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ACAA2 is a Ligand-dependent Coactivator for Thyroid Hormone Receptor β1

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Abstract

Thyroid hormones (THs) play a critical role in the metabolic phenotype of the heart; and most of the effects involve transcriptional regulation via thyroid hormone receptors (TRs). TRs ability to form combinatorial complexes with an array of partners accounts for TRs physiological flexibility in modulating gene expression. To identify proteins that associate with TRβ1 in the heart we performed a pull-down assay on cardiac tissue using GST-TRβ1 as bait and identified the bound proteins by LC MS/MS. ACAA2, a mitochondrial thiolase enzyme, was identified as a novel interacting protein. We confirmed ACAA2 localized to the nucleus and using a luciferase reporter assay showed ACAA2 acted as a TH-dependent coactivator for TRβ1. ACAA2 showed an ability to bind to TR recognition sequences but did not alter TRβ1 DNA binding ability. Thus, ACAA2 as a novel TRβ1 associating protein opens a new paradigm to understanding how TH/TRs may be manipulated by energetic pathway molecules.

1. Introduction

Thyroid hormone (TH) imbalance can have profound effects on cardiac function and is mediated by genomic and nongenomic mechanisms [1]. Most TH effects occur via its TH receptors (TRs) modulating gene transcription. Additionally, tissue-specific modulation of TH, TRs and the transcription apparatus enables enormous fluidity in TH signaling [2]. The transcription apparatus involves recruitment of cofactors to TRs, determining the functional state of the transcriptional complex with tissue specific expression profiles providing an additional control level. Two genes, TR alpha (TRα) and TR beta (TRβ), encode the numerous TR isoforms. In the heart, TRα1 and TRβ1 represent the predominant TRs

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expressed at 70% and 30%, respectively in the heart [3]. Therefore, most THs cardiac effects occur through the TRα1 isoform [4]. However, T3-induced cardiac hypertrophy growth is suggested to occur via TRβ1 signaling [3, 5] as a cardiac-specific TRβ (337T) dominant-negative mouse model showed a significant hypothyroid phenotype [6].

The minimal basal TR transcription factors are well established; however, the multiplicity of cofactors involved in the synergistic or antagonistic regulation of TR transcription still requires elucidation. The cofactors bind directly or indirectly to the receptor protein communicating regulatory cues effecting transcription. In this study we sought to identify cardiac TRβ1 interacting proteins to further understand the molecular mechanisms of TR signaling in the heart. Utilizing GST-tagged TRβ1 as bait, we identified acetyl-Coenzyme A acyltransferase 2 (ACAA2), an enzyme involved in medium-chain fatty acid metabolism as a potential binding partner.

2. Experimental methods

2.1. Ethics Statement

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health [7]. Studies were approved by the Office of Animal Care at Seattle Children’s Research Institute.

2.2. Strain and vectors

Adult male C57/BL6 mice used in this study were obtained from Charles Rivers Laboratories (Wilmington, MA). CV-1 cells (#CCL-70) were purchased from ATCC (Manassas, Virginia). HL-1 cells were a generous gift from Dr. W.C. Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA). The vectors used for bacterial expression: pET 28A (#69834-3, EMD Biosciences, Inc., San Diego, CA) and pGEX-4T-1 (#28954549, Cytiva, Marlborough, MA). For mammalian expression: pcDNA 3.1/myc-His and pCMV-MCS C-HA (#V800-20, #82018, Thermo Fisher, Waltham, MA), pGL3-promoter vector (#E1761, Promega, Madison, WI), and pRL-TK vector, (# E224, Promega, Madison, WI) were used.

2.3. Tissue Fractionation

Frozen mouse hearts were pulverized using a mortar and pestle. The crushed tissue was separated into a crude nuclear and cytoplasmic fraction by homogenization with a dounce homogenizer containing a cold hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCL_2$, 10 mM KCl, 0.5 mM DTT and protease inhibitors). The homogenate was centrifuged, and the cytoplasmic supernatant fraction retained. Nuclei enriched protein was isolated from the pellet after resuspension in hypotonic buffer plus 700 mM NaCl, and the recovered nuclear supernatant dialyzed into immunoprecipitation buffer (25 mM Tris, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, and 0.2 mM PMSF).

2.4. GST- TRβ1 pull-down and Nano LC MS/MS

Fractionated heart lysate (nuclear and cytoplasmic) was precleared with GST beads then subjected to GST- TRβ1 affinity purification pull-down. The bound proteins were

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eluted and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS; Applied Biomics, Hayward, CA). NanoLC fractionation and matrix-assisted laser desorption ionization-time of flight/time of flight (MALDI-TOF/TOF) were followed by a standard search of the National Center for Biotechnology Information and SwissProt databases, using MASCOT.

