Alternative mRNA Splicing of Corepressors Generates Variants That Play Opposing Roles in Adipocyte Differentiation*

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Background: The SMRT and NCoR corepressors play key biological roles in transcriptional repression.

Results: Alternative mRNA splicing produces corepressor variants that can exert opposite effects on adipocyte differentiation.

Conclusion: Corepressors are diversified by alternative mRNA splicing, allowing one locus to encode multiple proteins with distinct functions.

Significance: Changes in alternative splicing may help drive the differentiation and customize the physiology of specific cell types.

Many transcription factors function bidirectionally and are able to either activate or repress the target genes to which they are recruited. This transcriptional plasticity reflects the ability of these factors to recruit auxiliary proteins, denoted corepressors and coactivators, that mediate, in turn, the specific molecular events responsible for modulating gene expression up or down (1–3). Many coactivators function by inserting activation marks into chromatin, such as histone H3-K9 acetylation (4–10). Conversely, corepressors often recruit histone deacetylases and other enzymatic activities that generate a repressive histone code (11–15). Additional corepressor and coactivator mechanisms also contribute to the control of gene expression, including chromatin remodeling and interactions with the general transcriptional machinery (1, 16–19).

The bimodal nature of gene regulation is particularly evident in the actions of the nuclear receptor family of ligand-controlled transcriptional factors. Nuclear receptors play crucial roles in metazoan reproduction, development, and homeostasis and include receptors for a wide range of endocrine hormones, metabolic intermediates, and xenobiotics (20–25). Many nuclear receptors recruit corepressors and can repress target genes in the absence of a ligand; conversely, addition of a ligand agonist alters the receptor conformation so as to induce corepressor release, coactivator binding, and activation of target gene expression (2, 3, 9, 13, 26–31). Notably, the chemical nature of the ligand, the sequence of the DNA binding site, other cellular signaling pathways can influence the strength and the direction of the transcriptional response.

NCoR and SMRT are among the best characterized corepressors, and play critical roles in mediating transcriptional repression by a wide variety of nuclear receptors (11, 13–15, 32–34). NCoR and SMRT are encoded by two related genetic loci and share ~50% amino acid identity (35–39). Both serve as key nucleating proteins that assemble a series of architectural and enzymatic proteins, HDAC3, TBL1, TBLR1, and GPS2, into a larger corepressor holocomplex (40–49). The corepressor holocomplex is then tethered to its nuclear receptor partners through defined receptor interaction domains (RIDs)² present on the NCoR or SMRT subunit (50–58). Therefore it is the specificity of the SMRT or NCoR RIDs for a given nuclear receptor that determines whether that receptor can recruit the corepressor holocomplex or not (e.g. Refs. 18, 39, 59–62).

We and others have reported that both SMRT and NCoR are expressed by alternative mRNA splicing to generate a diverse series of corepressor protein variants (18, 63–71). These splice

2 The abbreviations used are: RID, receptor interaction domain; PPAR, peroxisome-proliferator-activated receptor; TR, thyroid hormone receptor; IBMX, 3-isobutyl-1-methylxanthine; MEF, mouse embryonic fibroblast; Dex, dexamethasone; LXR, liver X receptor.
variants differ in the number and sequence of their RID domains, in the presence or absence of interaction surfaces for additional components of the corepressor holocomplex, in their affinity for different nuclear receptor partners, and in their response to protein kinase signals operating in cells (18, 63–71). To better understand the impact of these alternative splicing events in a biologically relevant context, we turned to a study of adipocyte differentiation. The 3T3-L1 pre-adipocyte cell line can be induced to differentiate into terminal adipocytes in culture (72). A series of nuclear receptors plays key roles in this differentiation phenomenon, including peroxisome-proliferator-activated receptors (PPARs), Rev-Erb, COUP-TFII, and thyroid hormone receptors (TRs) (25, 28, 73–81). Agonists for certain of these receptors can accelerate the differentiation process (e.g. Refs. 82–86), suggesting that corepressors bound to unliganded receptors may serve to inhibit adipogenesis in the pre-adipocyte. Consistent with this hypothesis, a pan-specific siRNA knockdown of NCoR or SMRT in 3T3-L1 cells, or site-specific mutagenesis of these corepressors in mice, can enhance adipogenesis (87–89). However, none of these previous studies examined the contributions of the individual corepressor splice variants to this phenomenon.

