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The functional relationship between co-repressor N-CoR and SMRT in mediating transcriptional repression by thyroid hormone receptor α

Kyung-Chul CHOI*†, So-Young OH‡§, Hee-Bum KANG*†, Yoo-Hyun LEE*, Seungjoo HAAM‖, Ha-Il KIM*, Kunhong KIM*†, Young-Ho AHN*†, Kyung-Sup KIM*† and Ho-Geun YOON*†[1]

*Department of Biochemistry and Molecular Biology, Center for Chronic Metabolic Disease Research, Yonsei University College of Medicine, 134 Sinchon-dong, Seodaemun-gu, Seoul 120-752, South Korea, †Brain Korea 21 Project for Medical Sciences, Yonsei University College of Medicine, 134 Sinchon-dong, Seodaemun-gu, Seoul 120-752, South Korea, ‡Howard Hughes Medical Institute, 4000 Jones Bridge Road, Chevy Chase, MD 20815-26789, U.S.A., §Program in Gene Function and Expression, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, U.S.A., and ‖Department of Chemical Engineering, College of Engineering, Yonsei University, Seoul 120-749, South Korea

A central issue in mediating repression by nuclear hormone receptors is the distinct or redundant function between co-repressors N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor). To address the functional relationship between SMRT and N-CoR in TR (thyroid hormone receptor)-mediated repression, we have identified multiple TR target genes, including BCL3 (B-cell lymphoma 3-encoded protein), Spot14 (thyroid hormone-inducible hepatic protein), FAS (fatty acid synthase), and ADRB2 (β-adrenergic receptor 2). We demonstrated that siRNA (small interfering RNA) treatment against either N-CoR or SMRT is sufficient for the de-repression of multiple TR target genes. By the combination of sequence mining and physical association as determined by ChIP (chromatin immunoprecipitation) assays, we mapped the putative TREs (thyroid hormone response elements) in BCL3, Spot14, FAS and ADRB2 genes. Our data clearly show that SMRT and N-CoR are independently recruited to various TR target genes. We also present evidence that overexpression of N-CoR can restore repression of endogenous genes after knocking down SMRT. Finally, unliganded, co-repressor-free TR is defective in repression and interacts with a co-activator, p300. Collectively, these results suggest that both SMRT and N-CoR are limited in cells and that knocking down either of them results in co-repressor-free TR and consequently de-repression of TR target genes.

Key words: co-repressor, nuclear receptor co-repressor (N-CoR), repression, silencing mediator of retinoid and thyroid hormone receptor (SMRT), thyroid hormone receptor α.

INTRODUCTION

Thyroid hormones are known to play important roles in various biological processes, such as development, morphogenesis, growth, metabolism, body temperature and myocardial contractility [1–3]. TRs (thyroid hormone receptors) can bind to target promoters as monomers, homodimers or as heterodimers with RXRs (retinoid X receptors). TRs can positively or negatively regulate transcription depending on the presence or absence of hormone and the presence of a positive or negative thyroid hormone response element (TRE vs nTRE) in its target genes. In the absence of ligand, TR/RXR heterodimers bind to TREs and actively repress transcription. The repression by unliganded TR/RXR is largely mediated through the recruitment of co-repressor proteins such as SMRT (silencing mediator of retinoid and thyroid hormone receptor) and N-CoR (nuclear receptor co-repressor). A key progress in understanding the connection between the co-repressors SMRT and N-CoR with HDAC (histone deacetylase) came from the biochemical purification of SMRT and N-CoR complexes. The human SMRT complex purified from HeLa nuclear extracts by us and others contained HDAC3, TBL1 (transducin beta-like protein 1), TBLR1 (transducin beta-like related protein 1) and GPS2 (G-protein pathway suppressor 2) but not the components of the Sin3A-HDAC1/2 complex [4,5,17]. These studies demonstrate that SMRT and N-CoR complexes share a common set of subunits including HDAC3, GPS2, TBL1 and TBLR1. Consistent with the biochemical results that HDAC3 is the only HDAC identified in the purified complexes, knockdown of HDAC3 using siRNA (small interfering RNA) impaired repression by unliganded TR [6,7]. In contrast, knocking down HDAC1 or HDAC2 had little effect [6]. Together, these studies reveal the critical role of HDAC3 in repression by unliganded TR and have led to the conclusion that SMRT and N-CoR are class I HDAC-containing complexes primarily associated with HDAC3 [8].