2.5. Protein extraction, Immunoprecipitation and Western Blot Analysis

Heart tissue was homogenized in cold lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitor mixture. 300 μg of heart lysates were precleared with protein A/G beads (Roche Molecular Biochemicals) and incubated with TRβ1 antibody MA1-216 (Thermo Fisher, Waltham, MA) or AB5622 (Abcam, Cambridge, MA) and protein A/G-agarose beads overnight. Negative controls reactions used isotype IgGs. Complexed proteins were recovered by boiling beads in SDS sample buffer, standard SDS-PAGE and immunoblotting for ACAA2, (#GTX115417, GeneTex, Irvine, CA), was performed.

2.6. Cell Culture and Reagents

CV-1 cells were maintained in Minimal Essential Medium with 10% fetal bovine serum (FBS), 1X pen/strep solution and 2 mM L-glutamine. All reagents were from Corning Inc. (Corning, NY). HL-1 cells were maintained in Claycomb Medium (#51800C, Millipore Sigma, Burlington, MA), with 10% FBS, 1X pen/strep solution, 2 mM L-glutamine and 0.1 mM norepinephrine # (A0937, Millipore Sigma, Burlington, MA). For thyroid hormone depletion experiments, 5% hormone depleted FBS (# F6765, Millipore Sigma, Burlington, MA) was substituted for the 10% FBS. T3, (# T2877, Millipore Sigma, Burlington, MA) was dissolved in 0.1 N NaOH.

2.7. RNA isolation and cDNA synthesis and DNA Constructs

Heart tissue was homogenized in Trizol (Thermo Fisher, Waltham, MA) and RNA isolation performed per manufacturer's protocol. The cDNA and dsDNA were generated using SuperScript™ III One-Step RT-PCR System with Platinum™ *Taq* High Fidelity DNA Polymerase (Thermo Fisher, Waltham, MA). The PCR amplifications used oligonucleotides based on GenBank accession numbers to TRβ1 (NM_001113417) and ACAA2 (NM_177470.3).

2.8. Oligonucleotides

The PCR primers numbers are based on their location in the gene for the GenBank accession numbers stated above. For bacterial expression the full-length TRβ1 dsDNA was generated using the following two primers: TRβ1F: 5'-GGGCCC(GAATTC)ATGACTCCTAACAGTATGACA-3' (nucleotides 190–210) and TRβ1R: 5'-GGCGCC(GTCGAC)TTAGTCCTCAAATACTTCTAA-3' (nucleotides 1555–1575) and cloned into pGEX-4T-1 to create a Glutathione S-transferase (GST) tagged construct, pGEX-4T-TRβ1. The pGEX-4T-TRβ1 clone was the template for the other TRβ1constructs.

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The truncated TRβ1 constructs utilized PCR with primers designed to reintroduce the ATG or TAA codon and cloned into pGEX-4T-1. TRβ1(107): 5’- GGGCCC(GAATTC)ATGTGTGTMGTGTGTGGGGACA-3’ (nucleotides 508–526); TRβ1(174): 5’- GGGCCC(GAATTC)ATGGGCATGGCMAYRGACTTGGT-3’ (nucleotides 706–725); TRβ1(240): 5’-GGGCCC(GAATTC)ATGAARCAGARGCGRAAATTCCTGC-3’ (nucleotides 907–928); TRβ1(240R): 5’- GGCGCC(GTCGAC)TTACCAGTGGCTGCCCTGGGCATT-3’; TRβ1(420R): 5’- GGCGCC(GTCGAC)TTACACCTTCATCAGSAGRTTGGGCCA-3’ (nucleotides 1441–1464).

The PCR products for ACAA2 were cloned into pET 28A using oligonucleotides: ACAA2_5’: 5’-GGGCCC(GGATCC)AATATGGCCCTGCTACGAGGTGTGTTC-3’ (nucleotides 39–62); and ACAA2_3’_pET: 5’ GAAGAA(GCGGCCGC)TCAGGCTGTGTTCTGGATGATCAA-3’ (nucleotides 1209–1232).

For mammalian expression the full-length TRβ1 was also cloned into pcDNA 3.1/myc-His with the following primers: TRβ15’-EK: 5’-GGCGCC(GAATTC)AATATGGCCACTCCTAACAGTATGACA-3’ (nucleotides 190–210) and TRβ13’-X: 5’- GGCGCC(CTCGAG)GTCCTCAAATACTTCTAA-3’ (nucleotides 1555–1572).

The full-length ACAA2 was amplified and cloned into pCMV-MCS C-HA (#82018, Thermo Fisher, Waltham, MA) using oligonucleotides ACAA_5’ and ACAA2_3’_C-TAG: 5’ GAAGAA(GCGGCCGC)AGGCTGTGTTCTGGATGATCAA-3’ (nucleotides 1209–1229).

The luciferase reporter plasmid was obtained by inserting two copies of a direct repeat TR response elements (TREs) in tandem into the Mlu I and Bgl II sites of the pGL3-promoter vector.

