1676–1683.

22. Wilson BJ, Tremblay AM, DePrez G, Syvan-Drolet G, Gigaure V, et al. (2010) An acetylation switch modulates the transcriptional activity of estrogen-related receptor alpha. Mol Endocrinol. 24: 1349–1359.

23. Liu YY, Brent GA (2010) Thyroid hormone crosstalk with nuclear receptor signaling in metabolic regulation. Trends Endocrinol Metab. 21: 166–73.

24. Thakran S, Sharma P, Atitaa RR, Hori RT, Deng X, et al. (2015) Role of sirtuin 1 in the regulation of hepatic gene expression by thyroid hormone. J Biol Chem. 288: 407–418.

25. Yuan C, Lin JZ, Siegla DH, Ayers SD, Denoto-Reynolds F, et al. (2012) Identical gene regulation patterns of T3 and selective thyroid hormone receptor modulator GC-1. Endocrinology. 153: 501–11.

26. Lin JZ, Siegla DH, Yuan C, Su J, Arumanayagam AS, et al. Gene specific actions of thyroid hormone receptor subtypes. PLoS One. 8: e52407.

27. Velasco LF, Togashi M, Walfish PG, Pessahin R, Moser F, et al. (2007) Thyroid hormone response element organization dictates the composition of active receptor. J Biol Chem. 282: 12458–12466.

28. Lin HY, Hogkins R, Cao HJ, Tang HY, Alexander C, et al. (2005) Acetylation of nuclear hormone receptor superfamily members: thyroid hormone causes acetylation of its own receptor by a mitogen-activated protein kinase-dependent mechanism. Steroids. 70: 444–449.

29. Kitchens-Pacheco A, Martino-Condejas O, Mendoza-Pertuz M, Aranda A (2009) Residues K118, 132, and 134 in the thyroid hormone receptor-alpha are essential for receptor acetylation and activity. Endocrinology. 150: 5143–5152.

30. Lucas PC, Forman BM, Samuels HH, Grammer DK (1991) Specificity of a retinoic acid response element in the phosphoenolpyruvate carboxykinase gene promoter: consequences of both retinoic acid and thyroid hormone receptor binding. Mol Cell Biol. 11: 6343.

31. Park EA, Jerden DC, Balsouth SW (1995) Regulation of phosphoenolpyruvate carboxykinase gene transcription by thyroid hormone involves two distinct binding sites in the promoter. Biochem J. 309: 913–919.

32. Giralt M, Park EA, Gurney AL, Liu JS, Hakimi P, et al. (1991) Identification of a thyroid hormone response element in the phosphoenolpyruvate carboxykinase (GTP) gene. Evidence for synergistic interaction between thyroid hormone and cAMP cis-regulatory elements. J Biol Chem. 266: 21991–21996.

33. Feng W, Ribeiro RC, Wagner RL, Nguyen H, Apriletti JW, et al. (1998) Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science. 280: 1747–1749.

34. Baxter JD, Webb P (2007) Thyroid hormone mimetics: potential applications in atherosclerosis, obesity and type 2 diabetes. Nat Rev Drug Discov. 8: 308–320.

35. Du P, Kibble WA, Lin SM (2008) LAMP1: a pipeline for processing Illumina microarray data. Bioinformatics. 24: 1547–1548.

36. Smyth GK (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology. 3.

37. Team RDC (2009) R: A language and environment for statistical computing. R Foundation for Statistical Computing: Vienna.

38. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B (Methodological). 57: 289–300.

39. Hopp DR, SQL.<ins>50</ins>

40. Saed AI, Sharov V, White J, Li J, Liang W, et al. (2003) TM4: a free, open-source system for microarray data management and analysis. Biotechniques. 34: 374–376.

41. Cordeiro A, de Souza LL, Oliveira LS, Faustino LC, Santiago LA, et al. (2013) Thyroid hormone regulation of Sirt1 expression and implications to integrated responses in fasted mice. Journal of Endocrinology. 216: 181–193.

42. Feng X, Jiang Y, Meltzer P, Yen PM (2000) Thyroid hormone regulation of hepatic genes in vivo detected by complementary DNA microarray. Mol. Endocrinol. 14: 947–955.

43. Vujovic M, Nordstro¨m K, Gauthier K, Flamant F, Visser TJ, et al. (2009) Interference of a mutant thyroid hormone receptor alpha 1 with hepatic glucose metabolism. Nat Rev Drug Discov. 8: 2940–2947.

44. Qiang L, Wang L, Kong N, Zhao W, Lee S, et al. (2012) Brown remodeling of white adipose tissue by Sirt1-dependent deacetylation of PPARy. Cell. 150: 620–632.