SMRT/N-CoR complexes appear to be evolutionarily conserved. The yeast SET3 complex is probably the homologous complex of mammalian SMRT/N-CoR. The yeast SET3 complex has a role in repression of the sporulation gene program and contains Snt1, Sif2 and Hos2, which are the yeast homologues of mammalian SMRT/N-CoR, TBL1/TBLR1 and HDAC3 respectively [9]. This conservation in evolution emphasizes the functional importance of TBL1/TBLR1. Indeed, we have shown that TBL1 and TBLR1 have a redundant but essential role in the repression of the D1 (deiodinase I) gene by TRα. We found that targeting SMRT/N-CoR complexes to D1 requires at least two interactions, one between unliganded TR and SMRT/N-CoR and the other between TBL1/TBLR1 and hypoacetylated histones [10]. While this model nicely explains the conservation through

Abbreviations used: ADRB, β-adrenergic receptor; BCL3, B-cell lymphoma 3-encoded protein; ChIP, chromatin immunoprecipitation; CS-FCS, charcoal-stripped fetal calf serum; D1, deiodinase I; DMEM, Dulbecco's modified Eagle's media; FAM, 6-carboxyfluorescein; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPS, G-protein pathway suppressor; HDAC, histone deacetylase; N-CoR, nuclear receptor co-repressor; NR, nuclear receptor; P450R, P450 oxidoreductase; qRT–PCR, quantitative reverse transcription–PCR; RXR, retinoid X receptor; siRNA, small interfering RNA; RT–PCR, reverse transcription–PCR; SMRT, silencing mediator of retinoid and thyroid hormone receptor; SRC, steroid receptor co-activator; TBL, transducin beta-like protein; TBLR, transducin beta-like related protein; TAMRA, 6-carboxytetramethylrhodamine; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

[1] To whom correspondence should be addressed (email yhgeun@yuhs.ac)

evolution and the function of TBL1/TBLR1, whether this is the general mechanism for repression by unliganded TR remains to be tested.

A common theme in the transcriptional regulation by NRs (nuclear receptors) is the involvement of a highly related protein family. For instance, the highly related SMRT and N-CoR function as co-repressors, whereas three members of the SRC (steroid receptor co-activator) family proteins (SRC-1, SRC-2 and SRC-3) are important co-activators. Whether SMRT and N-CoR have a redundant or distinct function in mediating repression by unliganded TR has not been clearly addressed. To address the functional relationship between SMRT and N-CoR in TR repression in a broader sense, we have identified multiple TR target genes and demonstrate that either siRNA treatment against N-CoR or SMRT is sufficient for the de-repression of these target genes. Our data clearly suggest that SMRT and N-CoR are independently recruited to various TR target genes through the siRNA and ChIP (chromatin immunoprecipitation) experiments described herein. We present evidence that overexpression of N-CoR can restore the repression of endogenous genes after knocking down SMRT. Finally, unliganded, co-repressor-free TR is defective in causing repression and interacts with a co-activator, p300.