We report here a dissection of the specific roles that individual SMRT and NCoR splice variants play in regulating the 3T3-L1 adipogenesis pathway. Our results indicate that the different corepressor variants are expressed at different levels in pre-adipocytes and that the relative abundance of certain variants changes as these cells differentiate into terminal adipocytes. We determined that these different corepressor variants display disparate affinities for the nuclear receptors that regulate adipogenesis, and, most notably, certain corepressor variants accelerate, whereas others suppress 3T3-L1 differentiation. Consistent with these divergent effects on the overall adipogenesis program, different corepressor variants regulate distinct panels of target genes. Our results indicate that alternative splicing of the SMRT and NCoR corepressors serves to diversify their biological functions, with different splice variants serving different roles in adipogenesis. Furthermore, we propose that programmed alterations in corepressor splicing may help switch the transcriptional environment from one that maintains the pre-adipocyte in an undifferentiated state to a new transcriptional context that permits differentiation and helps establish the proper physiology of the mature adipocyte.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmid pFN21Ahg-EGFP was created by first introducing the enhanced green fluorescent protein coding sequence from pEGFP-C1 (GenBank™ accession number U55763) into the mammalian HaloTag expression plasmid, pFN21A (Promega, Madison, WI), using PCR to add the required SgfI and Pmel restriction sites to the open reading frame of each corepressor isoform and subsequently cloning into pFN21Ahg-EGFP, removing the GFP encoding DNA fragment.

Cell Culture and Stable Transfection of 3T3-L1 Cells—Undifferentiated murine 3T3-L1 cells were grown in DMEM containing 9% newborn calf serum (JR Scientific, Woodland, CA) at 37 °C and 5% CO₂. Cells were passaged or re-fed every 3 days and never allowed to reach >80% confluence prior to differentiation. Stable 3T3-L1 transformants were created by transfecting 5 × 10⁴ 3T3-L1 cells/35-mm plate with 2.5 μg of SwaI-linearized pFN21Ahg-EGFP, pFN21Ahg-NCoRΔ, or pFN21Ahg-NCoRw, using 7.5 μl of TransIT-LT1 (Mirus Bio, Madison WI) according to the manufacturer’s protocol. After 24 h the transfected cells were trypsinized and plated into a 100-mm plate. After an additional 24 h, hygromycin B was added to a final concentration of 500 μg/ml. Media (containing 500 μg/ml hygromycin B) was replenished every 7 days until visible colonies formed. After colony formation was observed, cells were passaged to a new 100-mm plate and grown until the cells reached ~80% confluence, at which point the cells were plated for differentiation.

3T3-L1 Differentiation—3T3-L1 cells were differentiated as previously described (90). Briefly, 3T3-L1 cells or stable transformants were plated at 2.5 × 10⁴ cells/cm². Once cells became confluent (2–3 days), medium was replaced with DMEM containing 9% fetal bovine serum (PPA Laboratories, Dartmouth, MA). Two days later (defined as differentiation day 0) the medium was replaced with medium containing DMI, the differentiation-inducing mixture (0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone (Dex), and 10 μg/ml insulin). At differentiation day 2 (after 48-h exposure to DMI) the medium was replaced with DMEM containing 9% fetal bovine serum and 10 μg/ml insulin. Cells were re-fed with medium containing insulin every 3 days (differentiation days 5 and 8). For 3T3-L1 cells differentiated with Rosiglitazone, DMEM containing 9% fetal bovine serum and 2.5 mM rosiglitazone was used in place of the DMEM containing insulin after DMI treatment.

Oil Red O Staining and Quantification—Differentiated 3T3-L1 transformants (day 8) were fixed and stained with Oil Red O to visualize lipid accumulation (91). Cells were then counterstained with hematoxylin. The extent of Oil Red O staining was quantified from multiple microscopic fields (n = 6) of each transformant using the Color Threshold and Analyze Particle functions of NIH ImageJ (version 1.45k) (92). The means ± S.E. of multiple microscopy fields from three independent transformants of NCoRΔ, NCoRw, and GFP were plotted.

Cell Proliferation Analysis—The growth rate of 3T3-L1 stable transformants was analyzed using CellTiter 96 Aqueous One Solution (Promega). Stable corepressor isoform (or GFP) 3T3-L1 transformants were plated at 1 × 10³ cells/well in replicate wells of a 96-well plate. Cell proliferation was measured 24 and 48 h after plating according to the manufacturer’s protocol. Media on the remaining wells was replaced after 48 h, and cell proliferation was measured in the remaining wells 72 and 96 h after plating.
GST Pulldown Assays—Glutathione S-transferase fusion proteins with the receptor interaction domains of SMRT (amino acids 1851–2473 of SMRTα (GenBank™ accession number AF113001) or equivalent) and NCoR (amino acids 1817–2453 of NCoRα (GenBank™ accession number U35312) or equivalent) were expressed in SF9 cells using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA). Whole cell extracts were prepared from 3 × 10^8 infected SF9 cells grown in 50 ml of suspension culture using Insect-XPRESS (Lonza, Walkersville, MD) medium containing 9% fetal bovine serum. Three days after infection, SF9 cells were collected, rapidly frozen in liquid nitrogen, and thawed on ice before resuspending in WCE buffer (20 mM HEPES (pH 7.9), 200 mM KCl, 1 mM EDTA, 0.5% IGEPAL CA-630, 10% glycerol, 1 mM dithiothreitol, 1 × Complete Protease Inhibitor mixture (Roche Applied Science)). Resuspended cells were again frozen in liquid nitrogen and thawed on ice. Lysates were cleared by centrifuging at 14,000 × g for 5 min at 4 °C. In vitro “GST pull-down” protein-interaction assays were preformed as previously described (93).

