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The Chicken Ovalbumin Upstream Promoter-Transcription Factors Modulate Genes and Pathways Involved in Skeletal Muscle Cell Metabolism*[S]

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The chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are "orphan" members of the nuclear hormone receptor (NR) superfamily. COUP-TFs are involved in organogenesis and neurogenesis. However, their role in skeletal muscle (and other major mass tissues) and metabolism remains obscure. Skeletal muscle accounts for ~40% of total body mass and energy expenditure. Moreover, this peripheral tissue is a primary site of glucose and fatty acid utilization. We utilize small interfering RNA (siRNA)-mediated attenuation of *Coup-TfI* and *II* (mRNA and protein) in a skeletal muscle cell culture model to understand the regulatory role of *Coup-Tfs* in this energy demanding tissue. This targeted NR repression resulted in the significant attenuation of genes that regulate lipid mobilization and utilization (including *Pparα*, *Fabp3*, and *Cpt-1*). This was coupled to reduced fatty acid β-oxidation. Additionally we observed significant attenuation of *Ucp1*, a gene involved in energy expenditure. Concordantly, we observed a 5-fold increase in ATP levels in cells with siRNA-mediated repression of *Coup-TfI* and *II*. Furthermore, the expression of "classical" liver X receptor (LXR) target genes involved in reverse cholesterol transport (*Abca1* and *Abcg1*) were both significantly repressed. Moreover, we observed that repression of the *Coup-Tfs* ablated the activation of *Abca1*, and *Abcg1* mRNA expression by the selective LXR agonist, T0901317. In concordance, *Coup-Tf*-siRNA-transfected cells were refractory to *Lxr*-mediated reduction of total intracellular cholesterol levels in contrast to the negative control cells. In agreement *Lxr*-mediated activation of the *Abca1* promoter in *Coup-Tf*-siRNA cells was attenuated. Collectively, these data suggest a pivotal role for *Coup-Tfs* in the regulation of lipid utilization/cholesterol homeostasis in skeletal muscle cells and the modulation of *Lxr*-dependent gene regulation.

Nuclear receptors (NRs)[3] comprise a large family of DNA-binding transcription factors that control many cellular and physiological processes crucial for development and survival. Dysregulation of nuclear receptor signaling leads to proliferative, reproductive, and metabolic disorders such as cancer, infertility, dyslipidemia, and insulin resistance/type II diabetes (1). Consequently, NRs are a major focal point of intense clinical and pharmaceutical studies. The NR superfamily includes receptors for steroid hormones, vitamin D, ecdysone, retinoic acids (all-*trans* and 9-*cis* isoforms), and thyroid hormones. In addition to these known receptors for known ligands, there are a number of NRs to which no ligand has been described, and thus, designated "orphan" receptors.

Of the orphan nuclear receptors, the chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) have received considerable attention for their role in neurogenesis, organogenesis, and embryogenesis (2–4). In mouse there are two highly related *Coup-Tf*s (*Coup-TfI* and *Coup-TfII*) encoded by distinct genes on chromosomes 13 and 7, respectively (5). Interestingly, *Coup-Tf*s can modulate the activity of genes that are fundamental in the orchestration of glucose and lipid metabolism. These include, bile acid synthesis (*Cyp7A*-cholesterol 7α-hydroxylase) (6), ketogenesis (mitochondrial hydroxymethylglutaryl-CoA synthase) (7), cholesterol transport (cholesterol ester transfer protein and apolipoprotein AI) (8, 9), and fatty acid β-oxidation (medium-chain acyl-coenzyme A dehydrogenase) (10). Furthermore, organ-specific (pancreatic β cells) *Coup-TfII* null mutants in mice demonstrated a role for *Coup-Tf* in glucose homeostasis and insulin sensitivity (11). In addition, the *Coup-Tf* ortholog in *Drosophila* (*seven up*) is required for the expression of alcohol dehydrogenase and type IV collagen, two genes that are essential for fat cell differentiation (12). Although *Coup-Tf*s are implicated in metabolism in these systems, little is known of the role of these NRs with respect to metabolism in skeletal muscle, and other major mass peripheral tissues.

Skeletal muscle is one of the most metabolically active tissues in the body that accounts for >30% of total energy expenditure. By virtue of its large mass (~40% of body weight) and signifi-

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[3] The abbreviations used are: NR, nuclear receptor; Q-RT, quantitative real time; ABC, ATP-binding cassette proteins; FABP3, fatty acid-binding protein 3; CPT-1, carnitine palmitoyltransferase 1; UCP3, uncoupling protein 3; CYP7A1, cholesterol 7α-hydroxylase; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; LXR, liver X receptor; DMEM, Dulbecco's modified Eagle's medium; siRNA, small interfering RNA; IL, interleukin; PPAR, peroxisome proliferator-activated receptor; PEPCK, phosphoenolpyruvate carboxykinase; DM, differentiation medium.

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cant energy demands, skeletal muscle provides a vital milieu for fatty acid and glucose homeostasis (13). Furthermore, it is also involved in cholesterol efflux (*i.e.* reverse cholesterol transport) (14). Consequently, skeletal muscle plays a considerable role in insulin sensitivity and the blood lipid profile. These characteristics alone suggest that skeletal muscle has an integral role in metabolism and provides an attractive target tissue to study the role of orphan nuclear receptors with respect to metabolism in this system.

Previous studies from this laboratory have identified a role for the orphan nuclear receptors (including *Ror*α, *Rev-erb*β, and *Nur77*) in the regulation of the genetic programs controlling lipid homeostasis in the mouse C2C12 skeletal muscle cell line (15–17). Moreover, other studies have successfully utilized this model system to study *Coup-Tfs*. For example, constitutive expression of *Coup-TfI* repressed the activity of the human muscle glycogen phosphorylase gene promoter in C2C12s (18). In fact, the C2C12 cell line has been used to study the functional role of many orphan nuclear receptors (14). The ability of this cell line to differentiate into multinucleated fibers in tissue culture (19), coupled to the acquisition of a contractile and metabolic phenotype, provides a robust system to study the role of COUP-TF in skeletal muscle metabolism.

In this study we tested the hypothesis that *Coup-Tfs* are implicated in the regulation of pathways controlling metabolism. Our studies suggest a role for *Coup-Tfs* in (i) the modulation of key genes involved in lipid utilization (*Ucp1*, *Ucp3*, *Fabp3*, and *Cpt-1*) and (ii) the regulation of total cholesterol levels, and liver X receptor (*Lxr*)-dependent expression of the ATP-binding cassette proteins that control cholesterol efflux (*Abca1* and *Abcg1*). The role of *Coup-Tfs* in this system presents an essential platform to further study lipid utilization and cholesterol efflux in skeletal muscle. Importantly, these processes have implications for disease states such as dyslipidemia.

MATERIALS AND METHODS

Cell Culture—Proliferating mouse C2C12 myoblasts were cultured and maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated Serum Supreme (Cambrex Bio Science, Victoria, Australia). Differentiation of myoblasts into post-mitotic, multi-nucleated myotubes was induced by mitogen withdrawal, *i.e.* DMEM supplemented with 2% horse serum (differentiation medium, DM) (Trace, Scientific, Victoria, Australia) for 4 days.

RNA Extraction and cDNA Synthesis—Total RNA was extracted from C2C12 cells using TRI-Reagent (Sigma) according to the manufacturer's protocol. Total RNA was then treated with 2 units of Turbo DNase I (Ambion, Austin, TX) for 30 min at 37 °C followed by purification of the RNA through an RNeasy purification column system (Qiagen, Victoria, Australia). RNA was electrophoresed to determine the integrity of the preparation. SuperScript III was used to synthesize cDNA from 4 μg of total RNA using random hexamers according to the manufacturer's instructions (Invitrogen). The cDNA was then diluted to 300 μl in nuclease-free water.

Protein Extraction—Total soluble protein was extracted from C2C12 cells by the addition of lysis buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA) containing protease "mixture" inhibitors (Sigma). Lysates were passed through a 26-gauge needle and centrifuged at 10,000 $\times$ g for 20 min. The supernatant was collected and total protein concentration was determined by the BCA as outlined by the manufacturer's instructions (Pierce).

Generation of Coup-TfI-siRNA Construct—A 21-mer siRNA duplex specific to the annotated mouse cDNA sequence of *Coup-TfI* (NM_010151) was generated with the siRNA target finder from Ambion. The target sequence, *Coup-TfI*-5′-AAGCACTACGGCCAATTCACC-3′, was chosen based on having no sequence similarity with other members of the nuclear receptor family and low sequence homology with non-target murine sequences. The sense and antisense siRNA oligomers were synthesized (Geneworks, SA, Australia) and cloned into the pSilencer 3.1 neomycin vector as described by the manufacturer (Ambion). Target sequences were confirmed by direct sequencing at the Australian Genome Research Facility, University of Queensland, Brisbane, Australia.

Transient Transfections of siRNA Constructs—C2C12 myoblasts were grown to ~80% confluence and three independent transfections of *Coup-TfI* (4 μg/10-cm dish) and the pSilencer 3.1 negative control siRNA sequence (a sequence not found in the rat, human, or the mouse genome, Ambion) were performed in the presence of Lipofectamine 2000 as outlined by the manufacturer's protocol (Invitrogen). Twenty-four h post-transfection, fresh DM was added for a further 24 h and RNA was collected as outlined above.