EXPERIMENTAL

Cell culture and siRNAs

Cell culture and siRNA treatment were carried out essentially as previously described [10–11]. For transfection of siRNAs, HeLa α2 (human cervical carcinoma) cells were first cultured in DMEM (Dulbecco's modified Eagle's media) supplemented with 10 % CS-FCS (charcoal-stripped fetal calf serum) (Gemini Bio-Products) for three days and then transfected at a cell confluency of $\sim$40–50 % using Lipofectamine™ 2000 (Invitrogen) with the appropriate amounts of siRNA as indicated by the manufacturer's instructions. Two days after transfection, cells were collected and processed for Western analysis, RT–PCR (reverse transcription–PCR), or ChIP as indicated. For experiments with T3, α2 cells were initially seeded at a density of 4×10^5 cells/100-mm-diameter tissue culture dish. After a 24 h incubation, the culture medium was replaced with DMEM with 10 % CS-FCS and incubated for 3 days, followed by replacement with fresh CS-FCS supplemented with 10 nM T3 for up to 6 h. For the experiments involving both siRNAs and T3, T3 was added 2 days after siRNA transfection and incubated for 1 h or as indicated in the ChIP assays and for 6 h for RT–PCR analysis. The siRNAs against N-CoR and SMRT have been previously described [7,10,11]. Various concentrations, between 20–80 nM, of each siRNA was transfected using Lipofectamine™ 2000 according to the manufacturer's instructions. Transfected cells were cultured for an additional 3 days before harvesting for further analyses. The efficiency of siRNA knockdown was determined by Western analysis using the corresponding specific antibodies.

ChIP assays

For ChIP assays, we first isolated chromatin as previously described [7,10,11]. Briefly, approx. 2×10^9 HeLa α2 cells in 150-mm-diameter dishes were first treated with PBS containing 1 % formaldehyde for 10 min, washed twice with PBS and then incubated with 100 mM Tris (pH 9.4) and 10 mM DTT (dithiothreitol) at 30 °C for 15 min. The cells were then rinsed twice with PBS and resuspended in 600 μl of SolA buffer [10 mM Hepes (pH 7.9), 0.5 % Nonidet P40, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT) by pipetting. After the sample was centrifuged for 5 min at 500 ***g*** at 4 °C, the pellets were resuspended in SolB [20 mM Hepes (pH 7.9), 25 % glycerol, 0.5 % Nonidet P40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) containing protease inhibitors, followed by vigorous pipetting in order to extract the nuclear proteins. After centrifugation at 6000 ***g*** for 30 min at 4 °C, the nuclear pellets were resuspended in IP buffer [1 % Triton X-100, 2 mM EDTA, 20 mM Tris/HCl (pH 8.0), 150 mM NaCl and protease inhibitors] and sonicated to break chromatin into fragments with an average length of 0.5–1 kb. ChIP assays were then performed with the indicated antibodies essentially as previously described but with SDS omitted in all buffers [11]. The antibodies against acetylated H3 and p300 were purchased from Upstate Biotechnology. The antibodies against N-CoR, SMRT, SRC-1 and SRC-3 have been described previously [7,10,11]. Primers used for ChIP analysis included the following: BCL3 (B-cell lymphoma 3-encoded protein) (TRE2): 5′-CCTGGCCCAGCAACACTTTCTTAACCTAAC-3′, 5′-GGCGCTGGCTTGTGGCCTCTACTC-3′; BCL3(TRE3): 5′-CTAGTCGGGAGGCTGAGGCCAGAAGATTGG-3′, 5′-GCACGCGAGCCTGGGTGACAGAGTAAGACT-3′; FAS (fatty acid synthase) (TRE5): 5′-TTACAGTCCATCACCTCCGCTATCTTCTCT-3′, 5′-AAAAATTAAATCTACAAACGGGGACAAA-3′; ADRB2 (β-adrenergic receptor 2) (TRE1): 5′-GAGCCTGCTTTTTCTTACATC-3′, 5′-CCTTTGCCCTCCAGCCTTGACGAA-3′; ADRB2(TRE2): 5′-GTCTATTCTTGATGGGCATTTGGGTTGGTT-3′, 5′-AGGTGGAGGTTGCAGTGAGCCGAGATT-3′. Spot14(TRE2): 5′-TCGGGAGGCTGAGGCAGGAGACTAAC-3′, 5′-TCCAGGGCAGAACAGGCAGCCAAATCCA-3′ Spot14(TRE3):5′-TCGGGAGGTGAGGCAGGAGACTAAC-3′, 5′-TGTAATAATGATAAGGGCAAACAATAACTGAAGA-3′; Spot14(TRE4): 5′-AGCAATCCTCCCACCTCAGCCACTT-3′, 5′-AGCCCACTCTGCACCCTCCCAA-CT-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase): 5′-CCCAGCCCGGAGAGA-3′, 5′-GGTTTCTGCACGGAAGGT-3′.