RT–PCR Analysis of Corepressor Isoform Expression—RNA for corepressor isoform RT–PCR and quantitative RT–PCR was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Complimentary DNA (cDNA) was synthesized from 1 μg of total RNA using a Quantitect Reverse Transcription kit (Qiagen). For corepressor isoform analysis, cDNA equivalent to 12.5 ng of RNA was amplified for 32 cycles using ethidium bromide and a 1.5% agarose/TAE gel and quantified using an Alpha Innotech Fluorchem 8900 and AlphaEase software (version 3.1.2).

Quantitative PCR Assay for Adipogenic Gene Regulation—Analysis of gene expression in pre- and post-differentiated 3T3-L1 cells was performed by quantitative RT–PCR using an Opticon2 real-time thermal cycler, SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) and the primer sets listed in supplemental Table S1A. PCR products were visualized using ethidium bromide and a 1.5% agarose/TAE gel and quantified using an Alpha Innotech Fluorchem 8900 and AlphaEase software (version 3.1.2).

Mouse Embryonic Fibroblast Isolation—Embryonic fibroblasts (MEFs) from wild-type C57BL/6 and transgenic mice engineered to only express the exon 37b – form of NCoR were isolated according to the method developed by Todaro and Green (94).

RESULTS

The Relative Abundance of the Different Corepressor Splice Variants Differs in Different Tissues and Cell Lines—The mRNA transcripts from both the SMRT and the NCoR loci are subject to alternative splicing (18, 63–70); the NCoR and SMRT splice variants that are the focus of this study are indicated in Fig. 1. These alternative splicing events are particularly extensive within the cRID regions of these corepressors and can generate NCoR variants that contain two or three distinct cRID domains, and SMRT variants that contain one, two, or three cRIDs, plus an additional SMRT variant in which the regions flanking RID1 have been deleted while preserving the interaction motif itself (Fig. 1). Additional alternative splicing events occur within the N-terminal and central regions of the corepressors that may alter docking surfaces for other components of the corepressor complex. Our nomenclature refers to both the specific splicing events that occur at the individual exons (e.g. whether SMRT exon 40b – or 40b+ is utilized) and to how these individual splice events are assembled together into the final mRNA/open reading frame (e.g. SMRTα is defined as the product of the assembly of exon 28+, 40b+, and 47b+). Notably, splicing at each splice site appears to not influence that at other splice sites (68).

We employed RT–PCR and primers that flank these alternative splice sites to determine the relative utilization of these different splicing events in different mouse tissues (Fig. 2). Certain alternative mRNA splicing events occurred at approximately the same frequency in all tissues examined; for example, the ratio of the SMRT 28+ to 28– splice form was near identical across all samples tested. Other alternative mRNA splicing events proved to be highly tissue-specific. For example, inclusion of the SMRT 40b+ exon (and its encoded RID3) prevailed in the splicing pattern in brain and testes but was relatively rare in other tissues (Fig. 2). Inclusion of the NCoR 37b+ exon, which encodes an RID3 related to that in the SMRT 40b+ exon, generally paralleled the SMRT 40b + tissue expression pattern (Fig. 2). Reciprocally, the SMRT 47b– splice (which deletes flanking sequences from the S1 CoNR box in RID1) was rare in brain and testes, but high in heart, spleen, lung, skeletal muscle, and adipose tissue (Fig. 2). Significantly, the SMRT and NCoR sequences that are generally considered to be the prototypes for these corepressors are not necessarily the most abundant or most widely expressed splice forms. For example, the 37b+ NCoRα variant, which encodes three cRIDs, is frequently cited and employed experimentally as the NCoR prototype; however, NCoRα is strongly expressed only in brain and testes, whereas NCoRδ, which contains only two cRIDs, predominates in all other tissues tested (Fig. 2).

Differentiation of Pre-adipocytes to Adipocytes Is Associated with a Shift from the 37b+ to 37b– NCoR Splice Variant—We next extended our RT–PCR analysis to 3T3-L1 cells, a widely used ex vivo model of adipogenesis (72) (Fig. 3). Although the pattern of corepressor variants expressed in the 3T3-L1 pre-adipocytes generally paralleled that observed in white adipose tissue in vivo, the ratio of the NCoR 37b+ splice to that of the 37b– splice was significantly higher in the former (Fig. 2). Notably, however, inducing differentiation of the 3T3-L1 cells into terminal adipocytes progressively reduced the ratio of the NCoR 37b+ splice to the 37b– splice over the 8-day differentiation period to more closely resemble that observed in differentiated white adipose tissue in vivo (Fig. 3A). This was also true in response to rosiglitazone, a PPARγ agonist known to enhance 3T3-L1 adipogenesis (Fig. 3B). The relative expression of the other corepressor variants examined was either minimally altered (e.g. SMRT 40b+/40b–) or not significantly altered under the same conditions. We conclude that adipocyte differentiation is accompanied by a decrease in the ratio of
NCoR corepressor splice forms that include RID3 versus those that do not.