Generation of C12C2 Stable Coup-Tf-siRNA Cell Lines—C2C12 cells were transfected at 50% confluence with 4 μg of pSilencer-3.1-*Coup-Tf* and 4 μg of pSilencer-3.1-negative control in 2$\times$ HEPES, 10 μl of *N*-[1(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium salt (DOTAP), and 10 μl of Metafectene (Scientifix, Victoria, Australia) per 25-cm^2 flask. Cells were subsequently passaged into 10-cm^2 round flasks followed by 8–12 days selection with 700 μg/ml G418 in DMEM. Following the selection process and the death of the non-transfected control population, viable siRNA-transfectant stable cell populations were maintained in DMEM supplemented with 300 μg/ml G418. The large number of stable transfectants (>100 colonies) were passaged into three independent polyclonal pools (to avoid clonal bias) and independently, passaged, cultured, and propagated for various experiments. Each independent stable myoblasts were then differentiated into myotubes as outlined above. Myoblasts and differentiated myotubes were analyzed on a Nikon Eclipse TS100 microscope (Coherent Scientific, SA, Australia) and digital images were captured on a Nikon Coolpix 4500 digital camera.

T0901317 Treatment of Stable C2C12 Cell Lines—C2C12 cell lines stably expressing the pSilencer negative 3.1 control and the *Coup-Tf*-siRNA were grown to confluence and then induced to differentiate over a 4-day period. At day 3 of differentiation, the cells were treated with either vehicle (Me_2SO) or LXR agonist, T0901317 (10 μM), until day 4 of differentiation. The cells were then collected for RNA processing as outlined above.

Quantitative Real-time PCR (Q-RT-PCR)—Q-RT-PCR was performed on an ABI Prism 7500 sequence detection system (Applied Biosystems) in triplicate on three independent RNA

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preparations. Target cDNA levels were analyzed in 25-μl reactions with either SYBR Green or TaqMan Technologies (Applied Biosystems). Primers (200 nM) used for the amplification of target gene sequences have been described in detail (15–17). PCR was performed with 5 μl of cDNA and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative level of target gene expression was normalized to *Gapdh* or 18 S ribosomal RNA as described under "Results" and associated errors were calculated using the guidelines described by Bookout and Mangelsdorf, on the Nuclear Receptor Signaling Atlas website (NURSA). Statistical analysis was performed on the average of three independent assays and a Student's *t* test was performed to calculate significance.

Western Blot Analysis—Total soluble protein from the pSilencer 3.1 negative control and pSilencer 3.1 *Coup-TfI*-siRNA stable C2C12 cell lines was resolved on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were blocked overnight or for 1 h in 5% skim milk in Tris-buffered saline/Tween 20 followed by an overnight incubation with either Coup-TfI (1:3000, sc-6577), Coup-TfII (1:2000, sc-5578), Abca1 (1:1000, sc-20794) (Santa Cruz Biotechnology, Inc.), or Gapdh (1:10,000) (R&D Systems, Minneapolis, MN) antibodies. Following 4 × 15-min washes the membrane was incubated with either anti-goat horseradish peroxidase (Coup-TfI and II) or anti-rabbit horseradish peroxidase (Gapdh and Abca1) (1:2000) for 1 h. Immunoreactive signals were detected using enhanced chemiluminescence Super-Signal West Pico Substrate (Pierce) and visualized by autoradiography on an X-Omat film developer (Kodak).

Luciferase Assay—C2C12 stable *Coup-Tf*-siRNA and the negative 3.1 control cells were grown in DMEM in 24-well plates to ~80% confluency. Transient transfection of the *ABCA1*-Luc promoter (20) (0.5 μg/well) was performed in the presence of Lipofectamine 2000 (Invitrogen). Twenty-four hours post-transfection, the cells were treated with either vehicle (Me_2SO) or LXR agonist, T0901317 (10 μM). Luciferase assays were performed using the LucLite luminescence reporter gene assay system as outlined by the manufacturer (PerkinElmer Life Sciences). Luminescence was recorded on a MicroBeta luciferase system (PerkinElmer Life Sciences) (20).

ATP Assay—A total of 10,000 C2C12 cells (counted by microscopy via the trypan blue exclusion method) per well (96-well plate) expressing the pSilencer 3.1 negative control and *Coup-Tf*-siRNA were seeded and maintained in DMEM. At 100% confluence, DM was added and the cells were induced to differentiate for 4 days. The measurement of ATP was performed using the ATPlite luminescence ATP detection assay system as outlined by the manufacturer (PerkinElmer Life Sciences). The results obtained are from three independent polyclonal pools, assayed in triplicate. ATP concentration was normalized to total soluble protein. A Student's *t* test was performed to calculate statistical significance.

Amplex Red Cholesterol Assay—The assay for free cholesterol and cholesterol esters was performed as described by the manufacturer's instructions (Invitrogen). Briefly, cell samples containing the pSilencer 3.1 negative control and the *Coup-Tf*-siRNA C2C12 stable cells were seeded at ~10,000 cells per well (96-well plate). The cells were then maintained in DMEM until confluent and differentiated in 10% charcoal-stripped DMEM prior to the addition of 10 μmol/liter of the LXR agonist, T0901317 for 24 h. The cells were then resuspended in Amplex Red reaction buffer (Invitrogen) and incubated at 100 °C for 30 min to inactivate endogenous cellular cholesterol esterase. Following a 30-min incubation at 37 °C with the Amplex Red assay reagents, fluorescence was measured at an excitation of 530 nm and emission of 590 nm. The results obtained are from three independent polyclonal pools, assayed in triplicate. Cholesterol concentration was normalized to total soluble protein and a Student's *t* test was performed to calculate statistical significance.

[9,10-^{3}H]Palmitic Acid Assay—The oxidation of [9,10-^{3}H]palmitate was measured on the basis of ^3H_2O released into the cell culture media. *Coup-Tf*-siRNA stable C2C12 cells were differentiated in 6-well plates for 4 days in DM. Following differentiation, the stable *Coup-Tf*-siRNA cells were serum-starved for 4 h and then incubated in 1 ml of fatty acid media (α-minimal essential medium, 2% fatty acid-free bovine serum albumin (w:v), 0.25 mmol/liter palmitate, 1 μCi/ml of [^{3}H]palmitate, pre-gassed) for 2 h at 37 °C. A total of 250 μl of media was mixed with 1.25 ml of CHC13:MeOH (2:1) for 15 min followed by the addition of 500 μl of 2 mol/liter KCl, 2 mol/liter HCl and further mixed for 15 min followed by centrifugation at ~2500 × *g* for 15 min. 200 μl of the aqueous phase was removed and mixed with 1.8 ml of scintillation fluid to use for β counting on a MicroBeta luciferase system (PerkinElmer Life Sciences). Data were expressed as ± S.E. and significance was calculated using a Student's *t* test.

RESULTS

The Coup-TfI and II mRNA and Protein Are Expressed during Skeletal Muscle Differentiation—To elucidate the role of *Coup-TfI* and *II* in skeletal muscle with respect to metabolism, we initially investigated the expression profile of both *Coup-TfI* and *II* mRNA relative to *Gapdh* in the mouse C2C12 myoblast cell line. Proliferating myoblasts can be induced to biochemically and morphologically differentiate into post-mitotic multinucleated myotubes by mitogen withdrawal over a 24–96-h period. This transition from a non-muscle to a contractile phenotype is associated with activation and repression of a structurally diverse group of genes responsible for contraction and the extreme metabolic demands placed on this tissue. During this period of differentiation, we observed by Q-RT-PCR that both *Coup-TfI* and *II* mRNA were expressed during skeletal muscle cell differentiation (Fig. 1, *a* and *b*). To assess the levels of Coup-TfI and Coup-TfII proteins during differentiation we performed Western blotting analysis. High levels of immunoreactive Coup-TfI and II were observed in the proliferating myoblasts and confluent myoblasts and down-regulated (relative to Gapdh) during myogenesis (Fig. 1, *a* and *b*).

To assess the differentiation status of the C2C12 cells and demonstrate that they had acquired a muscle-specific, contractile and metabolic phenotype, Q-RT-PCR was performed on several important marker genes encoding myogenin, a gene that encodes the hierarchical basic helix loop regulator and is specifically required for differentiation (21), the slow twitch (type I) and the fast twitch (type II) isoforms of the contractile

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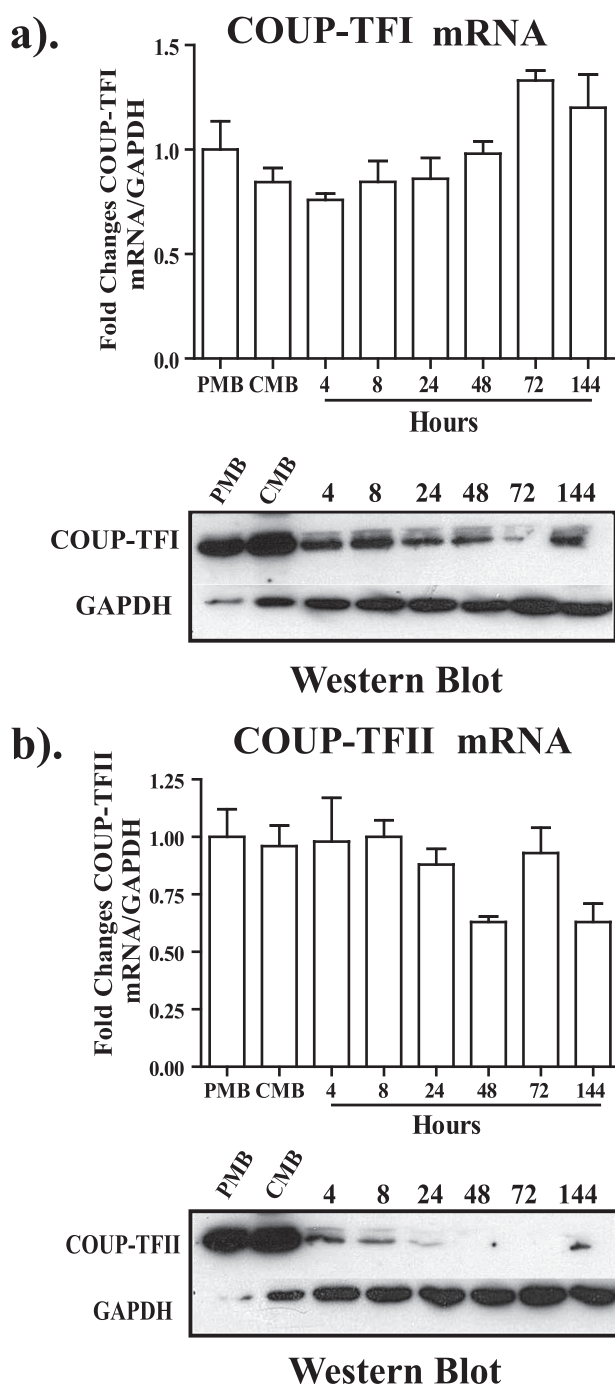

