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Thyroid Hormone Signaling *In Vivo* Requires a Balance between Coactivators and Corepressors

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Resistance to thyroid hormone (RTH), a human syndrome, is characterized by high thyroid hormone (TH) and thyroid-stimulating hormone (TSH) levels. Mice with mutations in the thyroid hormone receptor beta (TRβ) gene that cannot bind steroid receptor coactivator 1 (SRC-1) and *Src-1*$^{-/-}$ mice both have phenotypes similar to that of RTH. Conversely, mice expressing a mutant nuclear corepressor 1 (*Ncor1*) allele that cannot interact with TRβ, termed NCoRΔID, have low TH levels and normal TSH. We hypothesized that *Src-1*$^{-/-}$ mice have RTH due to unopposed corepressor action. To test this, we crossed NCoRΔID and *Src-1*$^{-/-}$ mice to create mice deficient for coregulator action in all cell types. Remarkably, NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have normal TH and TSH levels and are triiodothyronine (T_3) sensitive at the level of the pituitary. Although absence of SRC-1 prevented T_3 activation of key hepatic gene targets, NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice reacquired hepatic T_3 sensitivity. Using *in vivo* chromatin immunoprecipitation assays (ChIP) for the related coactivator SRC-2, we found enhanced SRC-2 recruitment to TR-binding regions of genes in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice, suggesting that SRC-2 is responsible for T_3 sensitivity in the absence of NCoR1 and SRC-1. Thus, T_3 targets require a critical balance between NCoR1 and SRC-1. Furthermore, replacement of NCoR1 with NCoRΔID corrects RTH in *Src-1*$^{-/-}$ mice through increased SRC-2 recruitment to T_3 target genes.

The maintenance of thyroid hormone (TH) levels is essential for development, metabolism, energy expenditure, and thermoregulation. TH levels are sustained by a negative-feedback loop within the hypothalamic-pituitary-thyroid (HPT) axis that operates to keep TH levels, both the predominant form, thyroxine (T_4), and the active form, triiodothyronine (T_3), within a precise range (1). Thyrotropin-releasing hormone (TRH) from the paraventricular nucleus of the hypothalamus (PVH) stimulates the release of thyroid-stimulating hormone (TSH) from the pituitary. TSH acts on the thyroid to synthesize and release TH, which then signals back to both the PVH and pituitary to suppress TRH and TSH levels, respectively (2). At the molecular level, T_3 mediates its actions through its nuclear receptor isoforms, thyroid hormone receptor alpha (TRα) and TRβ (encoded by *Thra* and *Thrb*), which function as ligand-dependent transcription factors to either increase or decrease expression of target genes (3–5). Classically, on positively regulated TH targets, the presence of T_3 allows the binding of coactivators, such as the steroid receptor coactivator family (SRC-1, -2, and -3) (6–8). These coactivators then recruit machinery to allow the activation of gene expression (9–12). In the absence of ligand, corepressors, such as nuclear corepressor 1 (NCoR1) or silencing mediator of retinoic acid (SMRT, or NCoR2), bind and recruit complexes to repress transcription (13–18). The processes by which negative TH targets are repressed or transcribed are not well understood, but active T_3 repression does require SRC-1 (19–22).

In previous reports, our laboratory has demonstrated that NCoR1 also mediates T_3 sensitivity and is critical in maintaining normal TH levels (23–25). NCoR1 binds to TR through two of its three nuclear-receptor-interacting domains, N2 and N3 (26–32). Mice globally expressing a conditional *Ncor1* allele lacking N2 and N3, termed NCoRΔID, either during embryogenesis or postnatally, have decreased levels of T_4 and T_3 and normal TSH levels (24, 25). In addition to altering the set point of the HPT axis, NCoRΔID increases the sensitivity of peripheral tissues, such as the liver, to T_3. This enhanced sensitivity leads to the increased activation of target genes in the presence of similar amounts of T_3 (24). The role of NCoR1 in determining ligand sensitivity in the context of nuclear receptor signaling has been demonstrated by other groups using a mouse model with disrupted interaction between NCoR1 and histone deacetylase 3 (HDAC3) (33, 34).

Recently, it was hypothesized that the dominantly inherited human disorder resistance to thyroid hormone (RTH) that is caused by mutations in *Thrb* is due to an imbalance of corepressor recruitment *in vivo* (35, 36). Indeed, many of the *Thrb* mutations that cause RTH are characterized by impaired ability to bind T_3 and therefore increased recruitment of corepressors (37). Therefore, we crossed the *Thrb*^{PV} mouse, characterized by a knock-in frameshift mutation in the ligand-binding domain of the *Thrb* gene that prevents *Thrb*^{PV} from binding T_3, with NCoR$^{\Delta ID/\Delta ID}$ mice (38). The *Thrb*^{PV} model faithfully recapitulates RTH syndrome, including high TH and TSH levels (38, 39). Notably, NCoR$^{\Delta ID/\Delta ID}$ *Thrb*^{PV} mice had lower TH and TSH levels than their *Thrb*^{PV} littermates, confirming that aberrant corepressor recruitment plays an important role in the development of RTH *in vivo* (40).

While RTH in humans is secondary to *Thrb* mutations in 85% of cases, two mouse models of RTH involve the coactivator SRC-1 (41). Mice null for SRC-1 (*Src-1*$^{-/-}$ mice) and mice with a mu-

Received 24 January 2014 Returned for modification 27 January 2014
Accepted 10 February 2014
Published ahead of print 18 February 2014
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doi:10.1128/MCB.00129-14

tated *Thrb* unable to interact with SRC-1 have high TH levels with inappropriately high TSH secretion (20, 21, 42). Thus, concurrent with the effects of NCoRΔID on $Thrb^{PV}$ mice, we hypothesized that both SRC-1 mouse models have RTH due to the unopposed recruitment of corepressors. We proposed that crossing $NCoR^{\Delta ID/\Delta ID}$ mice to $Src\text{-}1^{-/-}$ mice would correct the high TH and TSH levels. Indeed, we show here that $Src\text{-}1^{-/-}$ mice expressing the NCoRΔID allele have normal TH and TSH levels. Furthermore, the pituitary, which is resistant to T_3 in $Src\text{-}1^{-/-}$ mice, responds normally to T_3 in $NCoR^{\Delta ID/\Delta ID}$ $Src\text{-}1^{-/-}$ mice. Additionally, hepatic T_3 targets, which cannot be upregulated by T_3 in $Src\text{-}1^{-/-}$ mice, regain their sensitivity to T_3 in $NCoR^{\Delta ID/\Delta ID}$ $Src\text{-}1^{-/-}$ mice. Strikingly, $NCoR^{\Delta ID/\Delta ID}$ $Src\text{-}1^{-/-}$ mice have increased levels of the coactivator SRC-2 and greater SRC-2 recruitment to the TR-binding sites of T_3 target genes. Thus, in the absence of SRC-1 and NCoR1, SRC-2 is recruited to the TR-binding regions of T_3 target genes, suggesting that the presence of NCoR1 in the absence of SRC-1 prevents additional coactivator recruitment. This implies that TR target genes respond to T_3 based on the availability of specific corepressors and coactivators, explaining why individuals have different HPT axis set points and respond differently to similar amounts of T_3.

MATERIALS AND METHODS

Animals. All mouse breeding and housing were approved by the Beth Israel Deaconess Medical Center (BIDMC) Institutional Animal Care and Use Committee. Generation of mice containing the NCoRΔID allele ($NCoR^{\Delta ID}$) has been previously described (23, 24). $Src\text{-}1^{-/-}$ mice were rederived from previously described mice (a gift of B. W. O'Malley [42]). $NCoR^{\Delta ID/+}$ mice were crossed with $Src\text{-}1^{+/-}$ mice to create double-heterozygous ($NCoR^{\Delta ID/+}$ $Src\text{-}1^{+/-}$) mice, which were bred to produce the mice used in this study: $NCoR^{+/+}$ $Src\text{-}1^{+/+}$ (wild type [WT]), $NCoR^{\Delta ID/\Delta ID}$ $Src\text{-}1^{+/+}$ ($NCoR^{\Delta ID/\Delta ID}$), $NCoR^{+/+}$ $Src\text{-}1^{-/-}$ ($Src\text{-}1^{-/-}$), and $NCoR^{\Delta ID/\Delta ID}$ $Src\text{-}1^{-/-}$. The mice were maintained on a mixed B6-129S strain background. To ensure validity and reproducibility, we studied both male and female cohorts for the euthyroid studies ($n = 7$ to 13 per genotype; 4 genotypes) and then developed three additional experimental cohorts (euthyroid, hypothyroid, and PTU + T_3 [see below]) from the same parental lines. Importantly, NCoR1 and SRC-1 are not located on the same chromosome.

Animal experiments and sample collection. All mouse experiments were approved by the BIDMC Institutional Animal Care and Use Committee. The animals were housed on a 12-h light, 12-h dark cycle and given standard rodent chow (F6 Rodent Diet 8664; Harlan Teklad, Indianapolis, IN) and water *ad libitum* unless otherwise specified. At the end of the experiments, the mice were euthanized by asphyxiation with CO_2. Blood was drawn from the animals by cardiac puncture, and plasma was separated by centrifugation in EDTA-treated tubes and stored at −80°C until analysis. Tissues were rapidly collected, flash frozen in liquid nitrogen, and stored at −80°C until processed.