The annealed oligonucleotides sequences were: 5’-CCCGGGACGCGTGAATTCTA**AGGTCA**CTTC**AGGTCA**CTGGATCCTA**AGGTCA**CTTC **AGGTCA**CTAGATCTGCGCGC-3’ and 5’-GCGCGCAGATCTAGTGACCTGAAGTGACCTTAGGATCCAGTGACCTGAAGTGACCTT AGAATTCACGCGTCCCGGG-3’. The bold nucleotides represent the TR response elements.

The oligonucleotides used in the DNA pull-down assay were DR4_1: 5’-AGTGACCTGAAGTGACCTTAGGATCCAGTGACCTGAAGTGACCTTAGAATTCACGCG T-3’; DR4_2_TEG: 5’-5BiotinTEG/ACGCGTGAATTCTAAGGTCACTTCAGGTCACTGGATCCTAAGGTCACTTCAGGTCACT-3’; TRELAP_1_TEG: 5’-5BiotinTEG/AAGGGGATCCAGCTTGACCTGACGTCAGGTCAAGCTGATCTTCCT-3’; 5’-AGGAAGATCAGCTTGACCTGACGTCAGGTCAAGCTGGATCCCCTT-3’.

All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

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2.9. Transient Transfections

CV-1 cells were seeded into 96 well plates at 80% confluency 1 day prior to transfection. The reporter plasmid pRL-TK, used as a normalization control, was transfected with pcDNA3.1-TRβ1, pCMV-HA-ACAA2 and pGL3-DR4. Empty vectors were used as background control. Transfections were carried out using Lipofectamine 3000, (# L3000008, Thermo Fisher, Waltham, MA) according to manufacturer's protocol. After overnight incubation, culture medium was replaced with fresh MEM with 5% hormone depleted FBS ± 100 nM T3 for 24 hours before assay. Luciferase activity was assessed using the Dual-Glo Luciferase assay system, (# E2920, Promega, Madison, WI). All experiments were performed in triplicate with 6 well replicates/group. The results shown are the mean ± S.E.M.

2.10. In Vitro Binding Assay

The GST- TRβ1 and truncated forms were expressed from the pGEX-4T-1 vector. The fusion protein was expressed in Escherichia coli BL21 strain, induced by 1 mM isopropyl-α-D-thiogalactopyranoside for 3 hours at 30°C, and affinity-purified by glutathione-Sepharose 4B beads (Amersham Biosciences, Amersham, UK). [S^{35}]-Methionine labeled ACAA2 proteins were prepared by TNT-Quick Coupled Transcription/Translation reticulocyte lysate system (#L1170, Promega, Madison, WI) and incubated with a GST- TRβ1 (full or truncated form) in buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 5% glycerol and protease inhibitors) containing 0.5 mg BL21 soluble extract. The beads were rinsed in the same buffer and the bound proteins were extracted by boiling the beads in SDS sample buffer and separated on SDS-polyacrylamide gel and detected by autoradiography.

2.11. Immunocytochemistry

HL1 cells were transiently transfected with MYC-TRβ1 and HA-ACAA2. 48 hours post-transfection cells were fixed with cold methanol for 5 minutes, rinsed with phosphate buffered saline, treated with 10 mM sodium citrate buffer pH 6, rinsed in PBS and blocked with 10% normal donkey serum (NDS) for 1 hour. Cells were incubated overnight in 1.5% NDS with TRβ1, (# sc-737, Santa Cruz Biotechnology, Dallas, TX), ACAA2, or IgG isotype. The cells were washed with PBS and incubated with the secondary antibody Alexa Fluor 488 anti-mouse and Alex Fluor 546 anti-rabbit in 1.5% NDS, rinsed and coverslip mounted using Vectashield with DAPI, (# NC9524612, Thermo Fisher, Waltham, MA). Cells were imaged at 20X using a BZ-X series fluorescence microscope (Keyence, Osaka, Japan).

2.12. DNA Pulldown

The DNA pulldown assay was performed according to a protocol described by Chaparian and Kessel [8] with minor modifications. Briefly, 5'-TEG-biotin oligonucleotides were annealed to their non-labeled compliments to create DR4-TRE or PAL-TRE double stranded probes. BL21 bacterial cells were transformed with pET 28A-ACAA2, pGEX 4T- TRβ1 or empty vector. The BL21 cultures were grown to an optical density 600nm and induced with 0.1 mM IPTG for 2 hours at 30° C. The cultures were centrifuged and sonicated in

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BS/THES buffer (22 mM Tris-HCl, pH 7.5, 4 mM EDTA, 9% sucrose, 62 mM NaCl, 10 mM HEPES pH 7.5, 5 mM $CaCl_2$, 50 mM KCL and 12 % glycerol). The dsDNA probes were immobilized on Dynabeads MyOne Streptavidin C1 beads (#65001, Thermo Fisher, Waltham, MA). The lysate was precleared with streptavidin agarose beads followed by an incubation with the C1 beads for 1 hour in the presence of 25 μg salmon sperm DNA. The beads were washed repeatedly in BS/THES buffer and the bound proteins eluted off by heating at 95° C in SDS loading buffer. Eluted products were run on a 10% polyacrylamide gel, transferred to PVDF and western blotting performed using anti-HIS-tag, (#SC-8036, Santa Cruz, Dallas, TX) and anti-TRβ1 antibody.