FIGURE 1. **Expression of *Coup-TfI* and *II* mRNA and protein during skeletal muscle differentiation in C2C12 myoblasts and differentiated myotubes.** Total RNA and soluble protein from proliferating myoblasts (*PMBs*), confluent myoblasts (*CMBs*), and 4–144 h of differentiation were analyzed by Q-RT-PCR and Western blot analysis. *a* and *b*, Q-RT-PCR (*top panels*) and protein analysis (*lower panels*) of *Coup-TfI* and *Coup-TfII*, respectively. Gapdh protein was used as a loading control.

protein troponin I, and the metabolic genes *Abca1* and *Abcg1* (ATP-binding cassette proteins), *Fabp3* (fatty acid-binding protein 3), *Cpt-1* (carnitine palmitoyltransferase 1), and *Ucp3* (uncoupling protein 3).

Both the marker of differentiation (myogenin) and the contractile protein genes (types I and II, *Tnni1* and *Tnni2*, respectively) were dramatically induced during the myogenic process and thus confirmed the differentiated state of the C2C12 cell (Fig. 2, *a–c*). Furthermore, the expression of these key myogenic markers of differentiation are entirely consistent with previous studies (15, 17, 22). Additionally, genes involved in lipid metabolism (*Abca1*, *Abcg1*, *Cpt-1*, and *Fabp3*) and energy expenditure (*Ucp3*) were all induced during skeletal myogenesis, and confirmed that the muscle cells had acquired a metabolic phenotype (Fig. 2, *d–h*). Due to the availability of antibodies against Abca1, we assessed the levels of this protein across the developmental time course. From these data, the Abca1 protein correlated with the *Abca1* mRNA profile (Fig. 2*d*).

Coup-Tf-siRNA Expression Represses Endogenous Levels of Coup-TfI and Coup-TfII mRNA and Protein in Skeletal Muscle Cells—To elucidate the biological role of *Coup-Tfs* in skeletal muscle with respect to lipid utilization, we preceded to selectively ablate the expression of *Coup-TfI* and *II*. RNA interference was utilized to create stable cell lines expressing *Coup-Tf*-siRNA (having no sequence similarity to other nuclear receptors) in an attempt to attenuate mRNA levels of both *Coup-TfI* and *II*. As a control we produced stable C2C12 cells expressing the pSilencer 3.1 negative control (a sequence not found in the mouse, rat, or human genome). Accordingly, triplicate polyclonal C2C12 cell lines stably expressing the pSilencer 3.1 negative control siRNA (negative control) and the specific COUP-TF-siRNA (Fig. 3*a*) were subsequently differentiated for 4 days and total RNA and soluble protein was extracted. Representative images of the C2C12 myoblasts and myotubes are shown for the negative 3.1 control and the *Coup-Tf*-siRNA cells. Both negative 3.1 controls and the *Coup-Tf*-siRNA stable cell lines differentiated from a proliferating mononucleated phenotype to a post-mitotic multinucleated morphology indicative of a contractile phenotype (Fig. 3*b*).

Quantitative RT-PCR was then performed to measure the expression of both *Coup-TfI* and *Coup-TfII* mRNA normalized to both *Gapdh* and 18 S rRNA in the RNA isolated from the differentiated negative control and *Coup-Tf*-siRNA-transfected stable cell lines. We observed a significant attenuation of both the *Coup-TfI* (5.0-fold, $p < 0.0001$) and *Coup-TfII* (4.75-fold, $p < 0.0001$) mRNA when compared with the negative 3.1 control and normalized to *Gapdh* (Fig. 3, *c* and *d*). Moreover, the relative expression of *Coup-TfI* and *II* mRNA was similar when normalized to 18 S rRNA (*Coup-TfI*, 5.65-fold, $p = 0.0170$, and *Coup-TfII*, 4.38-fold, $p = 0.0114$) (Fig. 3, *c* and *d*). To determine that the attenuation of both *Coup-TfI* and *II* mRNA was not due to differential *Gapdh* mRNA expression, Q-RT-PCR was also performed on *Gapdh* mRNA normalized to 18 S rRNA. No change was observed in the levels of *Gapdh* mRNA expression in the negative control relative to the *Coup-Tf*-siRNA cell lines and thus, validated the attenuated expression of *Coup-TfI* and *II* mRNA in the C2C12 *Coup-Tf*-siRNA stable lines (Fig. 3*e*).

To determine whether the repression of *Coup-TfI* and *Coup-TfII* mRNA expression also correlated with Coup-Tf protein levels, Western blot analysis was performed using specific Coup-TfI and II antibodies. Western blot analysis also confirmed both Coup-TfI and II proteins were dramatically reduced in the C2C12 *Coup-Tf*-siRNA stables relative to the negative 3.1 control (Fig. 3, *f* and *g*). The effects of *Coup-Tf* attenuation by the specific siRNA (relative to the negative 3.1

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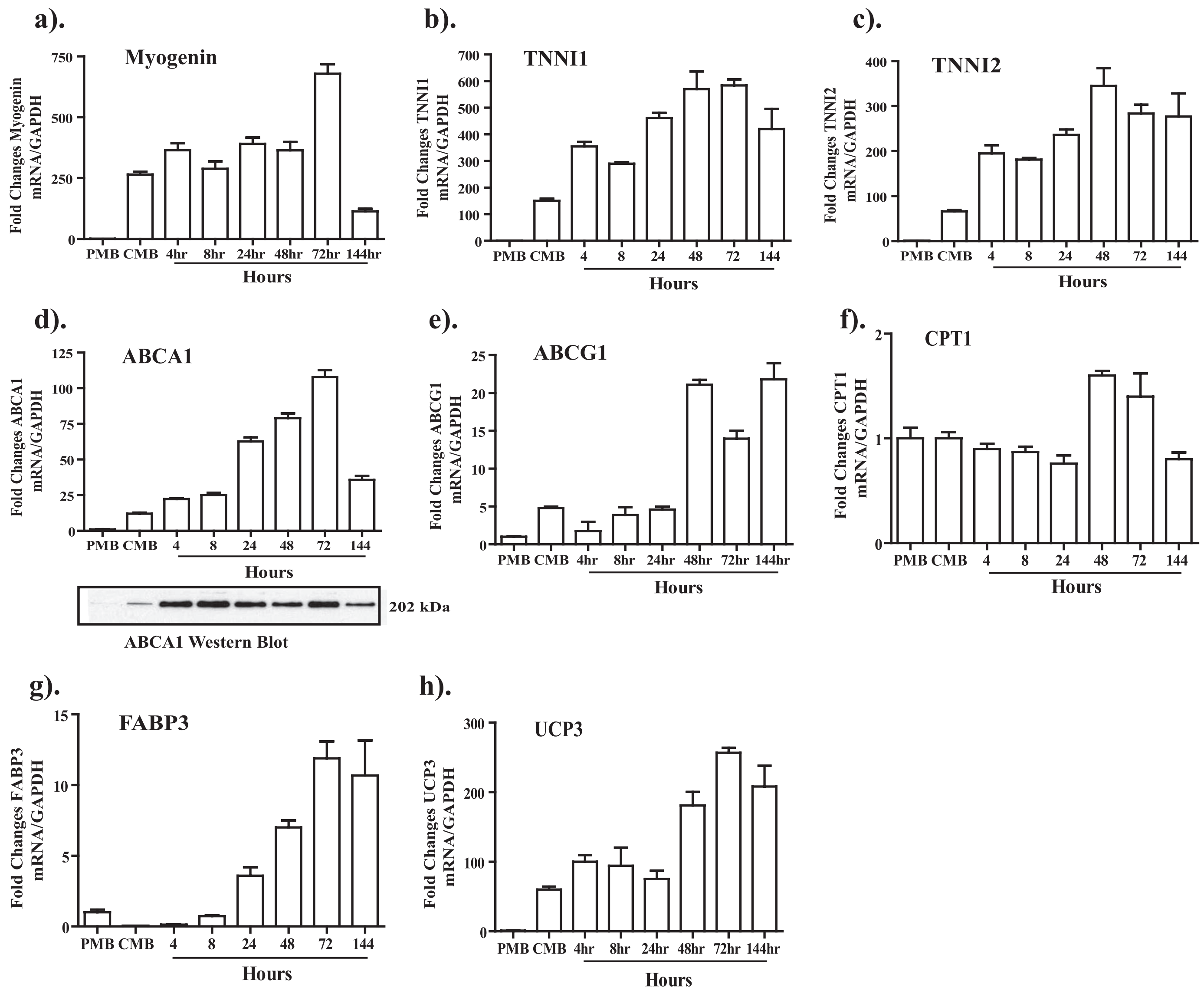