TSH suppression test. Control male mice (chow mice) were maintained on the chow diet until 9 to 12 weeks of age. At 6 weeks of age, a subset of mice (PTU mice) was fed a low-iodine diet supplemented with 0.15% propylthiouracil (LoI/PTU) (Harlan Teklad TD.95125) for a minimum of 21 days (PTU mice) to induce hypothyroidism. The PTU mice were sacrificed on day 22 of the LoI/PTU diet. On day 21 of the LoI/PTU diet, a subset of mice (PTU + T_3 mice) began to receive daily intraperitoneal (i.p.) injections of T_3 (Sigma, St. Louis, MO) while they were maintained on a LoI/PTU diet: a low dose (0.2 μg/100 g body weight [BW]/day) for 7 days, followed by a medium dose (0.5 μg/100 g BW/day) for 7 days, followed by a high dose (1.0 μg/100 g BW/day) for 7 days (21, 43). The animals were sacrificed 14 to 15 h following the last injection of T_3. The mice were euthanized as described above.

EchoMRI. At 9 weeks of age, chow mice were subjected to magnetic resonance imaging (MRI) using EchoMRI (Echo Medical Systems, Houston, TX) to determine body composition.

Plasma analysis. Total plasma T_4 (TT_4) and T_3 (TT_3) were measured by solid-phase radioimmunoassay (RIA) (Coat-a-Count; Diagnostic Products Corp., Los Angeles, CA) in 25 and 50 μl of plasma, respectively. Circulating TSH was measured via Milliplex MAP (rat thyroid hormone TSH panel; EMD Millipore, Billerica, MA) (25, 44).

Real-time quantitative PCR. Total RNA was extracted from frozen tissues with Stat-60 reagent (Tel-Test, Friendswood, TX). Then, 0.25 μg of total RNA was reverse transcribed using a SuperScript Vilo cDNA synthesis kit from Invitrogen (Carlsbad, CA). TaqMan gene expression assays for all mRNAs were purchased from Applied Biosystems (Carlsbad, CA). Quantitative PCR (qPCR) was performed in duplicate or triplicate using the 800HT thermal cycler (Applied Biosystems). Relative mRNA levels were calculated using the standard curve method and normalized to peptidylprolyl isomerase A (cyclophilin; *Ppia*) in the heart or 18S rRNA (*Rn18S*) in the liver, pituitary, and PVH (24, 25, 45).

Western blot analysis. Western blots were performed as previously described (24). Briefly, approximately 40 mg of liver was homogenized in 1 ml of cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor cocktail [Roche, Indianapolis, IN]). Samples were then sonicated four times for 5 s each time at power level 4 and centrifuged for 20 min at maximum speed. Twenty micrograms of total protein was resolved on 3 to 8% gradient Tris-acetate gels (Invitrogen) and blotted with the indicated primary antibodies, followed by appropriate horseradish peroxidase-conjugated secondary antibody, and developed using the ECL Plus Western blot detection system (Amersham, Piscataway, NJ). The primary antibodies used were an affinity-purified rabbit anti-NCoR1 antibody generated against the C-terminal portion of the NCoR1 molecule, anti-SRC-1 (128E7; Cell Signaling, Danvers, MA); anti-NCoR2 (PA1-843; Affinity Bioreagents/ThermoFisher); and anti-SRC-2 (A300-345A; Bethyl Laboratories, Montgomery, TX). The blots were then stripped (2% SDS, 62.5 mM Tris, pH 6.8, 0.8% 2-mercaptoethanol) and reblotted with anti-RNA polymerase II (05-952; EMD Millipore). The blots were scanned and quantified using ImageJ software (public domain; developed at the National Institute of Mental Health, Bethesda, MD), normalizing to anti-polymerase II.

ChIP. Chromatin immunoprecipitation assay (ChIP) methods were adapted from previous studies (24, 46, 47). ChIP was performed on mouse livers from PTU + T_3 mice of all genotypes. Thirty to 35 mg of minced mouse liver was double cross-linked by first cross-linking using 2 mM disuccinimidyl glutarate (DSG) in phosphate-buffered saline (PBS) at room temperature for 45 min. The livers were washed in PBS and then cross-linked using 1% formaldehyde for 5 min at room temperature. Cross-linking was stopped by adding 0.125 M glycine for 5 min, and the livers were washed again in PBS. We homogenized the livers in 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.3% NP-40 with protease inhibitors. Following homogenization, crude nuclei were pelleted by centrifugation at 1,000 × *g*, washed with PBS, and then sonicated in 1-ml Adaptive Focused Acoustics tubes using a sonicator (Covaris, Woburn, MA) in a buffer of 50 mM Tris, pH 8.0, 10 mM EDTA, 0.25% SDS with protease inhibitors. This process achieves a DNA shear size of 200 to 500 bp. Following sonication, samples were spun to remove debris. Chromatin from mouse livers ($n = 4$ per genotype) was pooled into a single sample. The samples were diluted in 0.5× RIPA buffer with protease inhibitors and 20 μg glycogen incubated with the appropriate antibody overnight at 4°C with rocking. SRC-2 ChIP was performed with anti-SRC-2 A300-345A (Bethyl Laboratories, Montgomery, TX). SRC-1 ChIP was performed with anti-SRC-1 SRC-1 128E7 rabbit monoclonal antibody (MAb) 2191 (Cell Signaling Technology). We used IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as a control for both ChIPs. Samples were precipitated with protein A-agarose beads (Thermo-

TABLE 1 ChIP-qPCR primer sequences

Set no.	Gene	Primer	
		Forward	Reverse
1	*Abcb11*	TCTCACCAGGCTCTCTACCA	TTGCTTATAGGTCAATGGCCT
2	*Thrsp A*	CCCTCCACTTAGGCCAGGAC	GCCCTAGGTCACCTCCAGAG
3	*Gpd2* Int1 A	GTCGTTGCTTTGGGCTGAGC	CCCATCGGCATACTCAGGTGG
4	*Gpd2* Int1 B	CAGGATTCTTACGGAGAACGC	CCCAGGACCTACTGTGGATC
5	*Gpd2* Int1 D	CCACTGACTTCCTGTCTCCC	GGCCACTGTGGCTGCATCAAG
6	β-*Globin (Hbb1)*	GTGTTGCCAAAAAGGATGC	GAAGCAAATGTGAGGAGCAACTG

Fisher). Cross-links were then reversed overnight at 65°C, and DNA was extracted using phenol-chloroform-isoamyl alcohol. DNA from 4 replicate precipitation reactions was used in quantitative PCR with SYBR green reaction chemistry (Applied Biosystems). The results are reported as fold enrichment (ChIP signal) normalized to the DNA input. Similar results were seen when we repeated the ChIPs with no antibody control (data not shown). Primer sequences were designed to regions of TR binding determined by *in vivo* chromatin affinity purification-DNA sequencing (ChAP-seq) using a biotinylated TRβ1 in mouse liver and are listed in Table 1 (47).

Statistical analyses. Data are presented as sample means and standard errors of the mean (SEM). Sample numbers ranged from 4 to 13 and are stated specifically within the text or figure legends. The text or figure legends highlight how differences between groups were measured, which included unpaired *t* test, 1-way analysis of variance (ANOVA) with Tukey's multiple-comparison *post hoc* test, 2-way repeated-measures (RM) ANOVA with a Bonferroni *post hoc* test, and 2-way ANOVA with a Bonferroni *post hoc* test.

RESULTS

NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice develop normally. To determine the roles of corepressors and coactivators in establishing the set point of the thyroid axis, we crossed mice that globally express NCoRΔID (NCoR$^{\Delta ID/\Delta ID}$ mice) with *Src-1*$^{-/-}$ mice (24, 42). Mice double homozygous for NCoRΔID and *Src-1*$^{-/-}$ (NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice) develop normally and are born at the expected Mendelian ratios. At 9 weeks of age, male and female NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have body weights, lean masses, and body fat percentages similar to those of WT, NCoR$^{\Delta ID/\Delta ID}$, and *Src-1*$^{-/-}$ mice (Fig. 1A to C). NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice also have normal pituitary expression of growth hormone (*Gh*) mRNA levels compared to WT, NCoR$^{\Delta ID/\Delta ID}$, and *Src-1*$^{-/-}$ mice (Fig. 1D). Previously, we reported that NCoR$^{\Delta ID/\Delta ID}$ mice weighed less than their WT littermates, but only at certain time points, which is consistent with the data reported here (24).