RNA extraction and RT–PCR

Total RNA was isolated from prostate tissues using an RNeasy Mini kit (Qiagen), according to the manufacturer's specifications. Total RNA from each sample was reverse-transcribed with random primers using a StrataScript™ reverse transcriptase kit (Stratagene) followed by quantitative PCR. Primers for amplification of Spot14 transcript were 5′-CGAGAAAGCCCAGGAGGTGACAAGGAAATA-3′ and 5′-GAGCGAGGGGAGCAGTGAATAGCAACAGA-3′. Primers for ADRB2 amplification were 5′-GGATCGCTACTTTGCCATTACTTCACCTTTCA-3′ and 5′-CCCCGTCCGCCCATCCTGCTCCAC-3′. Primers for BCL3 amplification were 5′-ACGCCGTGGAAAACAACAGCCTTAGCAT-3′ and 5′-GAGCGGCGTGTCGTTGTGGCAGTTCTTGAG-3′. Primers for FAS amplification were 5′-CATCGGGCACGTGGGCATTTTG-3′ and 5′-GGTCCCGCTGGCTGTCCCTGTCC-3′. Primers for P450R (P450 oxidoreductase) amplification were 5′-GCCCGCTACTACTCCATCGCCTCATCCT-3′ and 5′-GTCTCCCCCACCTCCTTGCCCTGCTGTCG-3′. Primers for D1 amplification were 5′-AACCCCCATTTCAGCCACGACAAC-3′ and 5′-ATTCAGCACCAGTGGCCTATTACCTT-3′. Primers for GAPDH amplification were 5′-CGCGGGGCTCTCCAGAACATCATCC-3′ and 5′-CTCCGACGCCTGCTTCACCACCTTCTT-3′. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Real-time PCR analysis

The RT–PCR analysis and quantification was performed with Taqman One-Step RT–PCR Master Mix reagents or SYBR

Green PCR Master Mix reagents (depending on the target RNA, see below) on an ABI Prism 7700 Sequence Detection System. The singularity and specificity of amplification were verified by Dissociation Analysis Software. All samples were normalized to human 18S rRNA (primer and probe set purchased from Applied Biosystems). Primer sequences for amplification of the ADRB2 RNA used in the qRT–PCR (quantitative RT–PCR) were: forward; 5′-TCTGCAGACGGTCACCAACTAC-3′, reverse; 5′-GCACCACTGCTAGGCCCAT-3′, and probe; 5′-FAM-CACTTCACTGGCCTGTGCTGATCTGGTTAMRA-3′, where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. Primer sequences for amplification of the D1 RNA used in the qRT-PCR were: forward; 5′-AGCCCATCTACTGCTGGCC-3′, reverse; 5′-TGTAGTTCCAAGGGCCAGATTT-3′, and probe; 5′-FAM-AGGCTCTACATAATCCAGGAGGGCAGGA-TAMRA-3′. All reactions were performed in triplicate. Relative expression levels and standard deviations (S.D.) were calculated using the comparative method. *P*-values were determined with the Student's *t* test.