2.13. Statistical Analysis

All reported values are expressed as mean ± standard error of the mean. For luciferase assays comparing control and experimental groups, we performed one-way ANOVA with Tukey post hoc analysis. Criterion for significance was $p < 0.05$ for all comparisons. We preformed statistical analysis using GraphPad Prism version 7.03 (GraphPad Software, San Diego, CA).

3. RESULTS

3.1. Identification of potential TRβ1 associated proteins

To identify heart proteins that interact with TRβ1, we performed a GST pulldown assay using GST- TRβ1 as bait. The heart tissue was fractionated to generate a nuclear-enriched and cytoplasmic fraction. LC MS/MS identified 109 proteins that potentially interact with TRβ1. The coimmunoprecipitation data has been deposited to the biological general repository for interaction database (BioGRID, https://thebiogrid.org) [9]. Table 1 shows a subset of the proteins identified that had a mascot score above 50. Of interest to our lab was the identification of ACAA2 (GenBank Accession No.: NM_177470.3) as a TRβ1 associating protein. ACAA2, acetyl-Coenzyme A acyltransferase or 3-ketoacyl-Co A thiolase, catalyzes the last step in the β-oxidation pathway, and our lab previously showed ACAA2 protein expression to be significantly decreased in male C57Bl/6 mice after thyroid hormone supplementation [10], suggesting ACAA2 is both the product of a TH responsive gene and an interactor with a TH receptor.

3.2. Interaction of TRβ1 with ACAA2

We tested the physiological relevance of the interaction between TRβ1 and ACAA2 by assessing whether the endogenous proteins interact in cardiac tissue. We immunoprecipitated cardiac lysate with TRβ1 antibody generated in 2 different species (Fig. 1a). The results showed that TRβ1 forms an endogenous protein complex containing ACAA2. The isotype IgG controls did not immunoprecipitate an immunoreactive band in the ACAA2 ~42 kD region.

3.3. GST pull-down assays

To determine whether TRβ1 and ACAA2 interact directly, a full-length mouse ACAA2 cDNA was cloned into the pET 28A vector to produce an [S^{35}]-labeled in vitro transcription/ translation product. The S^{35}-ACAA2 product was incubated with immobilized GST-

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TRβ1 or one its truncated forms illustrated in figure 1b. The proteins retained on the matrix were eluted, separated by PAGE and autoradiographed (Fig. 1c). The full length TRβ1showed the strongest binding to ACAA2. The fusion protein produced from clone pG-TRβ1-240R and pG- TRβ1-420R had the weakest interaction with ACAA2, suggesting a strong interaction site occurs between amino acids 420 and 461, the region involved in ligand-dependent transactivation. Considering deletion at no one location prevented binding, multiple interaction sites may exist between these two proteins.

3.4. Subcellular localization of ACAA2 and TRβ1

To determine where the association between TRβ1 and ACAA2 takes place we performed immunocytochemistry using the cardiac muscle cell line HL-1. ACAA2 and TRβ1 are predominantly cytoplasmic and mitochondrial (MitoTracker data not shown) (Fig 2a and 2b). However, ACAA2 and TRβ1 also colocalized within the nucleus (Fig. 2c) supported by the overlap with the DAPI stain (Fig. 2d), albeit at a lower intensity.

3.5. ACAA2 activate the T3-dependent transactivation activity of TRβ1

To assess the functional consequences of the physical interactions of TRβ1 with ACAA2, we used a promoter luciferase construct with a direct-repeat (DR4) thyroid hormone response element (TRE) inserted upstream. CV-1 cells were chosen due to a lack of endogenous TR activity [11]. CV-1 cells were transfected with a TRβ1 and ACAA2 expression plasmid alone or together and changes in transactivation activity assessed based on luciferase expression (Fig. 3a). Cells co-transfected with the empty vectors served as background control. Transfections of TRβ1 or ACAA2 individually or together minus TH (T_3) treatment did not show any change in transactivation. The addition of T_3 increased the transactivation of the single transformants 3-fold ($p < 0.04$); whereas the co-transfection of ACAA2 and TRβ1 increased transactivation activity 8-fold over background ($p<0.0001$). This suggest ACAA2 acts as a T3 dependent coactivator of TRβ1.