To assess the potential compensation by other nuclear cofactors for the loss of NCoR1 and SRC-1, expression of *Ncor1* and *Smrt* (*Ncor2*) was tested in male pituitaries and livers (Fig. 1E and F). The expression levels of *Smrt* and the 5′ region of *Ncor1* mRNA, which is common to both the WT allele and the NCoRΔID allele, were normal across all genotypes in both the pituitary and liver (23). Expression of the 3′ region of *Ncor1* mRNA, which contains the N2 and N3 domains deleted from the NCoRΔID allele, was more than 99% lower in the pituitaries and livers of NCoR$^{\Delta ID/\Delta ID}$ and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice than in WT mice, consistent with our previous study (23). We also tested the expression of the three coactivators *Src-1* (*Ncoa1*), *Src-2* (*Ncoa2*; TIF-2), and *Src-3* (*Ncoa3*) in the pituitaries and livers of all the mice (Fig. 1E and F). Expectedly, *Src-1* expression was more than 99% lower in both the pituitaries and livers of *Src-1*$^{-/-}$ and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice, respectively, than in those of WT mice. *Src-2* and *Src-3* expression remained unchanged compared to WT mice (Fig. 1E and F). Lastly, we measured mRNA expression of the different isoforms of *Thr* in both the pituitary (*Thra*,

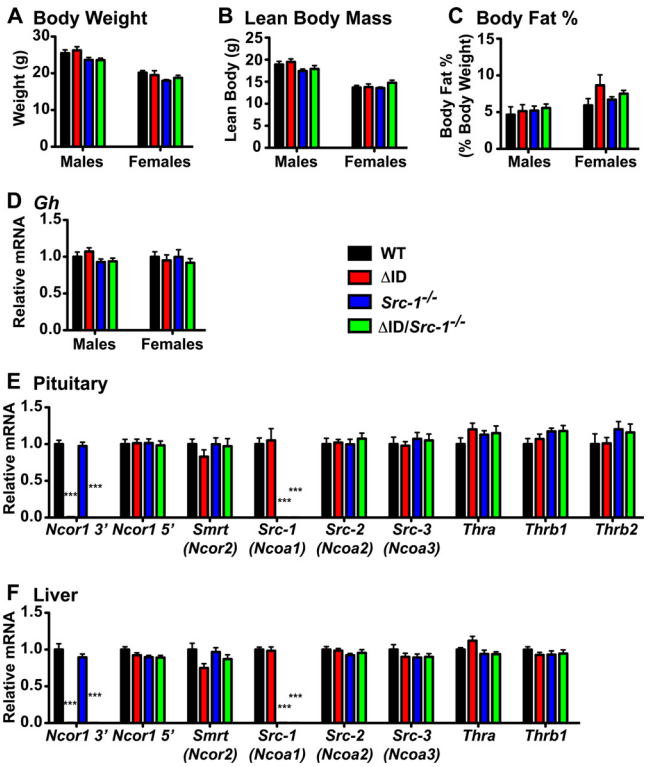

FIG 1 Deletion of *Src-1* and mutation of NCoR1 does not affect mRNA expression of other coregulators and thyroid hormone receptors. (A) Body weights of WT, NCoR$^{\Delta ID/\Delta ID}$ (ΔID), *Src-1*$^{-/-}$, and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (ΔID/*Src-1*$^{-/-}$) mice at 9 weeks of age were measured in male and female mice. (B and C) Lean body mass (B) and body fat percentage (C) were measured in male and female mice at 9 weeks of age by EchoMRI. (D) *Gh* mRNA was measured by qPCR in the pituitaries of male and female mice, normalized to 18S rRNA, and expressed relative to the WT group. (A to D) Data are presented as means and SEM ($n = 7$ to 13 per genotype). Significance was tested with 1-way ANOVA. (E) In pituitaries from male WT, ΔID, *Src-1*$^{-/-}$, and ΔID/*Src-1*$^{-/-}$ mice, qPCR was performed to quantify mRNA of the 3′ region of NCoR1 (*Ncor1* 3′), the 5′ region of NCoR1 (*Ncor1* 5′), SMRT (*Ncor2*), SRC-1 (*Ncoa1*), SRC-2 (*Ncoa2*), SRC-3 (*Ncoa3*), and thyroid hormone receptors (*Thra*, *Thrb1*, and *Thrb2*). (F) To quantify mRNA in the livers of the same male mice, qPCR was used to analyze the 3′ region of NCoR1 (*Ncor1* 3′), the 5′ region of NCoR1 (*Ncor1* 5′), SMRT (*Ncor2*), SRC-1 (*Ncoa1*), SRC-2 (*Ncoa2*), SRC-3 (*Ncoa3*), and thyroid hormone receptors (*Thra* and *Thrb1*). (E and F) mRNA was normalized to 18S rRNA and expressed relative to the WT group ($n = 7$ to 13 per genotype). Significance was tested by 1-way ANOVA with Tukey's multiple-comparison *post hoc* test. For *Ncor1* 3′, ***, $P < 0.001$ versus WT and *Src-1*$^{-/-}$ mice. For *Src-1*, ***, $P < 0.001$ versus WT and ΔID mice.

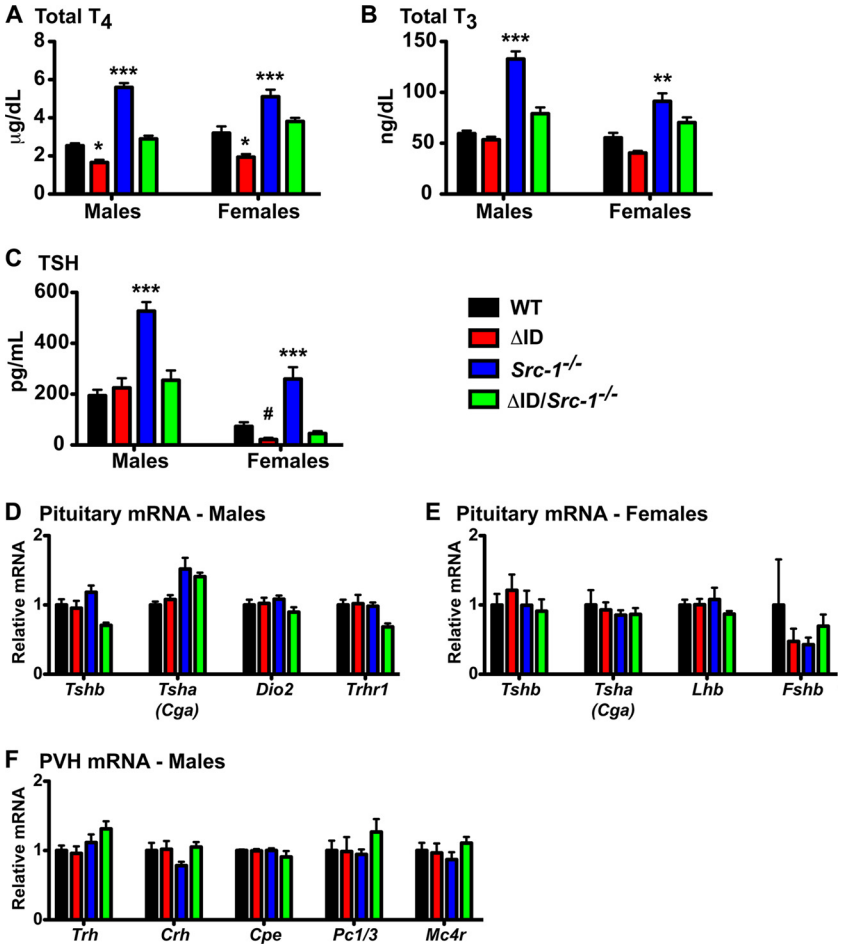

FIG 2 Disruption of the interaction between the TR and NCoR1 in *Src-1*$^{-/-}$ mice normalizes TH and TSH levels in male and female mice. Plasma total T_4 (A), total T_3 (B), and circulating TSH (C) were measured in male and female WT, NCoR$^{\Delta ID/\Delta ID}$ (ΔID), *Src-1*$^{-/-}$, and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (ΔID/*Src-1*$^{-/-}$) mice. (A to C) $n = 6$ to 12. The data are presented as means and SEM. Significance was tested by 1-way ANOVA with Tukey's multiple-comparison *post hoc* test. *, $P < 0.05$ versus all; **, $P < 0.01$ versus all; ***, $P < 0.001$ versus all; #, $P < 0.05$ versus WT and *Src-1*$^{-/-}$. (D) In pituitaries from male mice, mRNA expression was analyzed by qPCR for TSH subunits *Tshb* and *Tsha* (*Cga*), the type II deiodinase (*Dio2*), and the TRH receptor (*Trhr1*). (E) In pituitaries from female mice, mRNA expression was analyzed by qPCR for TSH subunits *Tshb* and *Tsha* (*Cga*), luteinizing hormone (*Lhb*), and follicle-stimulating hormone (*Fshb*). (F) In male mice, the PVH was microdissected, and mRNA was quantified by qPCR for thyrotropin-releasing hormone (*Trh*), corticotropin-releasing hormone (*Crh*), carboxypeptidase E (*Cpe*), prohormone convertase 1/3 (*Pc1/3*), and melanocortin 4 receptor (*Mc4r*). (D to F) mRNA was normalized to 18S rRNA and expressed relative to the WT group ($n = 6$ to 12). The data are presented as means and SEM. Significance was tested with 1-way ANOVA.

Thrb1, and *Thrb2*) and the liver (*Thra* and *Thrb1*) and found that the levels remained unchanged between genotypes (Fig. 1E and F).

Male and female NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have normal TH levels. To measure the impact of replacing NCoR1 with NCoRΔID in *Src-1*$^{-/-}$ mice on thyroid hormone levels, we measured thyroid function in all genotypes in males and females (Fig. 2). As previously reported, TT_4 levels were significantly reduced, by 34.6%, in male NCoR$^{\Delta ID/\Delta ID}$ mice (1.66 ± 0.14 μm/dl) compared to WT mice (2.54 ± 0.13 μm/dl) (Fig. 2A) (24, 25, 40). We found that TT_4 levels in male *Src-1*$^{-/-}$ mice (5.60 ± 0.23 μm/dl) were, as described previously, significantly increased, by 220% (20, 48–50). Strikingly, TT_4 levels in male NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice (2.90 ± 0.17 μm/dl) were normal compared to WT mice. Similar to male mice, female NCoR$^{\Delta ID/\Delta ID}$ mice had a significant (40%) decrease in TT_4 levels (1.93 ± 0.16 μm/dl) compared to WT mice (3.20 ± 0.33 μm/dl) (Fig. 2A). We found that TT_4 levels in female *Src-1*$^{-/-}$ mice (5.11 ± 0.36 μm/dl) were significantly increased, by 160% (20). Addition of NCoRΔID to *Src-1*$^{-/-}$ female mice normalized the TT_4 level (3.81 ± 0.19 μm/dl) to that of WT mice (Fig. 2A).