Different SMRT and NCoR Splice Variants Possess Different Affinities for the Different Nuclear Receptors Involved in Adipogenesis—The differentiation and subsequent function of adipocytes are tightly regulated through a network of nuclear receptors; we therefore next examined the ability of the different splice forms of SMRT and NCoR to interact with the different members of this network; we tested these first in the absence of ligand (Fig. 4, solid bars; additional statistical analysis is presented in supplemental Table S2). Notably, each nuclear receptor displayed its own unique pattern of interaction with the different corepressor variants tested. For example, in the absence of ligand, PPARγ interacted significantly more strongly with corepressor splice variants containing RID3 (the 37b+/NCoRδ and 40b+/SMRTγ) compared with otherwise identical corepressor splice variants lacking RID3 (the 37b−NCoRδ and 40b−SMRTα) (Fig. 4). In contrast, recognition of corepressor by LXRα appeared to be largely independent of the presence or absence of RID3 (e.g. compare LXRα binding to NCoRω versus NCoRδ, and SMRTγ versus SMRTα; Fig. 4). Conversely, excision of sequences could also increase the inter-

FIGURE 1. Alternative corepressor mRNA splicing. A, schematic of alternative mRNA splicing at the NCoR and SMRT loci (GenBank™ Accession numbers NM_011308.2 and NM_011424.2). Open reading frames are indicated by black lozenges; 5′ and 3′ untranslated regions are indicated by gray lines. Receptor interaction domains (RIDs) are shown as ovals. Vertical white lines indicate exon/exon boundaries (numbered in base pairs relative to the start of transcription). Exons removed (Δ) or added (Δ+) relative to the reference sequence by alternative splicing are indicated below each schematic. Note that our standardization on the RefSeq database in this report results in a change in the SMRT exon numbering system compared with our prior publications. B, schematic of individual corepressor proteins of interest. Exon sequences included in each corepressor protein are shown as thick lozenges; exon sequences deleted by alternative mRNA splicing are depicted as thin horizontal lines. RIDs are shown as ovals. Nomenclature has been described previously (69).
action between specific corepressor variants and specific nuclear receptors; for example, the 47b/SMRT variant, lacking a region flanking RID1, was bound more strongly by both PPAR and LXR than was the otherwise identical SMRT/containing this region (Fig. 4).

We also tested several of these nuclear receptors for the ability to release the different corepressor variants in response to ligand agonists (Fig. 4, open bars). In many cases the efficiency of release varied with the corepressor variant. For example, treating RAR with saturating concentrations of all-trans retinoic acid released SMRTγ, NCoRα, and NCoRβ, more completely than SMRTε, SMRTγ, or SMRTα. Similarly, TRα and PPARγ released certain corepressor variants more efficiently than others in response to cognate agonists. LXRα and Farnesoid X Receptor-α, however, displayed little or no release of any of the corepressor variants tested in response to the agonists employed. These divergent responses are likely to reflect differences in the conformational changes invoked in each nuclear receptor by its cognate agonist, and differences in the specific

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**FIGURE 2.** Relative expression of different corepressor splice variants in different murine tissues. Messenger RNA was isolated from the organs and tissues indicated and was subjected to RT-PCR using primers spanning the relevant splice sites (see Fig. 1A and supplemental Table S1A); the PCR products were resolved by gel electrophoresis and quantified to determine the percentage of each alternatively spliced mRNA produced at each splice site (total = 100%). The means ± S.E. (n ≥ 3) are presented. Skel Mscl, skeletal muscle; WAT, white adipose tissue; BAT, brown adipose tissue.

**FIGURE 3.** Relative expression of different corepressor splice variants during 3T3-L1 cell adipogenesis. Messenger RNA was isolated from 3T3-L1 cells at different times after inducing adipocyte differentiation with: A, Dex/insulin/IBMX followed by insulin, or B, Dex/insulin/IBMX followed by rosiglitazone. The two protocols differ beginning differentiation Day 3, so differentiation Days 0 and 2 in both panels represent the same cell populations. The mRNA was subjected to RT-PCR using primers spanning the relevant splice sites and was analyzed as in Fig. 2 to determine the percentage of each alternatively spliced mRNA produced at each splice site. The means ± S.E. (n ≥ 3) are presented.
interaction surfaces through which the different corepressor variants are bound.