FIGURE 2. **Expression of markers indicative of the acquisition of the muscle-specific (myogenin), contractile (*Tnni1* and *Tnni2*), and metabolic phenotypes (*Abca1*, *Abcg1*, *Cpt-1*, *Fabp3*, and *Ucp3*) during differentiation.** *a–h*, Q-RT-PCR mRNA expression of myogenin, *Tnni1*, *Tnni2*, *Abca1*, *Abcg1*, *Cpt-1*, *Fabp3*, and *Ucp3*, respectively. Data are expressed as the mean of triplicate samples ± S.D. *d*, Western blot analysis showing protein levels of Abca1. *PMB*, proliferating myoblasts; *CMB*, confluent myoblasts.

control) were further validated by analyzing the expression of both *Coup-TfI* and *II* mRNA after transient transfection of the *Coup-Tf*-siRNA. C2C12 cells were transiently transfected with the negative 3.1 control and *Coup-Tf*-siRNA and the endogenous levels of both *Coup-TfI* and *II* mRNA were measured in Q-RT-PCR experiments. Both *Coup-TfI* and *II* mRNA were clearly attenuated in the *Coup-Tf*-siRNA transfection (Fig. 3*h*). Moreover, these data show that the attenuation of both *Coup-TfI* and *II* is not a result of the G418 selection process for the generation of stable cell lines, and is specific to the expression of the siRNA.

Expression of Coup-Tf-siRNA Modulates the Expression of Genes Involved in Lipid Utilization—Genes involved in lipid utilization (from intramyocellular lipid depots) and catabolism, respectively, for example, *Fabp3* and *Cpt-1*) were dramatically attenuated (14-fold, $p = 0.0211$ and 10.5-fold, $p = 0.0199$, respectively) (Fig. 4, *a* and *b*). Consequently, we examined whether these changes in gene expression had functional consequences on lipid oxidation in these cells utilizing a ^3H_2O-based palmitate assay. In concordance with the dramatic attenuation of *Cpt-1*, we observed reduced fatty acid β oxidation in the *Coup-Tf*-siRNA (relative to the negative control) transfected cells (Fig. 4*c*). A number of other genes important in fatty acid homeostasis were analyzed (Table 1), however, minimal nonspecific changes were observed between the negative control and the *Coup-Tf*-siRNA cells.

We next analyzed a number of important nuclear receptors that are modulators of lipid metabolism and homeostasis, for example, *Ppars*, *Lxrα*, *Lxrβ*, *Rorα*, *Errα*, and *Rev-erbα* (14). From these studies, we observed a dramatic repression of *Pparα* (master regulator of lipid catabolism) mRNA of up to 30-fold ($p < 0.0001$) in the *Coup-Tf*-siRNA cells (Fig. 4*d*). Moreover, the specificity of *Pparα* attenuation was supported by the minimal changes observed in the expression of the other nuclear receptors in the *Coup-Tf*-siRNA-transfected cells (Table 2).

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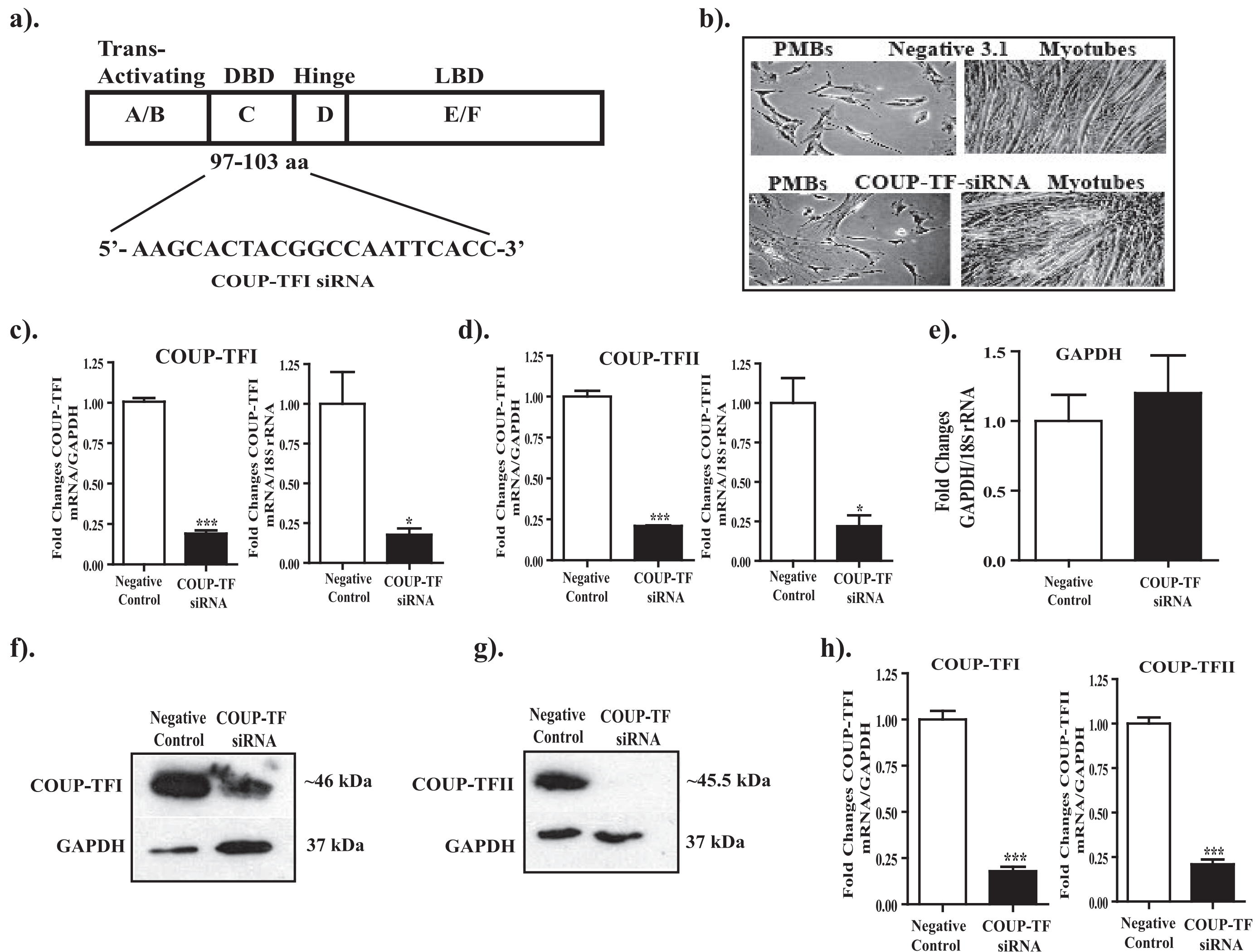

FIGURE 3. **Transfection of the *Coup-Tf*-siRNA attenuates *Coup-TfI* and *II* mRNA and protein expression.** *a*, schematic representation of the Coup-Tf receptor and position of the *Coup-Tf*-siRNA target sequence. The *Coup-Tf*-siRNA target sequence is shown between amino acids 97 and 103 in the C region of the DNA-binding domain (*DBD*). The other motifs represent the trans-activating domain (*A/B*), the hinge region (*H*), and the ligand-binding domain (*LBD*, *E/F*). Note: the sequence above was designed to target the *Coup-TfI* mRNA sequence but also shares 19/21-bp identity with *Coup-TfII*. *b*, photographic images of C2C12 PMBs and differentiated myotubes in stable C2C12 pSilencer negative 3.1 control and pSilencer 3.1 *Coup-Tf*-siRNA. *c* and *d*, *Coup-TfI* and *Coup-TfII* attenuation in C2C12 stable siRNA cell lines, respectively. -Fold changes of *Coup-TfI* and *Coup-TfII* are expressed relative to both *Gapdh* and 18 S rRNA. *e*, -fold change in *Gapdh* expression relative to 18 S rRNA. *f* and *g*, Western blot analysis of *Coup-TfI*, *II*, and *Gapdh* in the C2C12 stable expressing pSilencer negative 3.1 control and pSilencer 3.1 *Coup-Tf-siRNA*. COUP-TfI (~46 kDa), COUP-TfII (~45.5 kDa), and GAPDH (37 kDa) shown at their approximate molecular masses were used as a loading control. *h*, expression of both *Coup-TfI* and *II* in transient pSilencer 3.1 negative control and *Coup-Tf*-siRNA expressing C2C12 cell lines. For all experiments, the mean ± S.D. were derived from three independent RNA preparations (each analyzed in triplicate). Significance was calculated using the Student's *t* test; *, $p = 0.01–0.05$ and ***, $p < 0.0001$.

The myogenic cytokine, *Il-15* (Fig. 4*e*) was down-regulated 5.6-fold ($p = 0.0084$). *Il-15* has been demonstrated to function in a muscle to fat endocrine axis to modulate fat: lean body composition and insulin sensitivity (23). We also observed a dramatic attenuation of phosphoenolpyruvate carboxykinase (*Pepck-1*) a gene involved in gluconeogenesis. *Pepck-1* was undetectable in *Coup-Tf*-siRNA cells (Fig. 4*f*). Other genes involved in inflammation and glucose homeostasis were analyzed; however, minimal changes were observed (Tables 1 and 2).