3.6. ACAA2 weakly binds TREs

To assess the ability of ACAA2 to directly interact with TREs, we performed a DNA pull-down assay. Two TRE probes were designed to reflect either DR4 or palindromic (PAL) TRE sequences known to bind TRs. Lysate of bacterial BL21 cells induced to overexpress ACAA2 or TRβ1 protein was used to determine binding affinity to the DR4 or PAL probes. TRβ1 binding to both the DR4 and PAL probes acted as the positive control. The interaction between ACAA2 and the TREs, in comparison to the TRβ1-TRE binding, was weak and required prolonged exposure. To assess if ACAA2 affected TRβ1-TRE binding, ACAA2 lysate was incubated with the TRβ1-TRE lysate, but no change in intensity of TRβ1 binding to the TRE sequences was observed (Fig. 3b).

4. Discussion

Thyroid hormone is an important signaling molecule for cardiac function and predominantly acts through its TRs. We aimed to identify novel interacting proteins of the transcription factor, TRβ1, to better define the TH signaling networks in the heart. Using a GST-TRβ1 pulldown screen we identified 109 potential associating proteins. A search of BioGRID

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[9] and the literature revealed several of the proteins that we identified as known TRβ1-interacting proteins including p300, a histone acetyltransferase [12], NCOA6, nuclear receptor coactivator [13], and HADHA, mitochondrial trifunctional protein [14].

Previous work in our lab showed ACAA2 protein expression to be altered by TH status in male C57Bl/6 mice [10]. Therefore, it was of interest to identify ACAA2 as a potential TRβ1-interacting protein. ACAA2, a member of the thiolase 1 family, is involved in the final step of the fatty acid beta oxidation pathway [15]. Another member of the thiolase 1 family, mitochondrial trifunctional protein (MTP) has also been shown to associate with TRβ1 [14]. The authors of that study suggested that T_3 stimulated mitochondrial metabolism via TR-dependent stabilization of the MTP complex. We observed no such effect for ACAA2 (data not shown). However, we did observe a T_3 induced synergistic effect of ACAA2 on TRβ1 transcription ability, supporting a role for metabolic proteins beyond the substrate oxidation. Nuclear ACAA2 localizatin is not unprecedented. Choi et al. reported on an ACAA2 association with the histone H2A.Z-bound chromatin possibly explaining how gene expression is attuned to metabolite changes [16].

Interestingly, the ACAA2 single transfection stimulated the TRE luciferase factor in the presence of T3, although, CV-1 cells lack endogenous TR activity, suggesting actions through the TRE motif could also occur via other nuclear receptors retained in CV-1 cells. TRs are members of the nuclear receptor (NR) superfamily that share and compete for partners and receptor binding sites [17]. Additionally, TH has been shown to modulate other NRs actions via non-genomic mechanisms [18, 19]. This phenomenon suggests that AACA2 may be a promiscuous binding partner potentially interacting with other nuclear receptors, further emphasizing the need to examine and understand the intricacies of crosstalk in NR gene regulation.

Next, we characterized the protein-protein interaction sites. The binding assays showed the C-terminus of TRβ1, between amino acids 420–460, had a strong association with ACAA2. The C-terminus region of TRβ1 contains the hormone-dependent activation domain, possessing a hydrophobic cleft that can adopt conformational changes to bind the TH ligand and cofactor proteins [20]. Many nuclear receptors, like TRβ1, bind a short-conserved motif (LXXLL) found in many coactivators [21]. However, no such motif was observed in ACAA2; instead, the internal signature motif (I/L)XX(I/V)I was observed at the N- and C-terminus. This motif can form a predicted α-helical loop that can bind the TRβ1 hydrophobic cleft which is sufficient for receptor interaction [22]. However, the (I/L)XX(I/V)I motif has been associated with corepressors and ACAA2 in our study acted as a coactivator. One potential explanation is the importance of the flanking sequence of the (I/L)XX(I/V)I motif in affecting receptor conformation and activity [22, 23]. We, however, cannot rule out ACAA2 binding TRβ1 at alternate locations. Future investigation of the protein docking sites may provide a structural basis for drug design in thyroid disorders.

The ability of ACAA2 to bind the TRE sequence, albeit at highly reduced intensity, was of interest. Since ACAA2 did not affect TRβ1-TRE binding an alternative role may be as a recruiter to non-traditional TRE sites via cooperative binding [24], or ACAA2 binding to DNA may alter the DNAs conformational structure opening it up to TRβ1 activity [25].

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Hence, the identification of ACAA2 provides new avenues to understanding the signals in gene regulation.

TH influence on ACAA2 expression and ACAA2-TRβ1association underlies the complexities involved in the TH regulatory pathways. Outside of ACAA2 role in fatty acid metabolism, little is known about other mechanistic roles of which it might be involved. Gene expression profiles have shown ACAA2 to be altered in various disease states, like cardiac dysfunction [26]. Additionally, ACAA2 has been shown to complex with nucleosomes, potentially modifying histones [16], as well as having a role in apoptosis [27]. In our study we assign a new role to ACAA2, that of a T3-dependent TRβ1 coactivating protein; thereby continuing the further understanding of the intricacies of the transcriptional machinery and its regulation by metabolism.