**Different Corepressor Splice Variants Can Exert Opposite Effects on Adipogenesis**—Given their different affinities for different nuclear receptors, and their change in relative abundance during adipocyte differentiation, we chose to examine the roles of the NCoR/H9275 and NCoR/H9254 splice variants in greater detail. We created stable 3T3-L1 cell transformants expressing each splice variant, or a GFP control, from a stably integrated expression vector. Integration and expression were confirmed by PCR and RT-PCR, and a minimum of 10 individual hygromycin-resistant colonies was combined for each construct to minimize any potential clone to clone variability. The ectopically introduced NCoR/H9254 and NCoR/H9275 variants were expressed at comparable levels in their respective transformants and resulted in detectable changes in the overall ratio of NCoR/H9275 versus NCoR/H9254 compared with the control cells (Fig. 5, A and B); mRNA expression and splicing at the endogenous SMRT locus was unaffected (data not shown).

The GFP control, NCoR/H9254, and NCoR/H9275 transformants were indistinguishable by cell morphology and by most other criteria. Interestingly, however, quantitative proliferation studies revealed that transformants expressing the NCoR/H9254 variant propagated with a slower doubling time and achieved a lower...
Corepressor Splicing and Adipogenesis

The Divergent Effects of NCoRω and NCoRγ on Adipogenesis Are Also Observed Using a Splice-specific Knock-out Approach—
To further investigate the biological roles of the different NCoR splice variants, we created mice bearing an inactivating mutation of the 37b+ splice donor, thereby preventing expression of the NCoRω variant and replacing it with comparable expression of the NCoRδ variant. Mice homozygotic for the mutation were recovered. The absence of the NCoRω transcript, and the presence of the NCoRδ transcript, were confirmed in multiple organs by RT-PCR; splicing at the SMRT locus, or elsewhere in NCoR, was not detectably affected (data not shown). Although a comprehensive phenotyping of these animals at the organismal level remains in progress, we were able to recover MEFs from these versus matched wild-type animals and compare their ability to differentiate into adipocytes ex vivo. The loss of NCoRω in the MEFs resulted in a pro-adipogenesis phenotype, generating Oil Red O-positive cells at significantly higher incidence than was observed in wild-type MEFs (Fig. 7, A and B). This phenotype was reproducible in multiple MEF cultures from multiple embryos. NCoRδ, but not NCoRω expression was detected in the knock-out MEFs, and the expression of SMRT splice variants was unaffected (Fig. 7C and data not shown). These results, which are reciprocal to the inhibition of 3T3-L1 adipogenesis observed when NCoRω was overexpressed, support the proposal that NCoR splice variants containing RID3 inhibit adipogenesis, whereas NCoR splice variants lacking RID3 are pro-adipogenic.

Different Corepressor Variants Target Distinct Genes—Given their opposing biological effects in our Oil Red O experiments, we next examined the effects of the NCoRω versus NCoRδ splice variants at the transcriptional level. We analyzed the mRNA expression profiles of our GFP, NCoRω, and NCoRδ 3T3-L1 transformants at Days 0 and 8 after DMI induction. We used RT-PCR and focused on a panel of genes previously shown to be associated with adipogenesis. As expected from prior studies of adipogenesis, the majority of the genes in this panel were induced by Day 8 in our control transformants (Fig. 8; additional statistical analysis is provided in supplemental Table S3); the corresponding gene products include proteins involved in carbohydrate/lipid metabolism, storage, or transport (e.g. Acs12, ATGL, CD36, Fabp4, FAS, Hmgcl, HSL, Lpl, Me1, Pck1, Pcx, Plin, and Srebfl1), adipocyte-encoded cytokines (e.g. Adipoq and Retn), mitochondrial uncoupling proteins (e.g. UCP3), and a series of nuclear receptors and related transcriptional factors known to participate in adipocyte differentiation and function (e.g. Cebpα, LXRα, PGC1α, PPARγ, and Zfp423). Consistent with the ability of NCoRω to inhibit adipogenesis in our Oil Red O assay, the expression of the majority of these genes was reduced in the NCoRω transformants compared to the GFP control (Fig. 8). Similarly, given the enhanced Oil Red O phenotype of our NCoRδ transformants, the expression of many of these genes was elevated, or at least equal, in the NCoRδ transformants compared with the GFP control and were clearly distinct from the NCoRω transformants (Fig. 8). Nonetheless, the extent of repression by NCoRω, or of induction by NCoRδ, appeared to vary somewhat from gene to gene. These results indicate that the individual corepressor splice variants exert distinct, often opposing effects on gene expression that, by dif-

saturation density than did the NCoRδ or the GFP transformants (Fig. 5C).