The Attenuation of Coup-TfI and II Expression Modulates the Expression of the Uncoupling Genes and Steady State ATP Levels—Subsequently, we analyzed the mRNAs encoding the uncoupling proteins (*Ucp1–3*) and other genes (*Ampkα,β,γ*) involved in energy expenditure, and balance. For example, the expression of genes encoding *Ucp1* and *Ucp3* that are involved in energy uncoupling and preferential lipid utilization, respectively, were significantly modulated. Specifically, *Ucp1* mRNA was dramatically repressed (and undetectable), whereas in contrast, *Ucp3* mRNA expression was induced 6.24-fold ($p = 0.0024$) in the C2C12 *Coup-Tf*-siRNA cell line (Fig. 5, *a* and *b*). *Ucp2* and other genes involved in energy expenditure and balance were not substantially modulated or significantly changed in this system (Table 1). To assess the effect of the dramatic attenuation of *Ucp1* mRNA in the *Coup-Tf*-siRNA C2C12 stable cell lines, we measured and compared the levels of ATP in the negative control and *Coup-Tf*-siRNA-transfected C2C12 cells. In concordance with the striking reduction of *Ucp1* mRNA expression in the *Coup-Tf*-siRNA stable cells, we observed an approximate 5-fold increase in ATP levels in the

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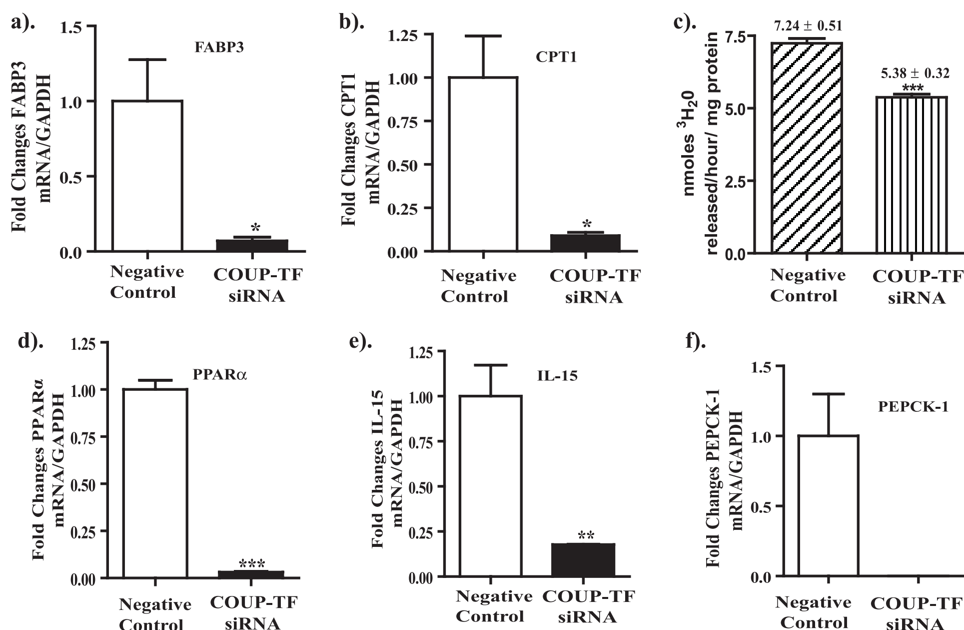

FIGURE 4. ***Coup-Tf* siRNA expression suppresses pathways involved in lipid utilization.** *a*, *Fabp3*; *b*, *Cpt-1*; *c*, palmitate oxidation; *d*, *Ppar*α; *e*, *Il-15*; and *f*, *Pepck-1*. -Fold changes of gene expression are relative to *Gapdh*. For all experiments, the mean ± S.D. were derived from three independent RNA preparations (each analyzed in triplicate). *c*, total nanomoles of ^3H_2O/h produced through β oxidation of [^{3}H]palmitic acid was calculated from $n = 9$ experiments according to a standard curve generated using increasing concentrations of [^{3}H]palmitic acid. pSilencer negative 3.1 controls (7.24 ± 0.51) and *Coup-Tf*-siRNA (5.38 ± 0.32) stable C2C12 cells. Significance was calculated using the Student's *t* test, where *, $p = 0.01–0.05$; **, $p = 0.001–0.01$; and ***, $p < 0.0001$. Note: *Pecpk-1* was undetectable in the pSilencer 3.1 *Coup-Tf*-siRNA stable cell lines.

TABLE 1

Relative expression of genes involved in energy expenditure and glucose/lipid metabolism

Gene	Relative expression C2C12 negative[a]	Relative expression C2C12 siRNA[a]	-Fold change and *p* value[b]
Energy expenditure and balance			
Ucp2	10132 ± 2622	3972 ± 15.7	2.55 $p = 0.0785$
Ampkγ*3*	57210 ± 5992	28310 ± 1148	2.0 $p = 0.0087$
Ampkγ*2*	22690 ± 2758	15100 ± 2013	1.5 $p = 0.0903$
Ampkα*2*	15050 ± 1721	7236 ± 1486	2.0 $p = 0.0429$
Ampkβ*2*	5586 ± 1176	5497 ± 639.4	1.0 $p = 0.9454$
Fatty acid homeostasis			
Cd36	1974 ± 375.3	920.2 ± 200.2	2.1 $p = 0.0685$
Fabp4	66.45 ± 17.68	49.10 ± 17.89	1.35 $p = 0.5585$
Acs4	4383 ± 332.4	2125 ± 242.3	2.0 $p = 0.0050$
Lpl	12670 ± 1211	12080 ± 1352	1.0 $p = 0.7618$
Mcad	4636 ± 156	2721 ± 333.2	1.7 $p = 0.0065$
ApoE	4242 ± 1097	2825 ± 224	1.5 $p = 0.2743$
Fas	3564 ± 281	1716 ± 409.9	2.0 $p = 0.0199$
Scd-1	90530 ± 1884	54520 ± 54520	1.6 $p = 0.0011$
Scd-2	52472 ± 1735	47637 ± 1156	1.1 $p = 0.1373$
Srebp1c	1019 ± 74	360.9 ± 71.95	2.8 $p = 0.0237$
Lipe	10892 ± 520.9	4796 ± 393.5	2.3 $p = 0.0007$
Glucose homeostasis			
Glut 4	0.5232 ± 0.109	0.4819 ± 0.0614	1.0 $p = 0.7856$
Glut 5	2469 ± 292.4	3644 ± 184.1	1.5 $p = 0.0356$
Pygm	97850 ± 13663	119388 ± 9549	1.2 $p = 0.0347$
Glycogexin	16.52 ± 1.16	23.42 ± 2.533	1.4 $p = 0.1318$
Pdk-2	2063 ± 218.2	1867 ± 109.4	1.1 $p = 0.4610$
Pdk-4	832.7 ± 87.7	424.9 ± 67.31	1.9 $p = 0.0210$

[a] Relative expression expressed as number of transcripts per GAPDH transcript ± S.D.

[b] -Fold change and associated *p* value.

Coup-Tf-siRNA (~36 μmol/liter) relative to the negative 3.1 control cells (~7 μmol/liter) (Fig. 5*c*).

Repression of the Coup-Tfs Attenuates the Expression of Genes Involved in Reverse Cholesterol Transport (Abca1 and Abcg1)—We further analyzed a number of important genes implicated in lipid efflux. We observed the mRNAs encoding the reverse cholesterol transporters, *Abca1* and *Abcg1*, were both significantly attenuated (4.5-fold, $p = 0.0073$, and 3.86-fold, $p = 0.0063$, respectively) (Fig. 5, *d* and *e*).

The Effects of Coup-Tf Repression on Lipid Homeostasis Are Independent of Differentiation—To evaluate whether the changes observed in the *Coup-Tf*-siRNA stable cell lines were not due to aberrant differentiation, the mRNA levels of the differentiation (myogenin) and contractile (*Tnni1* and *Tnni2*) markers were analyzed in the RNA samples derived from both the differentiated negative controls and the *Coup-Tf*-siRNA stable cell lines. Minimal non-significant changes in the expression of the mRNA encoding myogenin and troponin I were observed when compared with the negative 3.1 control (Table 3) relative to the dramatic significant changes observed in the metabolic genes reported above. Moreover, and more importantly, we observed both induction (*e.g. Ucp3*, Fig. 5*b*) and repression (*e.g. Cpt-1* and *Abca1*, Figs. 4*b* and 5*d*, respectively) of genes in the stable cells that are induced during the normal differentiation process (Fig. 2, *d*, *f*, and *h*). These data emphasize that the observed phenomenon are not differentiation-dependent and further validate that the modulating effects of *Coup-Tf* attenuation on metabolism were not due to aberrant differentiation.

Furthermore, to establish that the attenuation of *Coup-Tf* and the modulation of core metabolic genes was not an artifact of the G148 selection process, we performed Q-RT-PCR on these key genes from cells transiently transfected with the *Coup-Tf*-siRNA. In these studies we observed identical modulation of these key genes (for example, *Ppar*α, *Abca1*, *Ucp3*, and *Cpt-1* (supplementary materials Fig. S1, *a–d*) suggesting the effects observed in the *Coup-Tf*-siRNA cells was not inherent to the stable C2C12 cell lines.