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Highlights

- We identified ACAA2, a mitochondrial thiolase, as a TRβ1 associating protein.
- We determined ACAA2 acts as a thyroid hormone dependent coactivator of TRβ1transcriptional activity.
- We show ACAA2 can bind TRβ1 DNA recognition sequences.

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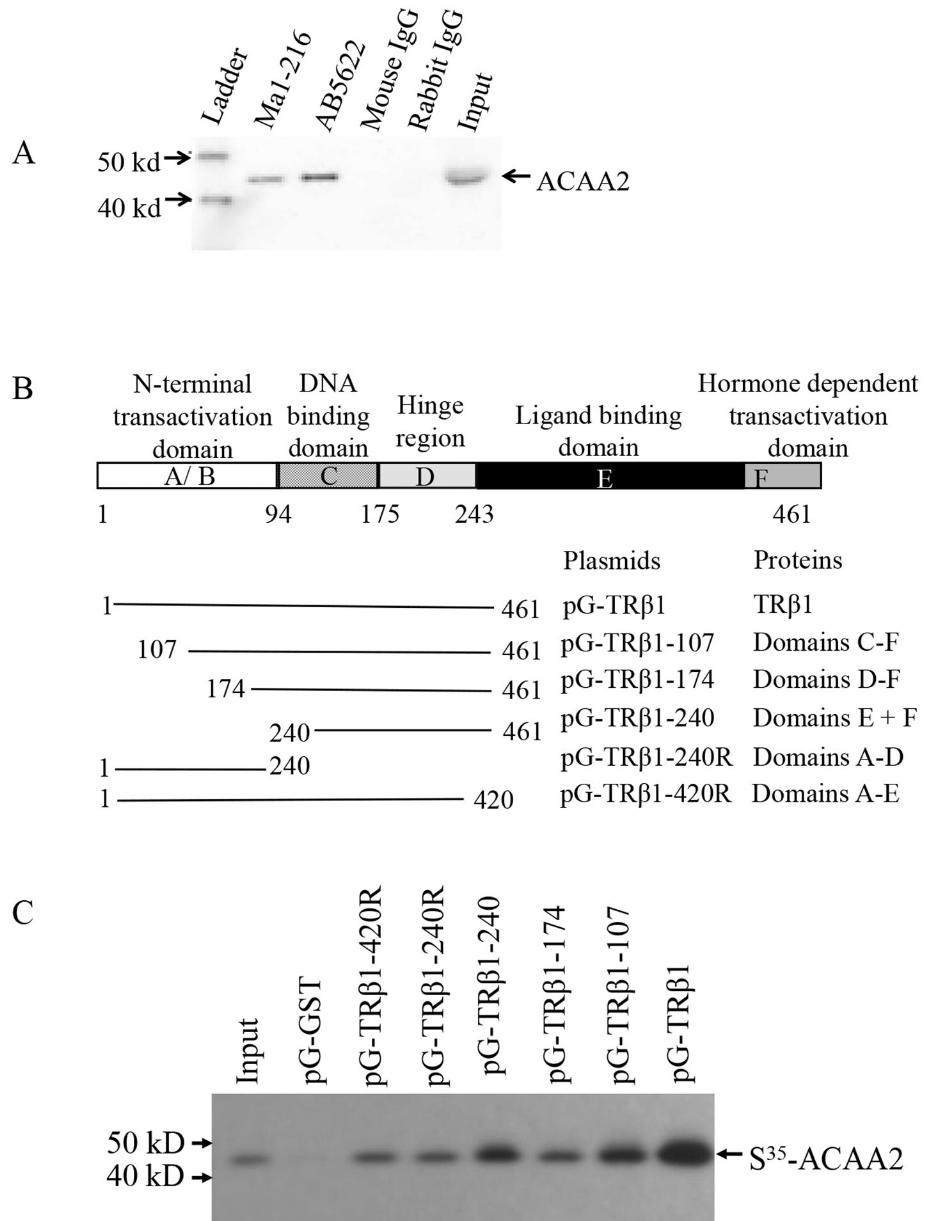

Figure 1.