We next induced the adipogenic program in these cells using the DMI protocol. The 3T3-L1 cells transformed by the GFP vector withdrew from the cell cycle and began accumulating lipids, as detected by Oil Red O staining. By day 8 after induction many or most of the empty vector transformants displayed the rounded morphology, weaker adhesion, and large Oil Red O-positive lipid droplets characteristic of terminally differentiated adipocytes (Fig. 6). Cells transformed by the NCoRω construct, in contrast, were significantly impaired in adipogenesis based on both morphological criteria and Oil Red O staining (Fig. 6). This result is consistent with the converse accelerated adipogenesis reported using pan-specific siRNAs to suppress all forms of NCoR in these cells (89). Unexpectedly, however, cells transformed by NCoRδ variant displayed the diametrically opposite effect, undergoing adipogenesis more rapidly and more completely than did the GFP vector control (Fig. 6). These results indicate that alternative splicing can either oppose or enhance 3T3-L1 adipogenesis depending on the incorporation, or excision, of exon 37b+ sequences from the final NCoR protein. Similar results were observed using HA- or Myc-tagged versions of NCoRω and NCoRδ (data not shown).

FIGURE 6. Inhibition of 3T3-L1 cell adipogenesis by NCoRω and enhancement by NCoRδ. A, representative microscopic fields. Cultured 3T3-L1 transformants expressing GFP, NCoRω, or NCoRδ were induced using Dex/insulin/IBMX and stained for lipid accumulation on differentiation Day 8 using Oil Red O. Cell morphology was visualized by hematoxylin counterstain. Representative photomicrographs are presented from three independent experiments. B, quantification. Oil Red O staining was quantified in the experiments described in A by digital imaging of microscope fields and the use of ImageJ software, as described under “Experimental Procedures.” The means ± S.E. from multiple microscope fields (n = 6 per transformant) from each of three independent experiments are shown. Oil Red O staining of the NCoRω and NCoRδ cells differed statistically from one another and from the control cells, all with a p value of <0.001.

44994 JOURNAL OF BIOLOGICAL CHEMISTRY
Complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapping sets of transcription factors that can assemble into near-identical corepressor complexes, interact with overlapin...
corepressor variants exhibit significant differences in their abilities to bind to different nuclear receptors. These differences, in part, reflect differences in the ability of the individual RID domains within each corepressor variant to bind to a given nuclear receptor. For example, TRs are known to bind strongly to an isolated RID3 construct and more weakly to isolated RID1 or RID2 constructs (39, 51, 52, 54–56, 58, 59, 62, 101–103). Therefore, it is not unexpected that TRα binds more strongly to corepressor splice variants that contain RID3 (such as NCoRα) compared with those that do not. Conversely, RARα is known to bind strongly to an isolated RID2 and weakly or not at all to isolated RID1 or RID3; given that all the corepressor variants tested include RID2 (53, 57), it is not surprising that RARα bound relatively broadly to multiple corepressor variants. In addition to splicing, sequence differences between the two corepressor loci themselves also have an effect on the nuclear receptor partnership. For example, RARα and PPARγ both interact more weakly with NCoRα than with SMRTγ, despite these two corepressor variants representing the products of very similar splicing events.

**Adipocyte Differentiation Is Accompanied by Specific Changes in Corepressor Splicing Pattern**—The mouse 3T3-L1 cell line has been extensively exploited as *ex vivo* models of pre-adipocyte to adipocyte differentiation. We report here that inducing adipogenesis in 3T3-L1 is paralleled by a significant shift in the splicing of NCoR from the 37b+ pattern to the 37b− pattern. This decreases the relative expression of NCoR variants containing three RIDS (such as NCoRω) but increases the relative expression of variants containing two RIDS (such as NCoRδ). The expression of the other corepressor splice variants either was not, or was only minimally, altered. Consistent with these findings, differentiated white adipose tissue *in vivo* expresses a low NCoR 37b+ to 37b− ratio. Given that the 37b+ NCoRω suppresses adipocyte differentiation, whereas the 37b− NCoRδ promotes it (below), it is tempting to speculate that this shift in NCoR splicing may help release the block to differentiation in the pre-adipocyte and/or may help establish the transcriptional program necessary for proper physiological function of the mature adipocyte.

**Different Splice Forms of the Same Corepressor Have Opposite Effects on Adipocyte Differentiation**—To examine the role of alternative corepressor splicing in adipogenesis, we tested the effect of overexpressing NCoRω versus NCoRδ in the 3T3-L1 cell system. Consistent with prior demonstrations that pan-specific knockdown of all NCoR (or all SMRT) mRNA enhances 3T3-L1 cell adipocyte differentiation (89), our reciprocal overexpression of ectopic NCoRω inhibited 3T3-L1 differentiation (monitored by Oil Red O staining). In contrast, however, expression of comparable levels of NCoRδ significantly enhanced 3T3-L1 adipogenesis under the same conditions. Therefore, different splice variants of the same corepressor class can mediate profoundly different effects on adipogenesis.