Repression of Coup-TfI and II Compromises LXR-mediated Activation of Abca1, Abcg1, and Srebp-1c mRNA Expression—We observed that suppression of *Coup-TfI* and *II* mRNA expression attenuated the expression of several "classical" *Lxr* target genes including *Abca1*, *Abcg1*, and *Srebp-1c*. Therefore, we explored the potential cross-talk between *Coup-Tf* and *Lxr* signaling in skeletal muscle cells and examined the effect of attenuated *Coup-Tf* expression on *Lxr* signaling. Consequently, we treated the negative control and the *Coup-Tf*-siRNA-transfected stable C2C12 cell lines with vehicle (Me_2SO), and the potent/selective LXR agonist, T0901317, and examined the activation of several classical LXR target genes. T0901317 treatment of the negative control cells resulted in a robust activation of the LXR target genes *Abca1*, *Abcg1*, and *Srebp-1c* (approxi-

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TABLE 2
Relative expression of members of the nuclear receptor superfamily and inflammatory factors

Gene	Relative expression C2C12 negative[a]	Relative expression C2C12 xiRNA[a]	-Fold change and *p* value[b]
Nuclear receptors			
Errγ	3.044 ± 0.521	1.691 ± 0.343	1.8 $p = 0.1624$
Errα	509.3 ± 103	286.1 ± 32	1.78 $p = 0.1085$
Lxrα	109.4 ± 15.40	44.13 ± 1.284	2.5 $p = 0.0134$
Lxrβ	3072 ± 133.6	2045 ± 176.6	1.5 $p = 0.0098$
Pparβ/δ	1917 ± 94.20	1004 ± 104.4	1.9 $p = 0.0012$
Pparγ	882.1 ± 81.33	323.3 ± 23.54	2.7 $p = 0.0027$
Rev-erbα	9319 ± 1610	7456 ± 770	1.2 $p = 0.3178$
Rev-erbβ	21645 ± 1508	7790 ± 965.8	2.8 $p = 0.0015$
Rorα	91.07 ± 3.638	76.06 ± 9.580	1.2 $p = 0.2169$
Rorγ	2199 ± 282.9	1760 ± 421.5	1.2 $p = 0.4779$
Nurr1	7983 ± 271.6	8483 ± 1486	1.06 $p = 0.7572$
Nur77	1843 ± 361.9	2237 ± 227.8	1.2 $p = 0.4090$
Inflammatory mediators			
Il-6	2482 ± 177.5	2838 ± 148	1.0 $p = 0.1990$
Iκ*b*α	212500 ± 15080	159700 ± 15210	1.3 $p = 0.0695$
Nf$\kappa\beta$*-p65*	330500 ± 32320	201100 ± 39470	1.6 $p = 0.0642$

mately, 30-, 7-, and 3.8-fold, respectively, relative to the vehicle, Fig. 6, *a–c*), and as reported previously (20). In contrast, the repression of *Coup-Tf* attenuated the *Lxr*-mediated/dependent transactivation of *Abca1*, *Abcg1*, and *Srebp-1c* (Fig. 6, *a–c*, respectively). These effects occurred in the absence of any significant changes in *Lxr*α and *Lxr*β mRNA expression (Fig. 6, *d* and *e*).

It has been well documented that activation of *Lxr*-dependent *Abca1* and *Abcg1* expression increases reverse cholesterol transport, and reduces intracellular cholesterol levels. In this context we had observed an ablation of the *Lxr*-dependent activation of *Abca1* and *Abcg1* mRNA expression. Therefore, we analyzed the effect of the selective LXR agonist, T0901317, on total intracellular cholesterol levels in the negative control and the *Coup-Tf*-siRNA-transfected cells. As expected, we observed a significant reduction in total intracellular cholesterol in negative control cells. In contrast, the LXR agonist did not reduce intracellular cholesterol levels in the *Coup-Tf*-siRNA-transfected cell lines (Fig. 7, *a* and *b*). This correlates with the attenuated induction of *Abca1* and *Abcg1* mRNA expression after T0901317 treatment in the *Coup-Tf*-siRNA-transfected cell line.

Repression of Coup-TfI and II mRNA Expression Ablates the Lxr-mediated Activation of the ABCA1 Promoter—Finally, we examined whether the attenuation of *Coup-Tf* affected the activity of the *ABCA1* promoter. T0901317 treatment relative to vehicle increased the activity of the *Lxr*-dependent *ABCA1* promoter in the negative control cells. In contrast, T0901317 treatment (relative to vehicle) did not activate the *ABCA1* promoter in the *Coup-Tf*-siRNA-transfected stable cells (Fig. 7*c*). This suggested the effects of *Coup-Tfs* on *ABCA1* expression are mediated by transcriptional mechanisms.

DISCUSSION

COUP-TFs are members of the orphan nuclear receptor superfamily that are implicated in neurogenesis and organogenesis. Sporadic reports suggested COUP-TFs regulate several genes involved in cholesterol homeostasis, fatty acid oxidation, and ketogenesis. However, the functional role of COUP-TFs with respect to the control of genetic programs associated with metabolism in skeletal muscle (or any other major mass peripheral tissue) has not been addressed. Here, we have utilized siRNA-mediated targeting of *Coup-TfI* and *II* to investigate whether the *Coup-Tfs* regulate critical pathways of metabolism in C2C12 skeletal muscle cells. We utilized the mouse C2C12 skeletal muscle cell culture model as previous data derived from this *in vitro* model with LXR and PPARδ agonists (20) were verified/validated in subsequent *in vivo* mouse studies (24, 25).

The *Coup-TfI* and *II* mRNAs are constitutively expressed during C2C12 skeletal muscle myogenesis. In contrast, Coup-Tf protein levels are down-regulated as the C2C12s exit the cell cycle and undergo myogenic differentiation. This suggests that during differentiation, and/or cell cycle withdrawal that Coup-Tf is regulated by translational control mechanisms. Whether this involves mechanisms targeting the 5′ and 3′ untranslated regions and/or subcellular localization is not currently known. However, sea urchin homologs of *Coup-TfI* are stored as maternal RNA in the egg, a common site of translational regulation (26).

Stable expression of the *Coup-Tf*-siRNA (relative to the pSilencer 3.1 negative control) in the C2C12 skeletal muscle cells significantly attenuated both *Coup-TfI* and *II* mRNA (5- and 4.75-fold, respectively) relative to two controls, *Gapdh* and 18 S rRNA. Moreover, these effects were independent of the differentiation status of the C2C12 cells.

Our study demonstrates that a subgroup of genes (including, *Fabp3*, *Cpt-1*, and *Ppar*α) involved in intramyocellular lipid mobilization/utilization and catabolism are dependent on the expression of *Coup-Tfs*. This is further highlighted by the fact that related genes such as *Fabp4*, *Ppar* β/δ, and *Ppar*γ (and genes in similar pathways for example, *Mcad*, *Lpl*, and several other NRs) were refractory to the attenuation of *Coup-Tf* expression (see Tables 1–3). The observed reduction of these key metabolic target genes is noteworthy for a number of reasons. First, correlation between *Fabp3* and *Ppar*α expression has been described during transcriptional adaptation of lipid homeostasis in the skeletal muscle of endurance trained human males (27). Second, the attenuated expression of *Cpt-1* correlates with reduced *Ppar*α expression. *Cpt-1* is a well characterized *Ppar*α (and δ) target gene (28). The roles of the above genes as important regulators of the lipolytic mobilization and catabolism of fatty acids (*Ppar*α (29), *Fabp3* (30), and *Cpt-1* (30)), and their modulation in our C2C12 cell line with attenuated *Coup-TfI* and *II* expression, suggests that *Coup-Tfs* plays a critical role in modulating lipolysis in this system. This is underscored by reduced pamitate/β-oxidation in the *Coup-Tf*-siRNA-transfected cells.

The role of the COUP-TFs in lipid utilization may appear contrary to the differentiation dependent regulation of *Coup-Tf* in the cell culture model. However, the *Coup-Tfs* are abundantly expressed in skeletal muscle tissue (4). Second, it is pertinent to point out that *Ppar*γ was described as a regulator of lipid storage that mediated the actions of insulin sensitizers in adipose tissue. Later studies with muscle-specific *Ppar*γ null mice underscored the role of this NR in skeletal muscle in the

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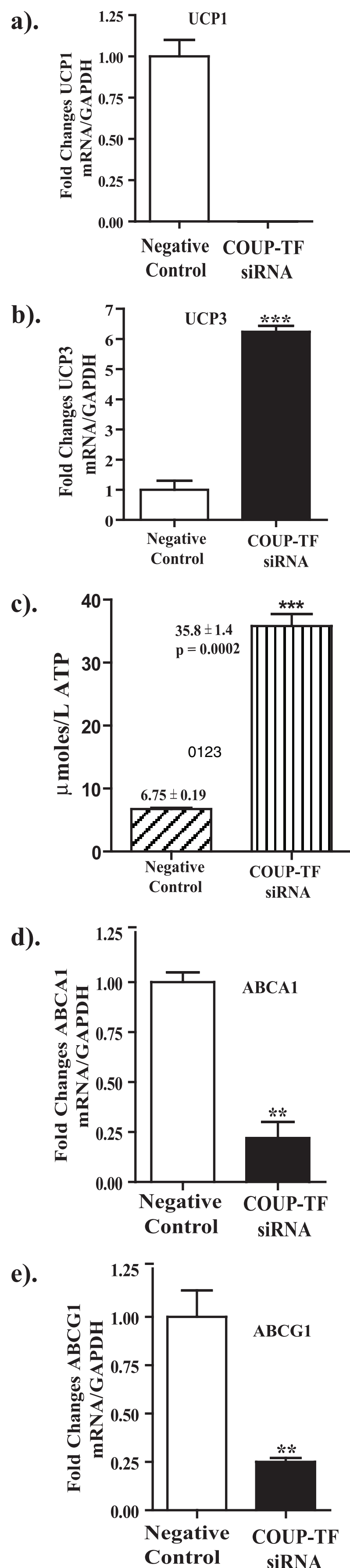

FIGURE 5. **Attenuation of *Coup-Tf* expression affects expression of the uncoupling genes and the reverse cholesterol transporter genes (*Abca1* and *Abcg1*).** *a*, *Ucp1*; *b*, *Ucp3*; and *c*, total ATP production. The *Coup-Tf*-siRNA produced an approximate 5-fold increase in total ATP (36 μmol/liter, $p = 0.0002$) relative to the pSilencer 3.1 negative control (7 μmol/liter) (*c*). *d*, *Abca1*; *e*, *Abcg1*. -Fold changes of gene expression are relative to *Gapdh*. Mean ± S.D. was derived from three independent RNA preparations (each analyzed in triplicate). Significance was calculated using the Student's *t* test; ***, $p < 0.0001$. Note: *Ucp1* was undetectable in the *Coup-Tf*-siRNA stable C2C12 cell lines (*a*).