Although we did not see decreased TT_3 levels in male NCoR$^{\Delta ID/\Delta ID}$ mice (53.5 ± 2.8 ng/dl) on this background, TT_3 levels in *Src-1*$^{-/-}$ mice (132.8 ± 7.6 ng/dl) were significantly increased, by 224%, compared to WT mice (59.4 ± 2.8 ng/dl), similar to previous reports (Fig. 2B) (20, 25, 48–50). Again, male NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice had TT_3 levels (79.1 ± 6.1 ng/dl) similar to those of WT mice (Fig. 2B). In females, NCoR$^{\Delta ID/\Delta ID}$ mice had TT_3 levels (40.4 ± 2.1 ng/dl) similar to those of WT mice (55.3 ± 4.9 ng/dl) (Fig. 2B). TT_3 levels in female *Src-1*$^{-/-}$ mice (91.1 ± 7.9 ng/dl) were significantly increased, by 165% (20). Female NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice had TT_3 levels (70.3 ± 5.2 ng/ml) similar to those of WT mice (Fig. 2B). Whereas TT_3 levels in male and female NCoR$^{\Delta ID/\Delta ID}$ mice in this study were not significantly lower than those of WT mice, as we have previously reported, the TT_3 assay used in this study is the same as we have used previously (24). We believe this difference is due to the back-

ground of the mice used in this study. Importantly, however, replacement of NCoR1 with the NCoRΔID allele in the *Src-1*$^{-/-}$ mouse normalizes TT_3 levels.

Other groups have demonstrated a large increase in circulating TSH in male *Src-1*$^{-/-}$ mice, and here, we show a significant 271% increase (526.9 ± 35.2 pg/ml) in circulating TSH compared to WT mice (194.2 ± 22.9 pg/ml), while the TSH level was normal in NCoR$^{\Delta ID/\Delta ID}$ mice (224.9 ± 38.0 pg/ml) (Fig. 2C). Male NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have normal TSH levels (254.5 ± 38.8 pg/ml) compared to both WT and NCoR$^{\Delta ID/\Delta ID}$ mice. In female mice, circulating TSH levels in NCoR$^{\Delta ID/\Delta ID}$ mice (21.9 ± 6.3 pg/ml) were 70.1% lower than in WT mice (73.3 ± 16.5 pg/ml) as opposed to the 354% increase in TSH levels in *Src-1*$^{-/-}$ mice (259.8 ± 46.7 pg/ml) (Fig. 2C) (20). NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ female mice had normal plasma TSH levels (45.3 ± 9.6 pg/ml) similar to WT levels.

In order to analyze the complete HPT axis, we looked at pituitary function in male and female mice. Gene expression analysis in the pituitaries of male mice revealed normal expression of the TSH subunits *Tsha* (*Cga*) and *Tshb*, the type II deiodinase (*Dio2*), and the TRH receptor (*Trhr1*) across all genotypes (Fig. 2D). Gene expression in female mouse pituitaries of the TSH subunits *Tsha* (*Cga*) and *Tshb* remained unchanged between genotypes (Fig. 2E). Additionally, we tested the expression of luteinizing hormone (*Lhb*) and follicle-stimulating hormone (*Fshb*), and no differences were detected between genotypes (Fig. 2E). Additionally, we analyzed the mRNA expression from microdissected PVHs of male mice and found that the expression of *Trh* was also normal across genotypes, including *Src-1*$^{-/-}$ mice, despite their increased TH levels (Fig. 2F). Additionally, expression of corticotropin-releasing hormone (*Crh*), carboxypeptidase E (*Cpe*), prohormone convertase 1/3 (*Pc1/3*), and melanocortin 4 receptor (*Mc4r*) was the same in all genotypes (Fig. 2F).

Circulating TSH responds normally to changes in TH levels in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice. By expressing the NCoRΔID allele in male and female *Src-1*$^{-/-}$ mice, we were able to normalize TT_4, TT_3, and TSH levels, suggesting that the resistance present in *Src-1*$^{-/-}$ mice is due to unopposed NCoR1. To investigate the dynamic response of the HPT axis to changes in circulating TH levels, we performed a TSH suppression test on all groups of male mice (Fig. 3) (43). To accomplish this, mice were placed on a LoI/PTU diet to render the mice hypothyroid (PTU mice). After 21 days, the mice had undetectable TT_4 levels (data not shown) and elevated plasma TSH concentrations greater than 24,000 pg/ml (Fig. 3A). The mice were then supplemented with daily injections of T_3 for the next 3 weeks in increasing doses (0.2, 0.5, and 1.0 μg/100 g BW/day), and the fall in TSH levels was measured weekly. As previously demonstrated, the fall in plasma TSH levels in *Src-1*$^{-/-}$ mice is resistant to increasing doses of T_3 compared to WT mice and also, as shown here, compared to NCoR$^{\Delta ID/\Delta ID}$ mice (2-way RM ANOVA; $P < 0.0001$) (Fig. 3A and C) (20, 51). To further validate the sensitivity of NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice to T_3, we averaged the area under the curve (AUC) for each mouse per genotype and found a significant difference between *Src-1*$^{-/-}$ mice (AUC, 0.65 ± 0.08) and the other genotypes of mice, WT (AUC, 0.10 ± 0.06), NCoR$^{\Delta ID/\Delta ID}$ (AUC, 0.03 ± 0.01), and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (AUC, 0.28 ± 0.12) ($P < 0.05$) (Fig. 3B). Strikingly, NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice were as sensitive to T_3 as WT mice, which carried through to the largest dose of 1.0 μg/100 g BW/day (*Src-1*$^{-/-}$ versus all, $P < 0.05$) (Fig. 3C). We also measured TT_3 levels in all the mice following the largest dose of T_3 (1.0 μg/100 g BW/day) and found that TT_3 levels in *Src-1*$^{-/-}$ mice were significantly higher than in all other genotypes (*Src-1*$^{-/-}$ mice versus all, $P < 0.01$) (Fig. 3D).

Next, we determined the response of pituitary gene expression to changes in circulating TH levels. Previous reports have found that expression of *Tshb* is resistant to T_3 in *Src-1*$^{-/-}$ mice compared to WT mice, whereas NCoR$^{\Delta ID/\Delta ID}$ mice are normal (24, 52). Mice of all genotypes were sacrificed after the final dose of T_3 (1.0 μg/100 g BW/day; PTU + T_3 mice) and compared to mice sacrificed after 21 days on a LoI/PTU diet (PTU mice) and control mice (chow mice). Expression of the TSH subunits *Tshb* and *Tsha* (*Cga*) increased following 21 days on a LoI/PTU diet and decreased with T_3 in all genotypes (Fig. 3E). *Tshb* expression in PTU-treated mice versus chow mice increased as much in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice as in WT and NCoR$^{\Delta ID/\Delta ID}$ mice (23-fold versus 22- and 25-fold, respectively, compared with *Src-1*$^{-/-}$ mice at 12-fold). The repression of *Tshb* was also resistant to T_3 in *Src-1*$^{-/-}$ mice, as expression decreased by only 66-fold between PTU and PTU + T_3 mice compared with the rest of the genotypes, which decreased by at least 135-fold ($P < 0.01$) (Fig. 3E, inset). This resistance in *Src-1*$^{-/-}$ mice is even clearer when one considers the elevation in total T_3 levels in the animals after T_3 administration. Expression of *Dio2*, *Trhr1*, and *Gh* all responded as expected to PTU and T_3, with no difference between genotypes, except for *Dio2*, where WT mice had a greater response to PTU than the other genotypes ($P < 0.01$) (Fig. 3E).

The hypothalamus and heart are normal in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice. To explore the sensitivity of the hypothalamus and heart to TH, we examined gene expression in mice after the final dose of T_3 (1.0 μg/100 g BW/day; PTU + T_3 mice) and compared it to that in mice sacrificed after 21 days on a LoI/PTU diet (PTU mice) and control mice (chow mice). In the PVH, expression of *Trh* was significantly increased in all genotypes after 21 days on a LoI/PTU diet (Fig. 4A). Following treatment with T_3, *Trh* levels were suppressed equally in all genotypes.

NCoR$^{\Delta ID/\Delta ID}$ mice show normal sensitivity to T_3 but slight derepression of *Hcn2* and *Serca-2* on a LoI/PTU diet, whereas *Src-1*$^{-/-}$ mice have normal expression of *Serca-2* and the α and β myosin heavy-chain genes (*Myh6* and *Myh7*, respectively) (24, 52). Here, we found no differences in the expression of *Hcn2*, *Serca-2*, *Myh6*, and *Myh7* between genotypes in response to a chow diet, to a LoI/PTU diet, or to T_3, suggesting that T_3 action in the heart does not require NCoR1 or SRC-1 (Fig. 4B).