To complement our overexpression approach, we also examined adipogenesis in MEFs isolated from a splice-specific 37b+ knock-out mouse model. We ablated the 37b+ splice donor in these mice by standard site-directed mutagenesis and ES cell techniques, abolishing their ability to make NCoRω, but leaving the 37b− splice donor, the shared splice acceptor, and the NCoRδ reading frame intact. Mice homozygous for the mutation fail to express NCoRω and display a corresponding shift to NCoRδ expression in all tissues examined; splicing of SMRT and elsewhere in NCoR are unaffected. The NCoRω−/ω− mice are viable and display a number of endocrine and metabolic phenotypes that remain under investigation.3 Notably, MEFs isolated from these NCoRω−/ω− mice display an enhanced adipocyte differentiation in culture compared with wild-type MEFs, a result fully consistent with the reciprocal ability of NCoRω overexpression to inhibit, and NCoRδ overexpression to enhance, 3T3-L1 adipogenesis.

**Different Corepressor Variants Generate Different Transcriptional Programs When Expressed in the 3T3-L1 Cells**—To determine if the divergent effects of the different corepressor splice forms on adipogenesis as a whole were re-capitulated at the individual gene level, we examined the expression of a panel of adipocyte-associated gene products in our 3T3-L1 transformants. The majority of these pro-adipogenic genes was strongly induced by Day 8 after Dex/insulin/IBMX treatment in the control and NCoRδ transformants, whereas this induction was severely muted in the NCoRω transformants.

A complex transcriptional network regulates adipocyte differentiation (25, 28, 73–81). Presumably any given corepressor variant associates with its specific nuclear receptor (and non-receptor transcription factor) partners within this transcriptional network, each contributing to the regulation of different target genes, and each contributing to the many individual changes in the overall transcription program that result in adipocyte differentiation. The influence of a given corepressor variant on adipogenesis as a whole is therefore the combinatorial result of these individual effects. It should therefore be noted that the late stage differences in gene expression we report here (on Day 8 after induction) probably reflect both the primary effects of the different corepressor variants and the secondary effects of the divergent differentiation phenotypes. A complete study of gene expression throughout the differentiation process, most likely combined with a chromatin immunoprecipitation assay, will be necessary to fully define which of these genes are directly targeted by which corepressor variant.

**Multiple Loci and Alternative mRNA Splicing Serve to Customize the Transcriptional Program for Specific Purposes in Specific Cell Types**—Some of the divergent effects of NCoRω and NCoRδ on adipogenesis are likely to reflect their different affinities for the nuclear receptors characterized in the current

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3 M. L. Privalsky and M. L. Goodson, unpublished observations.
study, such as PPARγ. Differences in the affinities of these splice variants for additional, as yet untested nuclear receptors and non-receptor transcription factors may also contribute to this phenomenon, as may aspects of corepressor function beyond that of transcription partner preference. For example, NCoR and SMRT are diversified and expressed. Both are frequently encoded by multiple genetic loci that are, in turn, alternatively spliced to generate a series of distinct corepressor variants and nuclear receptor isoforms that vary in relative abundance in different cell types. The gene duplication and divergence events that initiated this diversification appeared for both corepressors and nuclear receptors during the early vertebrate radiation ~300 to 500 million years ago. Both sides of this diversification have served to rewrite the partnership contract: alternative corepressor splicing can alter its affinity for a given nuclear receptor isoform, and alternative nuclear receptor splicing can alter its affinity for a given corepressor variant. Taken as a whole, these mechanisms allow a limited number of receptors and coregulators to don many guises, permitting them to play the many different roles assigned to them by evolution and to change the script as events require it.