TABLE 3

Minimal effect on biochemical markers of differentiation, myogenin, TNNI1, and TNNI2

Muscle-specific genes			
Myogenin	98.42 ± 2.506	240.2 ± 36.89[a]	2.4 $p = 0.0144$[b]
Tnni1	50362 ± 3873	52274 ± 9260	1.0 $p = 0.8576$
Tnni2	3039 ± 461.2	1578 ± 79.26	1.9 $p = 0.0355$

[a] Relative expression expressed as number of transcripts per GAPDH transcript ± S.D.
[b] -Fold change and associated *p* value.

context of insulin sensitivity, despite weak expression in this lean tissue (31).

The observed attenuation of *Il-15* in the *Coup-Tf*-siRNA was interesting. *Il-15* (and several other cytokines *Il-6* and *-8* (32, 33)) are expressed in skeletal muscle tissue and C2C12 cells and induced during myogenesis (34). Furthermore, it has been demonstrated that *IL-15* has an anabolic effect of skeletal muscle *in vitro* and *in vivo* (33). In contrast, adipogenic cells do not express *Il-15*. Moreover, IL-15 administered to rodents decreased white adipose tissue mass and induced adiponectin secretion (23). In fact, recent studies have suggested that *Il-15* functions in a muscle-to-fat endocrine axis to reciprocally regulate muscle/adipose tissue mass and to control insulin sensitivity (23). The repression of *Il-15* in the *Coup-Tf*-siRNA cell line provides further support for a critical regulatory role of this orphan NR in metabolism. Moreover, the link between *Coup-Tfs* and *Il-15* in muscle, and the role of *Il-15* in the muscle/fat axis and insulin sensitivity correlates with the effects of the pancreatic *Coup-TfII* "knock-out" on glucose intolerance (11). The attenuation of the *Pepck-1* mRNA in the *Coup-Tf*-siRNA cells is consistent with previous studies demonstrating *Coup-TfII*-dependent induction of *Pepck-1* transcription by glucocorticoids (2, 35).

We report that the attenuation of *Coup-TfI* and *II* in C2C12 cells modulates key genes involved in lipid utilization. Specifically, we observed that the mRNA encoding uncoupling proteins 1 and 3 (*Ucp1* and *Ucp3*) were significantly repressed and activated, respectively, in the *Coup-Tf*-siRNA stable cells. In this context the C2C12 *Coup-Tf*-siRNA cells expressed significantly elevated steady state levels of total ATP (~5-fold) relative to the negative 3.1 control cells. This may correlate with the reduction of *Ucp1* mRNA expression in these cells. Along these lines ectopic overexpression of *Ucp1* in HepG2 cells demonstrated a 23% reduction in ATP steady state levels (36). We should note that *Ucp1* is almost exclusively expressed in brown adipose tissue. However, studies have reported *Ucp1* mRNA expression is induced during myogenesis in C2C12 skeletal muscle cells (22, 37) and demonstrated that *Ucp1* expression in muscle increases in a pathophysiological context. For example, *Ucp1* is up-regulated in the soleus of the *kyphoscoliosis* (*ky*)

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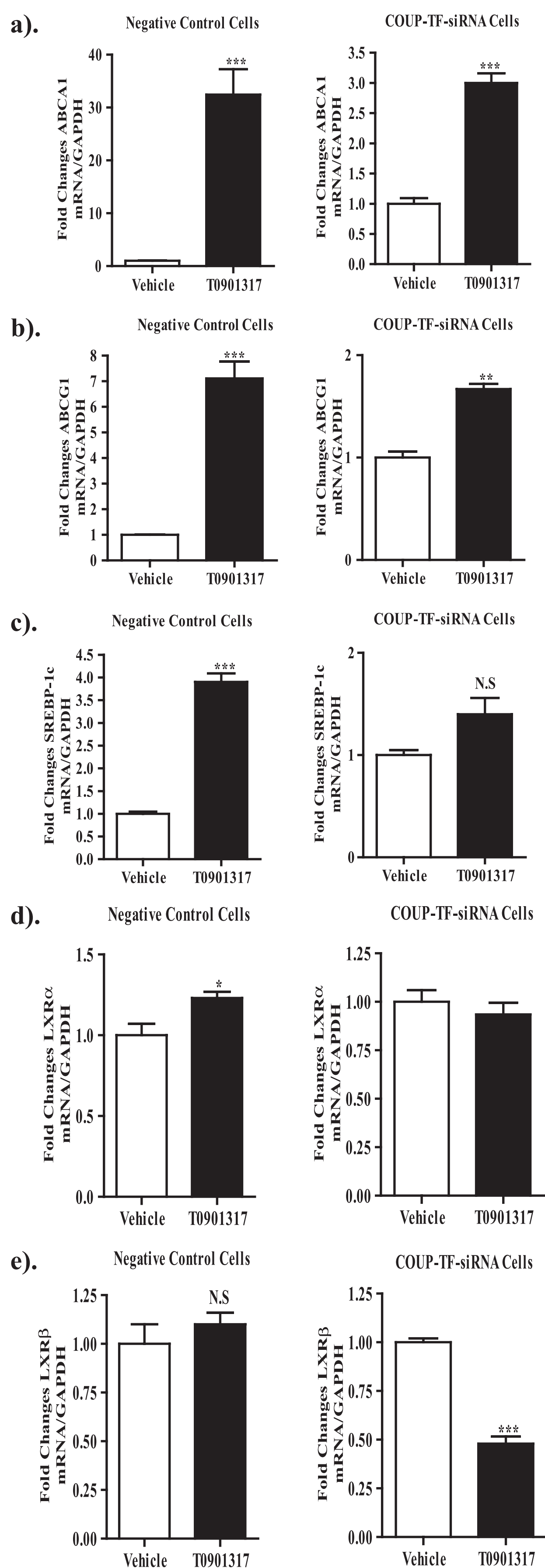

FIGURE 6. ***Coup-Tf*-siRNA expression represses *Lxr*-mediated activation of *Abca1*, *Abcg1*, and *Srebp-1c*.** C2C12 differentiated myotubes were treated with vehicle (Me_2SO) or 10 μmol/liter of the LXR agonist, T0901317, for 24 h. Total RNA was extracted and Q-RT-PCR was performed. *a–e*, *Abca1*, *Abcg1*, *Srebp-1c*, *Lxrα*, and *Lxrβ* mRNA expression, respectively, in the pSilencer 3.1 negative control and the *Coup-Tf*-siRNA. Mean ± S.D. of three independent stable cell preparations are shown. Significance was calculated using the Student's *t* test, where *, 0.01–0.05; **, 0.001–0.01; and ***, $p = < 0.0001$. *N.S.* indicates not significant. Note: for each experiment in the negative control and the *Coup-Tf*-siRNA, the Me_2SO vehicle was set at 1.

mouse mutant (38), a strain with muscular dystrophy localized to type I muscle fibers.

Ucp3 is highly expressed in skeletal muscle, in contrast to the role of *Ucp1* in β-oxidation associated energy expenditure; the role of the *Ucp3* is less understood. Ectopic and overexpression of *Ucp3* in transgenic mice and in cell culture (39–43) suggest that *Ucp3* is involved in adiposity, and preferential lipid utilization. Our study indicates that repression of *Coup-Tfs* leads to a significant increase in *Ucp3* mRNA expression. Although, a paradoxical observation in the context of reduced palmitate oxidation, *Fabp3*, *Cpt-1*, and *Pparα* expression; induction of *Ucp3* has been reported as a compensatory response to the attenuation of *Ucp1* expression in a non-obese *Ucp1* null mice model (44). From this perspective, *Ucp3* may be implicated in a more protective role in the mitochondria against lipid-induced oxidative damage. Second, the expression of the *Ucp3* (a *Pparα* target gene) is surprisingly maintained in the skeletal muscle of *Pparα* null mice (45). These reports are in concordance with our observations.