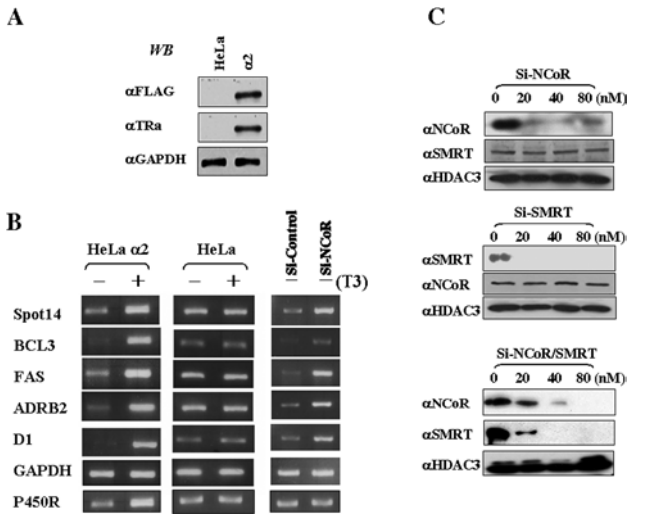

Figure 1 Confirmation of BCL3, Spot14, FAS and ADRB2 as TR target genes in HeLa α2 cells

(**A**) HeLa α2 and HeLa cells were harvested and the levels of the corresponding target proteins were analysed by Western blotting. (**B**) HeLa α2 and HeLa cells were transfected with or without 20 nM siRNAs as indicated. Three days after treatment, total RNAs were prepared and processed for RT–PCR analysis using gene-specific primers. (**C**) HeLa α2 cells were transfected with 20, 40 or 80 nM siRNAs as indicated. Three days after treatment, whole cell extracts were prepared. The levels of SMRT and N-CoR were determined by Western blot using SMRT and N-CoR antibodies. Western blot results for HDAC3 served as specificity controls.

RESULTS

Both N-CoR and SMRT are required for efficient repression of multiple TR target genes

The best characterized co-repressors, N-CoR and SMRT, commonly bind to unliganded nuclear receptors and repress transcription through recruitment of HDAC3. We have previously shown that knocking down either N-CoR or SMRT led to de-repression of the D1 gene in HeLa α2 cells, which stably express the FLAG tagged human TRα [7]. However, it has not been clearly addressed whether SMRT and N-CoR have a redundant or distinct function in mediating repression by unliganded TR. To investigate the distinct or redundant function of both SMRT and N-CoR in TR-mediated repression in a broader sense, we first attempted to identify additional TR target genes in HeLa α2 cells. As a control, the Western blot analysis confirmed that TRα is highly expressed in HeLa α2 cells but not in parental cells lacking TRα (Figure 1A). Several groups have previously used microarray experiments to identify TR target genes from tissue culture cells or mice [12–14]; however, such an experiment has not yet been conducted with human cells. Thus we tested a group of 18 genes, the expression of which was regulated by TR in mouse livers [12]. By RT–PCR analysis using primers specific for each gene transcript, we found that four of the genes, namely Spot14, BCL3, FAS and ADRB2, were significantly induced upon T3 treatment for 6 h (Figure 1B). As a control, these genes were not induced in the parental HeLa cell line lacking TRα (Figure 1B).

To test the effect and specificity of each siRNA, HeLa α2 cells were transfected with different amounts of siRNA against N-CoR and/or SMRT. Three days after transfection, HeLa α2 cells were collected, and the effect of siRNA treatment was analysed by Western blot. As shown in Figure 1(C), siRNA specific for N-CoR was able to reduce the level of N-CoR by more than 80 % when used at a concentration of 20 nM or higher. SMRT siRNA seemed to be even more effective, since knockdown was obvious, even at a concentration of 20 nM. Moreover, the combination of N-CoR and SMRT siRNAs led to knockdown of both SMRT and N-CoR. As a control, we measured the levels of HDAC3, which showed no significant change after siRNA treatments (Figure 1C). As shown in Figure 1(B), treatment with N-CoR siRNA also relieved the repression of selected TR target genes by unliganded TR, confirming the involvement of N-CoR in TR-mediated repression on selected TR-target genes. T3 also appeared to affect another TR target gene, P450R, which was identified by the cDNA microarray. However, the transcriptional level of P450R was not altered, even after treatment with siRNA against N-CoR, suggesting the possibility of the involvement of other co-repressors in TR-mediated transcriptional repression (Figure 1B).