(A) TRβ1 immunoprecipitated ACAA2 from mouse heart lysate. No bands observed in the isotype controls. MA1-216 and AB5622 - TRβ1 mouse monoclonal and rabbit polyclonal, respectively. (B) Schematic representation of full-length and TRβ1-truncated proteins used in the binding assay. The numbers indicate the position of amino acids. (C) ACAA2 complexes with TRβ1 *in vitro*. Immobilized GST or GST- TRβ1 fusion protein was incubated with *in vitro* translated ^{35}S-labeled ACAA2 as described under Experimental Methods 2.10. ($n = 3$)

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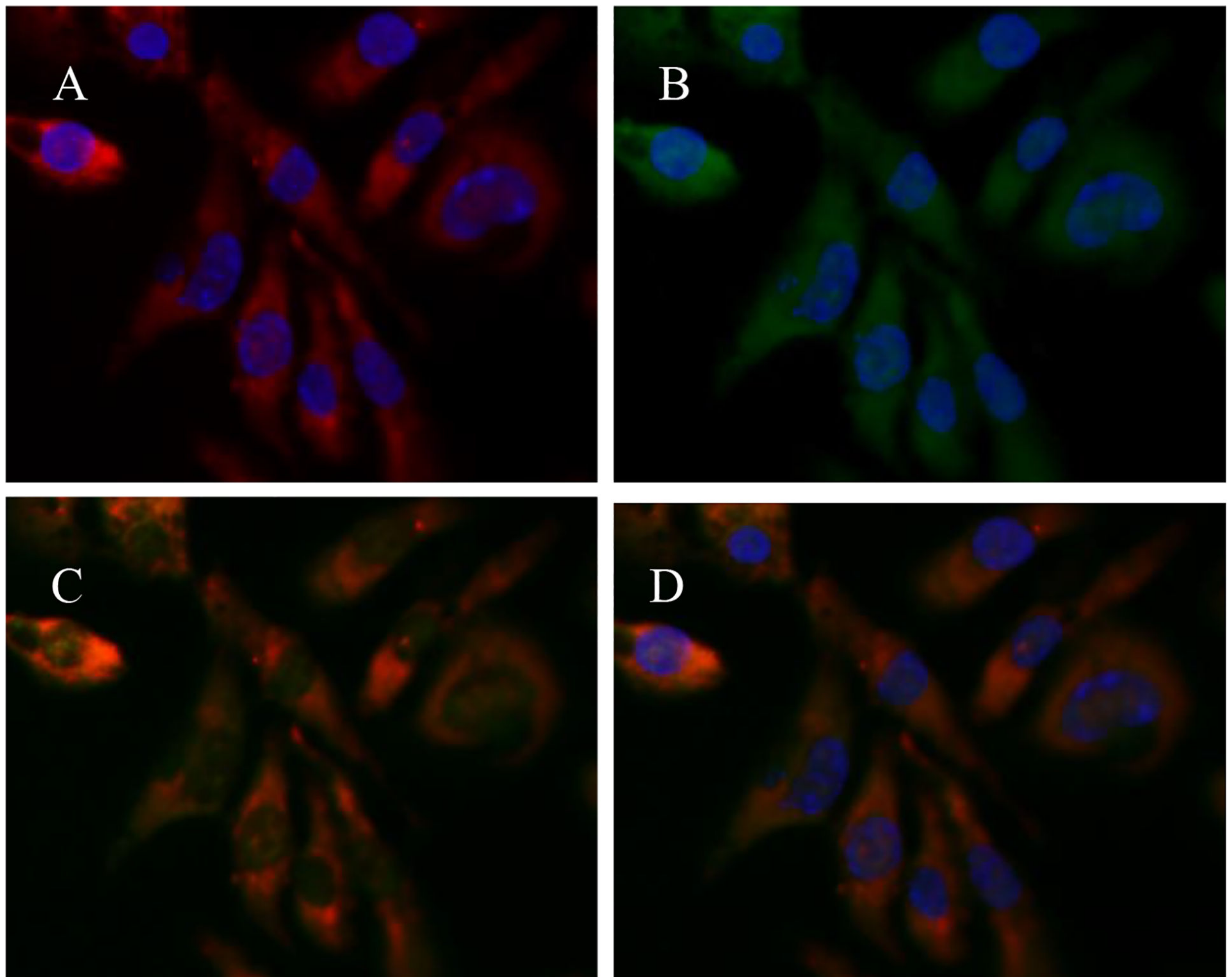

Figure 2.
ACAA2 colocalizes with TRβ1 in HL-1 cells. ACAA2 (A) and TRβ1(B) were detected mostly in the cytosol with low expression in the nucleus. Merged images show the overlap of ACAA2 and TRβ1 (C) in the nucleus and with the DAPI stain (D).