Expression of NCoRΔID in *Src-1*$^{-/-}$ mice reestablishes hepatic T_3 sensitivity. Unlike in the heart, mice expressing NCoRΔID have increased hepatic T_3 sensitivity, which is characterized by increased expression of T_3 target genes, such as the thyroid hormone-responsive SPOT 14 gene (*Thrsp*), the type I deiodinase gene (*Dio1*), the glycerol-3-phosphate dehydrogenase 2 gene (*Gpd2*), and the malic enzyme gene (*Mod1*), regardless of TH levels, and suggests that enhanced ligand-dependent activation occurs in the absence of NCoR1 (24). In contrast, *Thrsp* expression in *Src-1*$^{-/-}$ mice has previously been reported to be unresponsive to T_3 (52). We therefore analyzed the expression of several hepatic genes across genotypes and found that *Thrsp* fails to respond to T_3 administration in *Src-1*$^{-/-}$ mice (PTU+T_3 versus PTU, 1.1-fold increase) compared to WT mice (PTU + T_3 versus PTU, 4.6-fold increase) (Fig. 5A). Additionally, expression of *Gpd2* and *Mod1* is blunted in response to T_3 in *Src-1*$^{-/-}$ mice

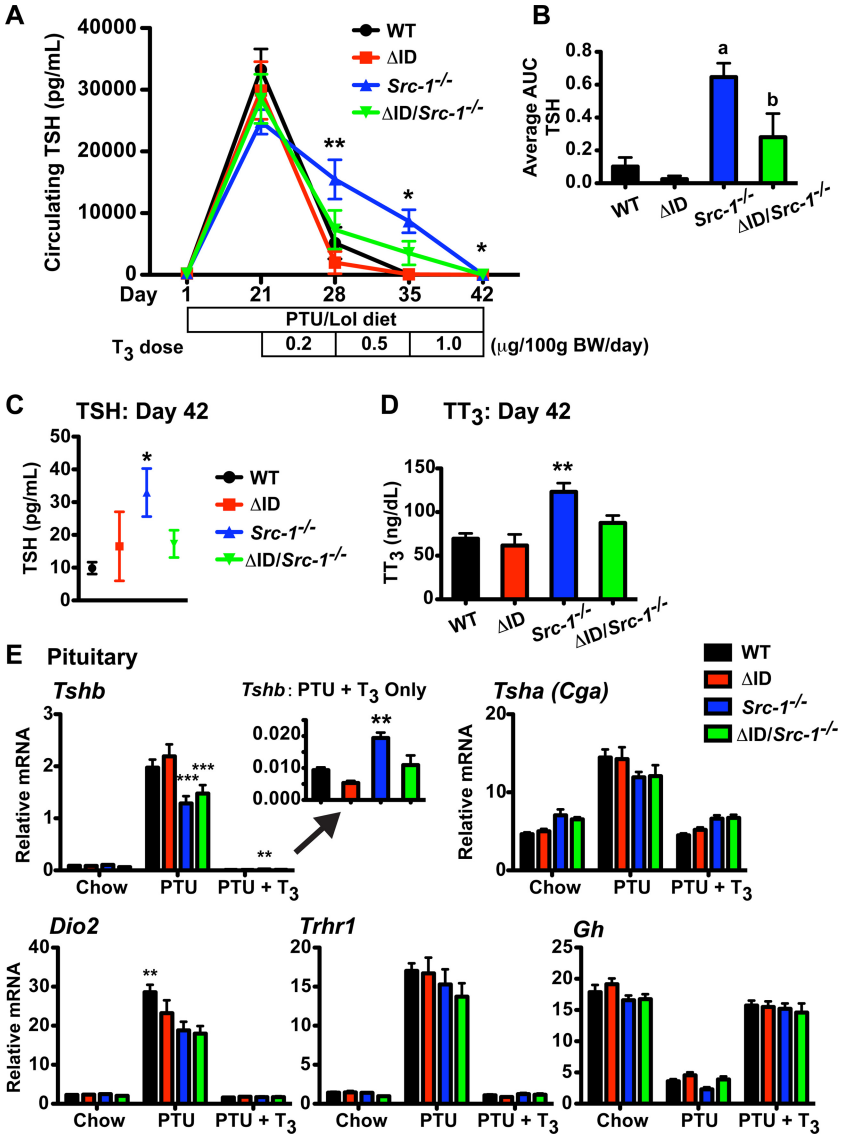

FIG 3 NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice suppress plasma TSH similarly to WT mice in response to T_3. (A) The plasma TSH concentration was repeatedly measured in WT, NCoR$^{\Delta ID/\Delta ID}$ (ΔID), *Src-1*$^{-/-}$, and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (ΔID/*Src-1*$^{-/-}$) male mice after 21 days of a LoI/PTU diet and consecutive 7-day treatments with increasing concentrations of T_3 (0.2, 0.5, and 1.0 μg/100 g BW/day; $n = 7$ or 8). The data are presented as means ± SEM and were analyzed by repeated-measures 2-way ANOVA with the Bonferroni *post hoc* test. *, $P < 0.05$ for *Src-1*$^{-/-}$ versus all; **, $P < 0.01$ for *Src-1*$^{-/-}$ versus all. (B) AUC of each mouse averaged by genotype ($n = 7$ or 8). The data are presented as means and SEM and were analyzed by 1-way ANOVA with Tukey's multiple-comparison *post hoc* test. a, $P < 0.01$ for *Src-1*$^{-/-}$ versus WT and ΔID; b, $P < 0.05$ for *Src-1*$^{-/-}$ versus ΔID/*Src-1*$^{-/-}$. (C and D) Circulating TSH levels and TT_3 levels at day 42 following 7 days of treatment with the largest dose of T_3. (C) $n = 7$ or 8. The data are presented as means ± SEM and were analyzed by repeated-measures 2-way ANOVA with a Bonferroni *post hoc* test. *, $P < 0.05$ for *Src-1*$^{-/-}$ versus all. (D) $n = 7$ or 8. The data are presented as means and SEM and were analyzed by 1-way ANOVA with Tukey's multiple-comparison *post hoc* test. **, $P < 0.01$ for *Src-1*$^{-/-}$ versus all. (E) Analysis of mRNA expression of TH-responsive targets in the pituitary by qPCR in the indicated groups. Expression of *Tshb*, *Tsha*, *Dio2*, *Trhr1*, and *Gh* was normalized to 18S rRNA ($n = 7$ to 12). The inset highlights *Tshb* mRNA expression at day 42 following 7 days of treatment with the largest dose of T_3. The data are presented as means and SEM and were analyzed by 2-way ANOVA with a Bonferroni *post hoc* test. **, $P < 0.01$ versus all; ***, $P < 0.001$ versus WT and ΔID.

(PTU + T_3 versus PTU, 2.6-fold and 2.1-fold increases, respectively) compared to WT mice (PTU + T_3 versus PTU, 6.7-fold and 4.7-fold increases, respectively). Replacing NCoR1 with NCoRΔID in *Src-1*$^{-/-}$ mice reestablishes the sensitivity of these genes' expression to T_3 both in the context of the fold increase (PTU + T_3 versus PTU, 2.6-fold increase [*Thrsp*], 3.5-fold increase [*Gpd2*], and 3.8-fold increase [*Mod1*]) (Fig. 5A) and overall expression. Interestingly, expression of *Dio1*, another positive TH target, is normal across genotypes (Fig. 5B). Furthermore, expression of negative TH targets, such as the glutathione *S*-transferase A2 gene (*Gsta2*) and the F-box protein 21 gene (*Fbxo21*), is unaffected by removal of normal NCoR1, SRC-1, or both (Fig. 5C). Thus, specific T_3-positive targets in the liver are regulated by the amount of NCoR1 or SRC-1 present.

To understand how the loss of NCoR1 allows SRC-1-dependent positive TH targets to regain sensitivity in the absence of SRC-1, we looked at the expression of other coregulators. Expression levels were similar between genotypes for *Ncor1*, SMRT

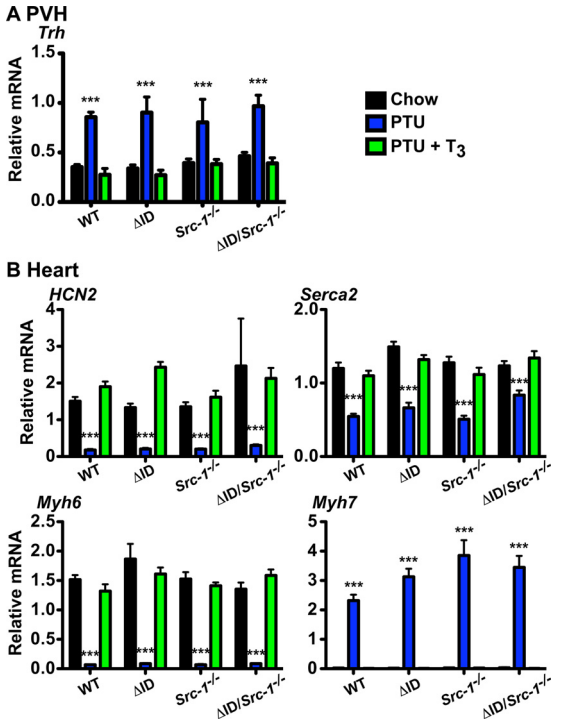

FIG 4 Gene expression in the PVH and the heart. mRNA levels were measured by qPCR in WT, NCoR$^{\Delta ID/\Delta ID}$ (ΔID), *Src-1*$^{-/-}$, and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (ΔID/*Src-1*$^{-/-}$) mice following 21 days of a LoI/PTU diet (PTU) and a LoI/PTU diet with T_3 replacement for 21 days with increasing doses of T_3 (PTU + T_3) compared with the control (Chow). (A) *Trh* mRNA was measured in the PVH microdissected from mouse brain and was normalized to expression of 18S rRNA. (B) mRNA expression of known positive and negative TH targets in the heart. *HCN2*, *Serca2*, *Myh6*, and *Myh7* were normalized to expression of cyclophilin. (A and B) $n = 5$ to 7. The data are presented as means and SEM and were analyzed by 2-way ANOVA with a Bonferroni *post hoc* test. ***, $P < 0.001$ versus chow and PTU + T_3.