The observed significant repression of classical LXR target genes involved in reverse cholesterol efflux and lipogenesis (*Abca1*, *Abcg1*, and *Srebp-1c etc.*) in the *Coup-Tf*-siRNA-transfected cells is of interest for several reasons. First, *Coup-TfII* has been demonstrated to activate the cholesterol 7α-hydroxylase (*CYP7A1*) (6, 46), and the cholesterol ester transfer protein (*CETP*) gene promoters (8), important genes in cholesterol homeostasis. Second, the *Cyp7a1* and *Cetp* genes have also been demonstrated to be directly trans-activated by LXR agonists (47, 48). Indeed, studies of $Lxr\alpha^{-/-}$ null mice failed to induce the expression of the *Cyp7a1* (49), which has also been demonstrated to be dependent on *Coup-Tf* expression (6, 46, 50, 51). This suggested to us the existence of cross-talk between and *Lxr* and *Coup-Tf* signaling in muscle. In concordance with this hypothesis we clearly observed that repression of the *Coup-Tfs* significantly diminished the classical and very reproducible activation of *Abca1* (mRNA and promoter), *Abcg1*, and *Srebp-1c* mRNA (20, 52) expression by the selective LXR agonist, T0901317, independent of major changes in *Lxrα* and *Lxrβ* expression. Moreover, this was consistent with no change in intracellular cholesterol levels in the *Coup-Tf*-siRNA cell line (in contrast to the negative control) after treatment with the LXR agonist (T0901317). In humans, mutations in the *ABCA1* gene have been linked to Tangier disease, where these patients have a decreased ability to efflux cholesterol and consequently accumulate cellular cholesterol (53). Recently, it has been shown that a *Pparα-Rxr-Lxr* axis in mouse liver modulates an overlapping set of genes involved in both fatty acid catabolism and synthesis (54). This may also be true in our C2C12 cell model

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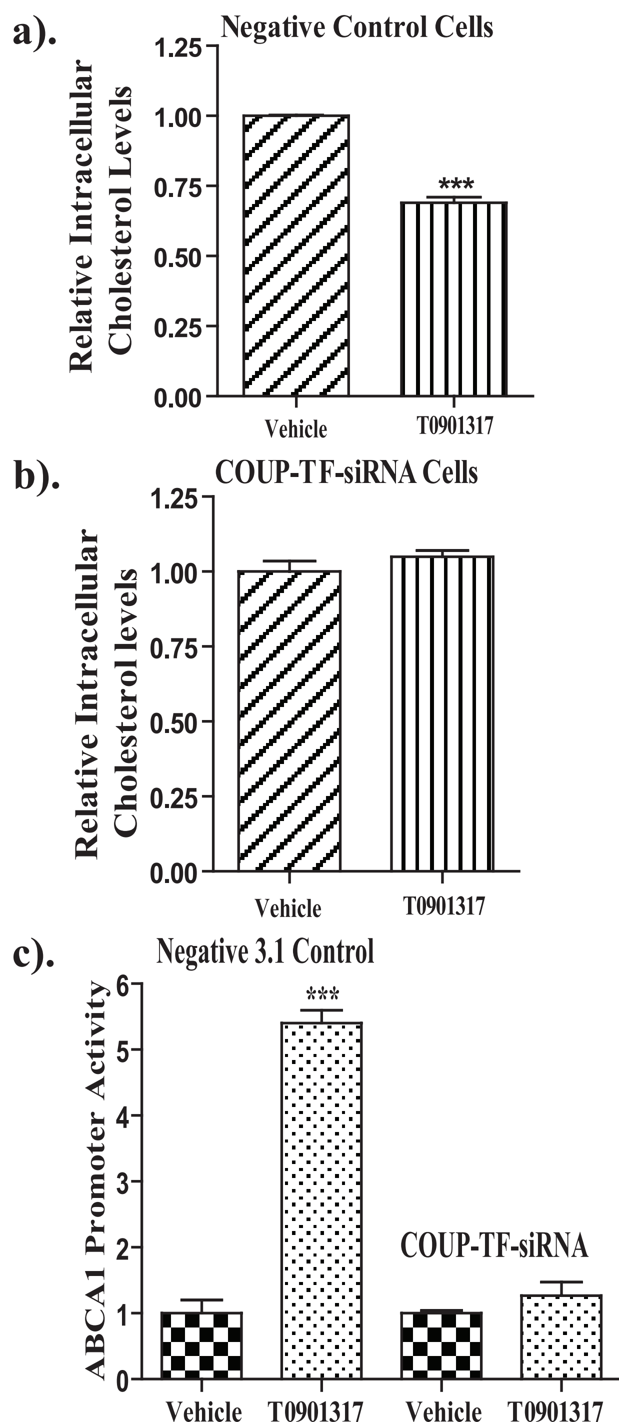

FIGURE 7. ***Coup-Tf*-siRNA expression represses *Lxr*-mediated changes in cholesterol levels and ablates the activation of the *ABCA1* promoter in the *Coup-Tf*-siRNA cells.** *a* and *b*, C2C12 differentiated myotubes were treated with vehicle (Me_2SO) or 10 μmol/liter of the LXR agonist, T0901317, for 24 h. Total intracellular cholesterol and cholesterol esters were measured as previously described. Relative intracellular cholesterol levels in the negative 3.1 control (*a*) and *Coup-Tf*-siRNA cells (*b*), respectively. *c*, -fold changes in *ABCA1* promoter activity following T0901317 treatment in the negative control and the *Coup-Tf*-siRNA-transfected cells was recorded. Mean ± S.D. was derived from three independent cell preparations (each analyzed 6 times). Significance was calculated using the Student's *t* test; ***, $p < 0.0001$.

whereby the ablation of *Coup-Tf* and subsequent *Pparα* attenuation results in the modulation of LXR-dependent genes involved in metabolism.

A number of studies have demonstrated a role for *Coup-Tfs* in cholesterol metabolism. For example, *Coup-TfII* was shown to abrogate the transactivation of reporter constructs containing the *ApoA-II*, *ApoB*, and *ApoC-III* gene promoters (55). These proteins are major components of the high density lipoproteins and are critical for their role in transporting cholesterol from extrahepatic tissues to the liver for processing and excretion in the form of bile acids. Moreover, they are intrinsically linked with the pathogenesis of atherosclerosis. The ability of *Coup-Tf* to modulate *Lxr*-inducible gene expression involved in cholesterol homeostasis may have utility in the future (if novel small molecule regulators are identified) in the context of cholesterol homeostasis.

In summary, these links between the *Coup-Tfs*, *Lxr*, and cholesterol efflux in skeletal muscle are consistent with other studies on *Coup-Tf-* and *Lxr*-mediated gene regulation and cholesterol homeostasis in several other major mass tissues. Moreover, these studies underscore the role of *Coup-Tfs* in skeletal muscle lipid utilization and mobilization, and provide a robust system to further study the molecular mechanisms involved in Coup-Tf/Lxr-dependent control of cholesterol homeostasis. Further studies are underway to examine the role of these orphan NRs in tissue-specific animal models.

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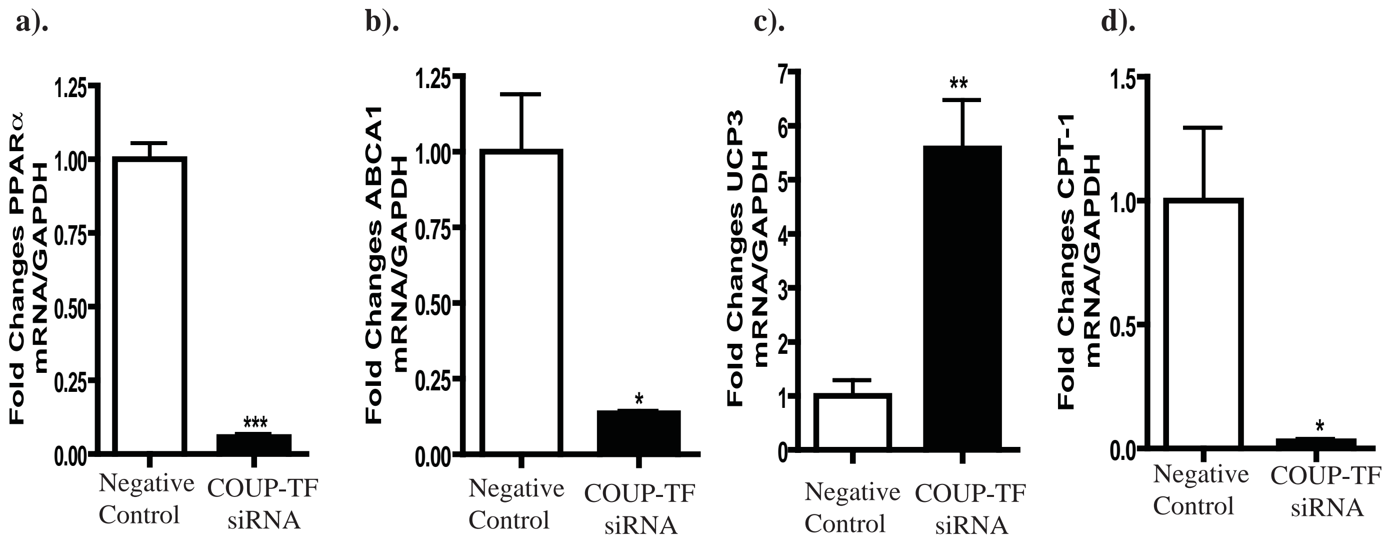
Supplementary Figure 1.



Supplementary Figure 1: Transient transfection of COUP-TF-siRNA in C2C12 cells invokes similar responses to those observed in the stable C2C12 COUP-TF-siRNA cells. To establish that the modulation of key genes in the C2C12 stable COUP-TF-siRNA cells was not an artifact of the G418 selection process, Q-RT-PCR was performed on a number of these genes from transient studies. **a.** PPAR alpha, **b.** ABCA1, **c.** UCP3, and **d.** CPT-1. Fold changes of gene expression are relative to GAPDH. The mean +/- S.D of three independent cell preperations are shown. Significance was calculated using the Student's t-test where * p = 0.01-0.05, ** p = 0.001-0.01 and *** p < 0.0001.

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