A previous study demonstrated that unliganded TR could partially activate transcription of the *D1* gene if not associated with co-repressor proteins in a yeast experimental system [15]. To examine whether de-repression by unliganded TR after knocking down N-CoR and SMRT expression is a general mechanism or a gene-specific event, we analysed the effect of the siRNAs against the co-repressors on the transcriptional level of additional TR target genes in HeLa α2 cells. For this purpose, HeLa α2 cells were treated with siRNA against SMRT, N-CoR or both, to knock down their expression individually or in combination. Three days after siRNA treatment, the cells were harvested and the levels of *BCL3*, *FAS*, *ADRB2*, *D1* and *Spot14* gene expression were determined by quantitative real time PCR. While treatments with siRNAs against SMRT, HDAC3 and/or N-CoR did not alter the transcription level of GAPDH, a significant effect was observed for most of the TR target genes (Figure 2, left panels). In this case, knocking down either N-CoR or SMRT de-repressed the transcription of D1 and ADRB2 from ∼8- to ∼10-fold, whereas knocking down both N-CoR and SMRT induced a ∼12-fold de-repression. Thus the knockdown of SMRT and N-CoR in general led to a de-repression of the unliganded TR-mediated transcription levels from all three TR target genes, although the extent of increased expression is gene-dependent. The effects of knockdown of N-CoR or SMRT are mediated via TRα, since the knockdown of SMRT or N-CoR had no effect on TR target genes expression in parental HeLa cells lacking TR (Figure 2, right panels). As expected, the effect of each siRNA is specific for repression and had no effect on activation induced by liganded TRα (Figure 2). Together, we conclude, at

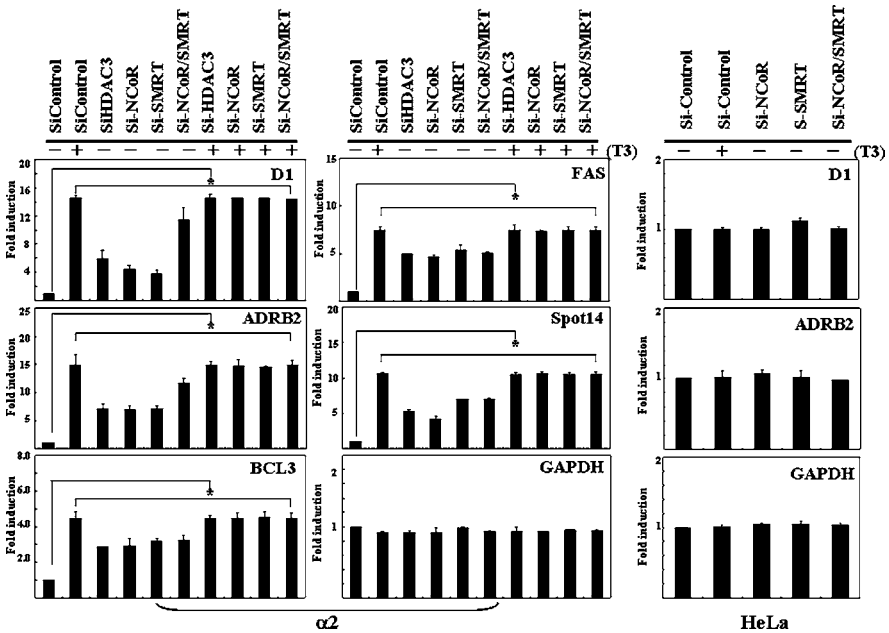

Figure 2 Both SMRT and N-CoR are required for mediating TR repression

HeLa α2 cells were cultured with charcoal-treated serum medium and treated with siRNAs as indicated. Three days later, total RNAs were prepared and processed for real-time PCR analysis using gene specific primers. Each PCR reaction also contained a control PCR product (marked by *) from GAPDH mRNA. The results are shown as means $\pm$ S.D. calculated from three independent experiments. $^*P < 0.05$.

least for the genes tested, that SMRT and N-CoR to a large extent are required for efficient repression for multiple TR target genes.