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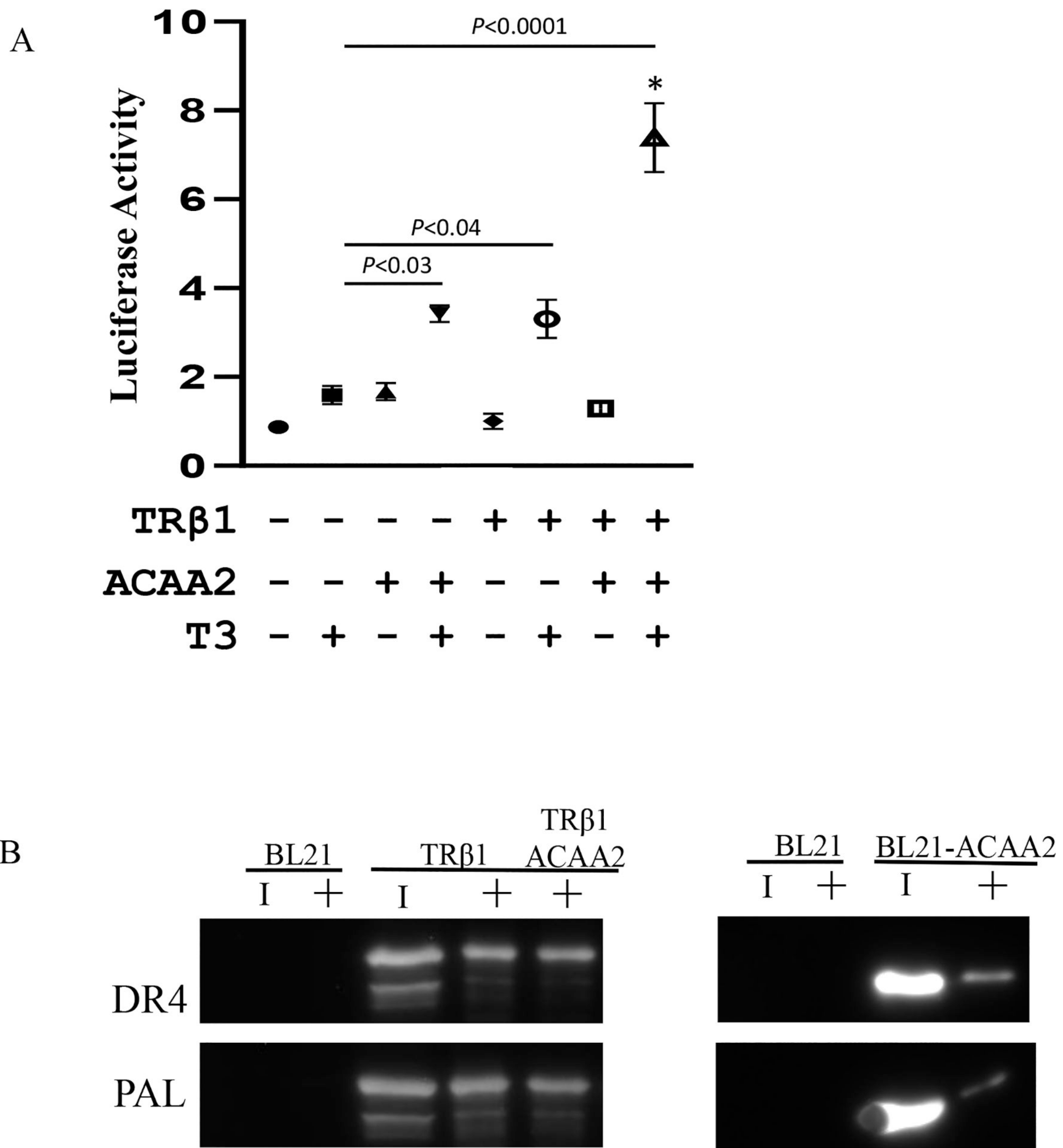

Figure 3.

(A) ACCA2 increased TRβ1 transcriptional activity in transfected TH-treated CV1cells. Data (Tukey) show mean ± S.E.M. ($n = 3$). (B) Representative blot showing ACAA2 weakly binds to TRβ1-TREs. The biotin-labeled TRE was incubated with BL21 unprogrammed lysate or BL21-TRβ1 or BL21-ACAA2 programmed lysate alone or together, as stated under Experimental Methods 2.12. I, Input protein. ($n = 3$).

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TABLE 1.

Subset of proteins identified by coimmuniprecipitation and LC-MS/MS analysis

Protein Name	UniProtKB	Mascot Score				Molecular Function
		2–3 months		18 months		
		Nuclear	Cyto	Nuclear	Cyto	
Elongation factor 1-alpha 2	P62631	42	164	53	217	nucleotide binding, translation elongation
Elongation factor 1-gamma	Q9D8N0	184	392	202	431	translation elongation
Thyroid hormone receptor alpha	P63058	124	74	122	66	transcription factor
Ankyrin repeat domain-containing protein 26	Q811D2	39	46	53	43	protein binding
Keratin, type II cytoskeletal 5	Q922U2	96	44	85	34	scaffold protein binding
Elongation factor Tu, mitochondrial	Q8BFR5	--	74	53	97	nucleotide binding, translation elongation
Keratin, type I cytoskeletal 10	P02535	75	31	108	--	scaffold protein binding
ATP synthase subunit alpha, mitochondrial	Q03265	37	68	34	--	nucleotide and protein binding
3-ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	--	--	61	45	transferase activity