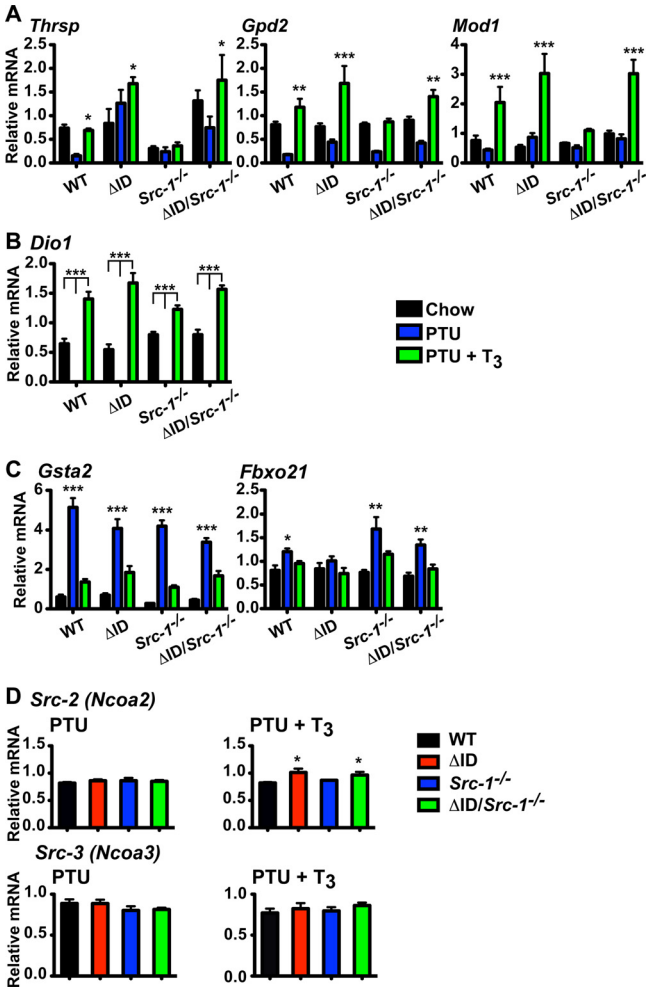

FIG 5 Loss of TR-NCoR1 interaction in *Src-1*$^{-/-}$ mice reestablishes the T_3 response in some positive TH targets in the liver. mRNA levels were measured by qPCR in livers from WT, NCoR$^{\Delta ID/\Delta ID}$ (ΔID), *Src-1*$^{-/-}$, and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (ΔID/*Src-1*$^{-/-}$) mice following 21 days of a LoI/PTU diet (PTU) and a LoI/PTU diet with T_3 replacement for 21 days with increasing doses of T_3 (PTU + T_3) compared with the control (Chow). (A to C) All genes were normalized to 18S rRNA ($n = 6$ to 13). The data are presented as means and SEM and were analyzed by 2-way ANOVA with a Bonferroni *post hoc* test. (A) T_3 response in positive TH targets *Thrsp*, *Gpd2*, and *Mod1* was reestablished with the addition of NCoRΔID. *, $P < 0.05$ versus PTU; **, $P < 0.01$ versus PTU; ***, $P < 0.001$ versus PTU. (B) Positive TH target type I deiodinase (*Dio1*) is not affected by changes in NCoR1 or SRC-1. ***, $P < 0.001$. (C) Expression of negative TH targets *Gsta2* and *Fbxo21*. *, $P < 0.05$ versus chow and PTU + T_3; **, $P < 0.01$ versus chow and PTU + T_3; ***, $P < 0.001$ versus chow and PTU + T_3. (D) Responses of SRC-2 (*Ncoa2*) and SRC-3 (*Ncoa3*) mRNAs to changes in TH levels under PTU and PTU + T_3 conditions. The data were normalized to 18S rRNA ($n = 6$ to 13). The data are presented as means and SEM and were analyzed by 1-way ANOVA with Tukey's multiple-comparison *post hoc* test. *, $P < 0.05$ versus chow.

(*Ncor2*), and SRC-1 (*Ncoa1*) under chow, PTU, and PTU + T_3 conditions (data not shown). However, we did find a small but significant increase in expression of SRC-2 (*Ncoa2*) in response to T_3 in NCoR$^{\Delta ID/\Delta ID}$ (1.23-fold increase; $P < 0.05$) and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (1.18-fold increase; $P < 0.05$) mice compared to WT mice, which suggests increased availability of SRC-2 in the presence of T_3 and in the absence of NCoR1 and/or SRC-1 (Fig. 5D). In comparison, hepatic SRC-3 (*Ncoa3*) mRNA levels in mice do not change between genotypes under PTU or PTU + T_3 conditions (Fig. 5D).

Given the small but elevated increase in *Src-2* mRNA levels under PTU + T_3 conditions, we next examined protein levels of coregulators across all genotypes. Western analysis of NCoR1 confirmed the presence of NCoRΔID in NCoR$^{\Delta ID/\Delta ID}$ and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice; however, the levels of NCoR1 and/or NCorΔID did not change among genotypes (Fig. 6A). We also confirmed the absence of SRC-1 in *Src-1*$^{-/-}$ and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice, but the levels of SRC-1 between WT and NCoR$^{\Delta ID/\Delta ID}$ mice were not different (Fig. 6A). Using an antibody that recognizes SMRT, NCoR1, and NCoRΔID, we assessed the levels of SMRT and found no difference between genotypes that lacked NCoR1, where the remaining 270-kDa band is SMRT (Fig. 6A). Upon examination of SRC-2 protein levels in all the genotypes under chow and PTU + T3 conditions, we found no significant difference in SRC-2 protein levels between genotypes under chow-fed conditions (Fig. 6B). However, under PTU + T_3 conditions, SRC-2 protein levels trend toward increased levels in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice compared to WT and *Src-1*$^{-/-}$ mice when quantified and normalized to RNA polymerase II levels (1-way ANOVA, $P < 0.05$; Tukey's multiple-comparison *post hoc* test, NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ versus WT, $P = 0.092$, and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ versus *Src-1*$^{-/-}$, $P = 0.093$) (Fig. 6B). We

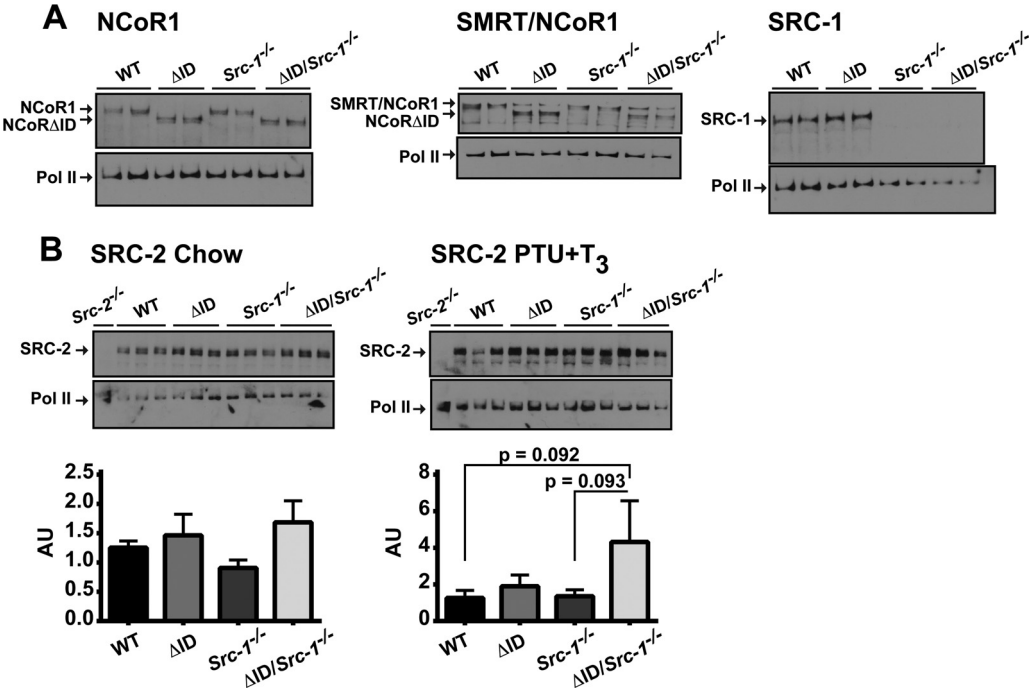

FIG 6 Under PTU + T_3 conditions, SRC-2 protein levels trend toward increased levels in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice compared to WT and *Src-1*$^{-/-}$ mice. (A) Whole-cell protein extracts were isolated from the livers of male WT, NCoR$^{\Delta ID/\Delta ID}$ (ΔID), *Src-1*$^{-/-}$, and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (ΔID/*Src-1*$^{-/-}$) mice and subjected to Western analysis with anti-NCoR1, SMRT/NCoR1, and SRC-1. The blots were stripped and reprobed with anti-RNA polymerase II (Pol II). (B) Western analysis of SRC-2 protein levels in the livers of all genotypes under chow and PTU + T_3 conditions compared to RNA polymerase II. The blots were scanned and quantified with ImageJ and normalized to RNA polymerase II levels (1-way ANOVA, $P < 0.05$; Tukey's multiple-comparison *post hoc* test, ΔID/*Src-1*$^{-/-}$ versus WT, $P = 0.092$, and ΔID/*Src-1*$^{-/-}$ versus *Src-1*$^{-/-}$, $P = 0.093$). Extracts from *Src-2*$^{-/-}$ mice were used to confirm SRC-2 antibody specificity.

confirmed the specificity of the SRC-2 antibody by examining SRC-2 levels in WT and *Src-2*$^{-/-}$ mice (the livers were a gift from Bert O'Malley and Brian York).

NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have increased recruitment of SRC-2 to the promoters of T_3-sensitive genes. Mice null for SRC-2 have normal thyroid function (53). However, when mice are null for both SRC-1 and SRC-2, T_4, T_3, and TSH levels are even higher than in *Src-1*$^{-/-}$ mice, suggesting a partial compensatory role for SRC-2 in the regulation of the thyroid axis (53). Because of this and the trend of increased mRNA and protein levels of SRC-2 in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice under PTU + T_3 conditions, we asked if SRC-2 could serve as a substitute for SRC-1 in T_3 gene activation. To confirm the performance of our SRC-2 antibody in ChIP, we analyzed the previously measured SRC-2 target *Abcb11*, which also binds TR (47, 54). *Abcb11* is also a T_3 target gene (Fig. 7A). Surprisingly, *Abcb11* mRNA levels do not respond to T_3 in *Src-1*$^{-/-}$ mice (Fig. 7A). Using ChIP, we show that SRC-2 is present at the *Abcb11* promoter site compared to the IgG control in both WT and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice given PTU + T_3 but not in *Src-1*$^{-/-}$ mice, where *Abcb11* expression is also blunted (Fig. 7B) (47).

To validate the role of SRC-2 in the reacquired T_3 sensitivity of NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice, we performed ChIP for SRC-2 and examined its recruitment to a TRβ binding site upstream of the *Thrsp* promoter, to three intragenic TRβ binding sites within the first intron of *Gpd2*, and to a control site on the β-globin gene that does not bind TR, all previously described (47). All TRβ binding sites were identified using *in vivo* hepatic ChAP-seq in mice expressing a biotinylated-TRβ1 isoform in the liver only (47). Strikingly, there is increased recruitment of SRC-2 to the TRβ binding sites of these genes in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice compared to the IgG control (Fig. 7C). In contrast, SRC-2 is not recruited in WT or *Src-1*$^{-/-}$ livers, suggesting that the presence of NCoR1 prevents its recruitment. Furthermore, SRC-2 is not recruited to the β-globin gene (*Hbb1*) (Fig. 7C). We also examined the recruitment of SRC-1 to these sites and found that NCoR1 allows recruitment of SRC-1 to the *Abcb11* and *Thrsp* promoter in WT mice compared to IgG, but clearly, this is lost in mice that lack SRC-1 (Fig. 7D). SRC-1 is not recruited to TRβ binding sites on the *Gpd2* and β-globin genes. Taken together, these data suggest that SRC-2 can promote T_3 sensitivity in mice null for SRC-1 and expressing NCoRΔID.

DISCUSSION

The HPT axis is regulated through a feedback mechanism by which the hypothalamus releases TRH to increase TSH production in the pituitary, which stimulates TH release from the thyroid. In turn, TRH and TSH are suppressed by circulating TH (2). Recent studies by our group and others have shown that the circulating levels of TH and TSH are established by an interaction between TRβ isoforms and NCoR1 (24, 25, 40). Indeed, the correction of RTH by expressing NCoRΔID in *Thrb*^{PV} mutant mice emphasizes the role of corepressors in establishing TH levels and the set point of the axis (40). Mice expressing a mutant form of *Thrb* that is unable to bind the coactivator SRC-1 are similar to this *Thrb*^{PV} mutant, with high TH and TSH levels and increased

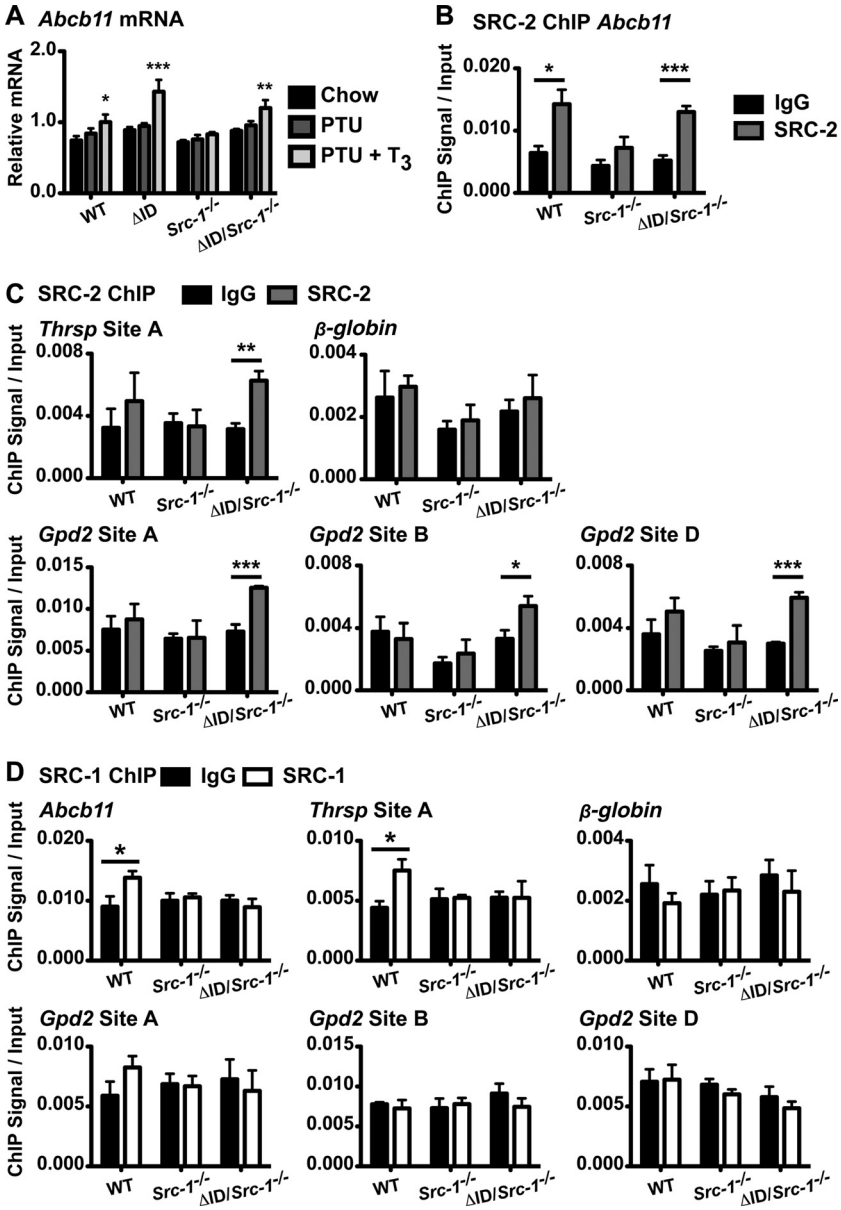

FIG 7 SRC-2 binds to T_3 target gene promoters in the absence of SRC-1 and NCoR1. (A) In livers from male WT, $NCoR^{\Delta ID/\Delta ID}$ (ΔID), *Src-1*$^{-/-}$, and $NCoR^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ (ΔID/*Src-1*$^{-/-}$) mice, mRNA levels were measured by qPCR following 21 days of a LoI/PTU diet (PTU) and a LoI/PTU diet with T_3 replacement for 21 days with increasing doses of T_3 (PTU + T_3) compared with control (Chow). Expression of the SRC-2 target gene *Abcb11* was normalized to the expression of 18S rRNA ($n = 6$ to 13). The data are presented as means and SEM and were analyzed by 2-way ANOVA with a Bonferroni *post hoc* test. Chow versus PTU + T_3, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (B) SRC-2 ChIP was validated by ChIP-qPCR analysis using an anti-SRC-2 antibody (SRC-2) versus IgG for the proximal promoter of the SRC-2 target gene *Abcb11* on the chromatin from livers of WT, *Src-1*$^{-/-}$, and ΔID/*Src-1*$^{-/-}$ mice under PTU + T_3 conditions. (C) Using the same chromatin, SRC-2 ChIP-qPCR analysis was performed for a TRβ binding site (site A) upstream of the *Thrsp* promoter, three intragenic sites (sites A, B, and D) within the first intron of the T_3 target gene *Gpd2*, and a site on the β-globin gene (*Hbb1*) that does not bind TRβ. (D) ChIP-qPCR analysis was performed using an anti-SRC-1 antibody (SRC-1) or IgG on the same chromatin from panels B and C for the proximal promoter of the SRC-2 target gene *Abcb11*; *Thrsp* site A; *Gpd2* sites A, B, and D; and a site in the β-globin gene (*Hbb1*) that does not bind TRβ. (B to D) $n = 4$. The results are reported as fold enrichment (ChIP signal) normalized to the DNA input. The data are presented as means and SEM and were analyzed by unpaired *t* test for antibody versus IgG. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

pituitary *Tshb* mRNA levels (21). The proposed role of the coactivator SRC-1 and the corepressor NCoR1 in regulating TSH repression and establishing T_3 sensitivity led us to hypothesize that the balance between coactivators and corepressors is important in determining the set point of the thyroid axis. Thus, we sought to express the mutant NCoRΔID allele in mice null for SRC-1 to create a mouse that does not have functional NCoR1 or SRC-1 in the context of TH signaling. Here, we show that these $NCoR^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have normal T_4, T_3, and TSH levels, demonstrating that the replacement of NCoR1 with NCoRΔID alleviated the functional loss of SRC-1 and reestablished a balance between corepressors and coactivators, which is necessary to set basal TH and TSH levels (40).