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REFERENCES

1. Buranapramest, M., and Chakravarti, D. (2009) Prog Mol. Biol. Transl. Sci. 87, 193–234
2. Lonard, D. M., and O’Malley, B. W. (2007) Mol. Cell 27, 691–700
3. Savkur, R. S., Bramlett, K. S., Clawson, D., and Burris, T. P. (2004) Vitam. Horm. 68, 145–183
4. Freiman, R. N., and Tjian, R. (2003) Cell 112, 11–17
5. Kraus, W. L., and Wong, J. (2002) Eur. J. Biochem. 269, 2275–2283
6. Lee, J. W., Lee, Y. C., Na, S. Y., Jung, D. J., and Lee, S. K. (2001) Cell. Mol. Life Sci. 58, 289–297
7. Oetting, A., and Yen, P. M. (2007) Best Pract. Res. Clin. Endocrinol. Metab. 21, 193–208
8. Rice, J. C., and Allis, C. D. (2001) Curr. Opin. Cell Biol. 13, 263–273
9. Tsai, C. C., and Fendell, J. D. (2004) Vitam. Horm. 68, 93–122
10. Wang, C., Powell, M. J., Popov, V. M., and Pestell, R. G. (2008) Mol. Endocrinol. 22, 539–545
11. Jones, P. L., and Shi, Y. B. (2003) Curr. Top. Microbiol. Immunol. 274, 237–268
12. Karagianni, P., and Wong, J. (2007) Oncogene 26, 5439–5449
13. Lazar, M. A. (2003) Nucl. Recept. Signal. 1, e001
14. Perissi, V., Jepsen, K., Glass, C. K., and Rosenfeld, M. G. (2010) Nat. Rev. Genet. 11, 109–123
15. Stanyia, K. J., and Kao, H. Y. (2009) Cell Div. 4, 7
16. Alenjath, T. Y., Yu, J., and Lazar, M. A. (2006) EMBO J. 25, 3966–3974
17. McKenna, N. J., and O’Malley, B. W. (2002) Endocrinology 143, 2461–2465
18. Muscat, G. E., Burke, L. J., and Downes, M. (1998) Nucleic Acids Res. 26, 2899–2907
19. Wong, C. W., and Privalsky, M. L. (1998) Mol. Cell. Biol. 18, 5500–5510
20. Chawla, A., Repa, J. J., Evans, R. M., and Mangelsdorf, D. J. (2001) Science 294, 1866–1870
21. Flamant, F., Baxter, J. D., Forrest, D., Refetoff, S., Samuels, H., Scanlan, T. S., Vennström, B., and Samarut, J. (2006) Pharmacol. Rev. 58, 705–711
22. Glass, C. K., and Saijo, K. (2010) Nat. Rev. Immunol. 10, 365–376
23. Hager, G. L. (2001) Prog Nucleic Acids Res Mol. Biol. 66, 279–305
24. McEwan, I. J. (2009) Methods Mol. Biol. 505, 3–18
25. Sonoda, J., Pei, L., and Evans, R. M. (2008) FEBS Lett. 582, 2–9
26. Cheng, S. Y., Leonard, J. L., and Davis, P. J. (2010) Endocr. Rev. 31, 139–170
27. Duong, V., Augereau, P., Badia, E., Jalaguir, S., and Cavailles, V. (2008) Adv. Exp. Med. Biol. 617, 121–127
28. Feige, J. N., and Auwerx, J. (2007) Trends Cell Biol. 17, 292–301
29. Hsia, E. Y., Goodson, M. L., Zou, J. X., Privalsky, M. L., and Chen, H. W. (2010) Adv. Drug Deliv. Rev. 62, 1227–1237
30. Privalsky, M. L. (2004) Annu. Rev. Physiol. 66, 315–360
31. Smith, C. L., and O’Malley, B. W. (2004) Endocr. Rev. 25, 45–71
32. Moehren, U., Eckey, M., and Baniahammad, A. (2004) Essays Biochem. 40, 89–104
33. Ordentlich, P., Downes, M., and Evans, R. M. (2001) Curr. Top. Microbiol. Immunol. 254, 101–116
34. Privalsky, M. L. (2008) in: NR Coregulators and Human Diseases (Kumar, R., and O’Malley, B. W., eds) pp. 243–280, World Scientific Publishing, Ltd., Singapore
35. Chen, H., and Privalsky, M. L. (1995) Proc. Natl. Acad. Sci. U.S.A. 92, 422–426
36. Hörlein, A. I., Nää, A. M., Heinzl, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Söderström, M., and Glass, C. K. (1995) Nature 377, 397–404
37. Ordentlich, P., Downes, M., Xie, W., Genin, A., Spinner, N. B., and Evans, R. M. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 2639–2644
38. Sande, S., and Privalsky, M. L. (1996) Mol. Endocrinol. 10, 813–825
39. Seol, W., Mahon, M. J., Lee, Y. K., and Moore, D. D. (1996) Mol. Endocrinol. 10, 1646–1655
40. Alenjath, T., Meyers, K., Mullican, S. E., Leitner, K., Adeniji-Adele, A., Avila, J., Buan, M., Ahima, R. S., Kaestner, K. H., and Lazar, M. A. (2008) Nature 456, 997–1000
41. Downes, M., Ordentlich, P., Kao, H. Y., Alvarez, J. G., and Evans, R. M. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 10330–10335
42. Guenther, M. G., Barak, O., and Lazar, M. A. (2001) Mol. Cell. Biol. 21, 6091–6101
43. Ishizuka, T., and Lazar, M. A. (2003) Mol. Cell. Biol. 23, 5122–5131
44. Li, J., Wang, J., Wang, J., Nawaz, Z., Liu, M. J., Qin, J., and Wong, J. (2000) EMBO J. 19, 4342–4350
45. Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997) Cell 89, 373–380
46. Perissi, V., Aggarwal, A., Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (2004) Cell 116, 511–526
47. Wen, Y. D., Perissi, V., Staszewski, L. M., Yang, W. M., Krones, A., Glass, C. K., Rosenfeld, M. G., and Seto, E. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 7202–7207
48. Yoon, H. G., Chan, D. W., Huang, Z. Q., Li, J., Fondell, J. D., Qin, J., and Wong, J. (2003) EMBO J. 22, 1336–1346
49. You, S. H., Liao, X., Weiss, R. E., and Lazar, M. A. (2010) Mol. Endocrinol. 24, 1359–1367
50. Burke, L. J., Downes, M., Lauvet, V., and Muscat, G. E. (1998) Mol. Endocrinol. 12, 248–262