In the hypothyroid state, TSH levels are increased many hun-

dredfold, and the ability of various doses of T_3 to reduce TSH levels is a superb indicator of thyrotroph T_3 sensitivity (43). Previous work has demonstrated that mice lacking a functional TRβ–SRC-1 interaction or null for SRC-1 are unable to suppress TSH levels to the same degree as WT mice (20, 21). In our studies, we saw the same resistance to T_3 in *Src-1*$^{-/-}$ mice. Interestingly, the level of central resistance in *Src-1*$^{-/-}$ mice is more apparent when serum TSH rather than expression of the known T_3 target genes *Tshb* and *Trh* is used as a readout. This raises the possibility that NCoR1 and SRC-1 are exerting their effects on other target genes that control TSH production translationally and posttranslationally. It is also important to stress that *Src-1*$^{-/-}$ mice have levels of *Tshb* and *Trh* mRNA expression similar to those of WT mice, which are inappropriately high considering the elevated T_4 and T_3 levels in the basal state. Thus, *Src-1*$^{-/-}$ mice are resistant to TH. The normalization of TH levels in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice accompanied by now appropriate *Tshb* and *Trh* mRNA levels that are equivalent to those of the WT control animals demonstrates the need for balance between corepressors and coactivators in their regulation.

When NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice were given increasing doses of T_3 after the induction of hypothyroidism, circulating TSH levels were suppressed to a degree similar to that in WT mice as opposed to the increased TSH levels in *Src-1*$^{-/-}$ mice. This correction was also seen in the context of pituitary gene expression, as *Tshb* mRNA levels fell with T_3 administration equally in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ and WT mice compared to the elevated *Tshb* mRNA levels in *Src-1*$^{-/-}$ mice. This is particularly striking considering that *Src-1*$^{-/-}$ mice had elevated TT_3 levels following the 3 weeks of T_3 therapy, demonstrating that these animals are indeed resistant to thyroid hormone at the level of gene expression. At the same time NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have normal sensitivity consistent with SRC-1 and NCoR1 having a direct role in the regulation of the *Tshb* gene and demonstrating that the balance of coregulators is important for interpreting the dynamic changes in thyroid hormone levels.

At the level of the hypothalamus, *Trh* and other genes involved in the processing of the TRH peptide were relatively unaffected by the removal of functional NCoR1, SRC-1, or both. However, as discussed above, in the context of the elevated thyroid hormone levels, *Src-1*$^{-/-}$ mice have inappropriately elevated *Trh* mRNA levels whereas NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice have normal *Trh* mRNA and TT_3 levels. We know that TRH is important to the set point of the HPT axis, as mice null for TRH have decreased T_4 levels, slightly increased TSH levels, and reduced TSH bioactivity (55). The results from this study suggest that NCoR1 and SRC-1 are important to the regulation of *Trh* in the hypothalamus and also at the level of the pituitary (24, 25). This is indeed consistent with our recent findings, demonstrating that expression of NCoRΔID in the pituitary alone can alter TSH regulation by T_3 (25).

In addition to the pituitary, *Src-1*$^{-/-}$ mice are also unable to maintain normal levels of expression of TH targets in the liver despite the presence of increased circulating levels of TH. (Fig. 8A to C shows a model). In contrast, we have previously demonstrated that NCoR1 is critical for the T_3 sensitivity of hepatic TR targets, such as *Dio1*, *Gpd2*, *Mod1*, and *Thrsp*, so that without available NCoR1, there is increased response to a set amount of T_3 (23, 24). These data strongly suggest that the hepatic TRs may recruit both SRC-1 and NCoR1 in the presence of T_3 and that the

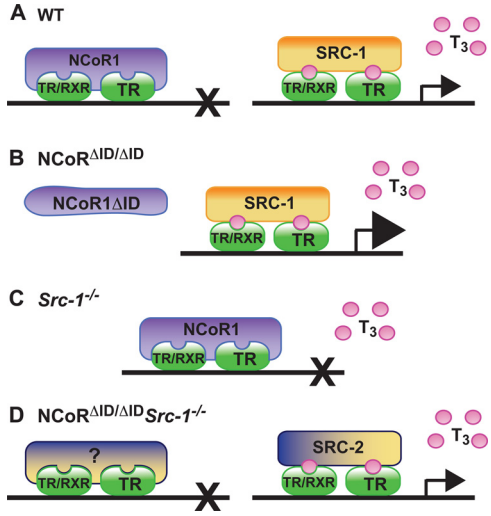

FIG 8 In peripheral tissues, the balance of corepressors and coactivators is most important in determining T_3 sensitivity in the liver. (A) On classic positive T_3 targets in WT mice, NCoR1 is recruited to nuclear receptor heterodimers (TR/RXR) or homodimers (TR/TR) in the absence of ligand, and transcription is repressed. In the presence of T_3, NCoR1 is released, and SRC-1 is recruited to mediate transcriptional activity. (B) When NCoR1 is not readily available, as exemplified in NCoR$^{\Delta ID/\Delta ID}$ mice, there is increased response to T_3, suggesting that recruitment of SRC-1 is also increased. (C) In *Src-1*$^{-/-}$ mice, when SRC-1 is absent, genes do not respond to T_3, which is consistent with decreased sensitivity and suggests prioritized recruitment of NCoR1. (D) Replacement of NCoR1 with NCoRΔID in *Src-1*$^{-/-}$ mice reestablishes hepatic T_3 sensitivity. Our work here describes the increased binding of SRC-2 in the liver in the absence of both SRC-1 and NCoR1 under increased T_3 conditions, suggesting a compensatory role for SRC-2. NCoR1, nuclear corepressor 1; NCoR$^{\Delta ID/\Delta ID}$, mice expressing NCoRΔID; NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$, mice expressing NCoRΔID and also null for SRC-1; RXR, retinoid X receptor; SRC-1, steroid receptor coactivator 1 (Ncoa1); *Src-1*$^{-/-}$, SRC-1 knockout mice; SRC-2, steroid receptor coactivator 2 (Ncoa2); T_3, triiodothryonine (thyroid hormone); TR, thyroid hormone receptor.

absence of NCoR1 prioritizes the recruitment of SRC-1, which then leads to increased gene expression (24). Here, we demonstrate that *Src-1*$^{-/-}$ mice fail to appropriately activate *Thrsp*, *Gpd2*, and *Mod1* in the presence of increasing amounts of T_3, consistent with decreased sensitivity. The replacement of NCoR1 with NCoRΔID in *Src-1*$^{-/-}$ mice reestablishes hepatic T_3 sensitivity, which highlights the fact that the balance of NCoR1 and SRC-1 is critical. Moreover, once NCoR1 action is removed in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice, target genes regain their T_3 sensitivity, possibly by recruiting another coactivator, such as SRC-2, which has been shown to have a direct role in the regulation of hepatic genes, including *Thrsp*, *Gpd2*, and *Mod1* (56). Therefore, we measured the expression of *Src-2* mRNA in our mice and found that expression was higher in NCoR$^{\Delta ID/\Delta ID}$ and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice in response to T_3. Furthermore, measurement of SRC-2 protein levels highlighted a trend toward increased SRC-2 in NCoR$^{\Delta ID/\Delta ID}$ and NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice receiving T_3. Finally, and most importantly, there was increased enrichment of SRC-2 in TR-binding regions of T_3-sensitive genes in NCoR$^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice in the presence of T_3. Thus, in the absence of NCoR1 and SRC-1, positive regulation of key hepatic TH targets is mediated by SRC-2. Although we cannot rule out a compensatory role for SRC-3 or other coregulators when NCoR1 and SRC-1 are absent, we did not see changes in *Src-3* mRNA expression between genotypes with T_3. Moreover, previous studies have detected little

overlap in the roles of SRC-1 and SRC-3 in hepatic metabolism (56). Interestingly, NCoR1 and SRC-1 do not appear crucial to the regulation of negative TH targets in the liver, as expression of *Gsta2* and *Fbxo21* responded similarly in all genotypes. Clearly, the roles of NCoR1 and SRC-1 in gene regulation appear to vary by tissue and gene target. Further work will be required to understand this specificity *in vivo*.

While NCoR1 and SRC-1 have been shown to play critical roles *in vivo* in thyroid hormone and nuclear receptor action, their combinatorial role in ligand activation by TH has not been shown previously. Clearly, the set point of the HPT axis can be determined by a balance between corepressors and coactivators, suggesting that genetic differences in disease states could be modified depending upon the relative expression levels of these coregulators (57). The normalization of TSH secretion in $NCoR^{\Delta ID/\Delta ID}$ *Src-1*$^{-/-}$ mice demonstrates that other coregulators, such SRC-2, can substitute for NCoR1 and SRC-1 *in vivo* but that NCoR1 and SRC-1 are likely the primary drivers of the set point. This balance of coregulators is also crucial to the regulation of T_3 action in the liver and supports the notion that the primary role of corepressors and coactivators is to regulate the hormone sensitivity of nuclear receptors rather than to play a unique role in their ligand-independent or -dependent action (Fig. 8). In the presence of NCoRΔID, increased SRC-2 recruitment allows T_3 sensitivity of genes in the absence of SRC-1. Thus, therapeutics targeting coregulators like NCoR1 and SRC-1 in a tissue-specific fashion could potentially be used to regulate T_3 sensitivity to treat metabolic disease. Whether the effects on ligand sensitivity could be limited to T_3 would need to be determined.

ACKNOWLEDGMENTS

We thank Bert O'Malley for the *Src-1*$^{-/-}$ mice and Bert O'Malley and Brian York for the *Src-2*$^{-/-}$ mouse liver samples. We thank Benjamin Rodwin, Nicholas F. O'Neill, and Paul D. Same for technical assistance.

This work was supported by National Institutes of Health grants DK078090 and DK056123 to A.N.H. and DK091403 and T3207516 to K.R.V.

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