The identification of TR-binding sites in TR target genes

To examine the association of SMRT and N-CoR with these TR target genes, we also attempted to identify TR-binding site(s) in these genes. We first used a computer program (TRANSFAC) to search for putative TREs within a 10 kb region upstream of the transcriptional start sites (Figure 3). This program searches and identifies TREs with palindromic or directed repeats of the consensus AGGTCA (with a mismatch of up to three nucleotides) separated by 4 bp. To test the binding of TRα to each of these putative TREs, we performed ChIP experiments for TRα using samples derived from HeLa α2 cells that had been deprived of T3 for 6 h. Specific PCR primers were designed to amplify sequences (100–150 bp) surrounding each of the putative TREs. As shown in Figure 3, the binding of TRα could be detected at TREs in *BCL3*, *FAS*, *ADRB2* and *Spot14* genes at positions $-2216/-2526$, -6062, -3401 and $-2152/-2572$ respectively. Under similar experimental conditions, we failed to detect the binding of TR to other putative TREs (results not shown).

To substantiate the ChIP experiment with a TRα antibody, we also performed ChIP assays using an N-CoR-specific antibody under similar experimental conditions. ChIP assay using an N-CoR antibody revealed a correlation between the binding of TRα and the presence of N-CoR. Consistent with the lack of TRα binding to the putative TRE in the tested genes, we also failed to detect the recruitment of N-CoR to those TREs. Thus the recruitment of N-CoR is strictly correlated with the binding of TR and was observed under conditions lacking T3. A similar result was observed for SMRT (results not shown). Taken together, we conclude that the co-repressors SMRT and N-CoR are recruited to various TR target genes by unliganded-TR.

SMRT and N-CoR are independently recruited to various TR target genes

Previous antibody injection experiments from Rosenfeld and co-workers as well as siRNA experiments from our laboratory showed that inactivation of either SMRT or N-CoR led to depression by TRs [7,16]. To analyse in more detail the role of SMRT and N-CoR in mediating repression by unliganded TR using HeLa α2 cells and the *D1* gene, we observed the effect of siRNA directed against the co-repressor N-CoR on the recruitment of SMRT. For this experiment, we transfected 20 nM of N-CoR siRNA, SMRT siRNA or both into HeLa α2 cells, and then performed ChIP assays using anti-TR, -N-CoR and -SMRT antibodies. The results in Figure 4 show that knocking down either N-CoR or SMRT, as expected, diminished their association with the D1 promoter but had no effect on the binding of the other. The above experiments are focused on the *D1* gene. The identification of the additional TR target genes provides us an opportunity to address the functional relationship between SMRT and N-CoR in TR repression in a broader sense. ChIP analysis revealed that knockdown of SMRT had no effect on the recruitment of N-CoR and also that the recruitment of SMRT to various TR target genes was not altered in the absence of N-CoR, suggesting that SMRT and N-CoR are independently recruited to various TR target genes (Figure 4).

Unliganded, co-repressor-free TR is defective in repression and interacts with co-activators

We next focused on why knocking down either SMRT or N-CoR led to de-repression of the D1 gene. Based on the results using siRNA directed against N-CoR and SMRT on multiple TR target genes, we hypothesized that unliganded TR could activate transcription if it was not associated with co-repressor proteins. To test this, we first compared the histone acetylation status over the D1 and GAPDH promoter from cells treated with a control

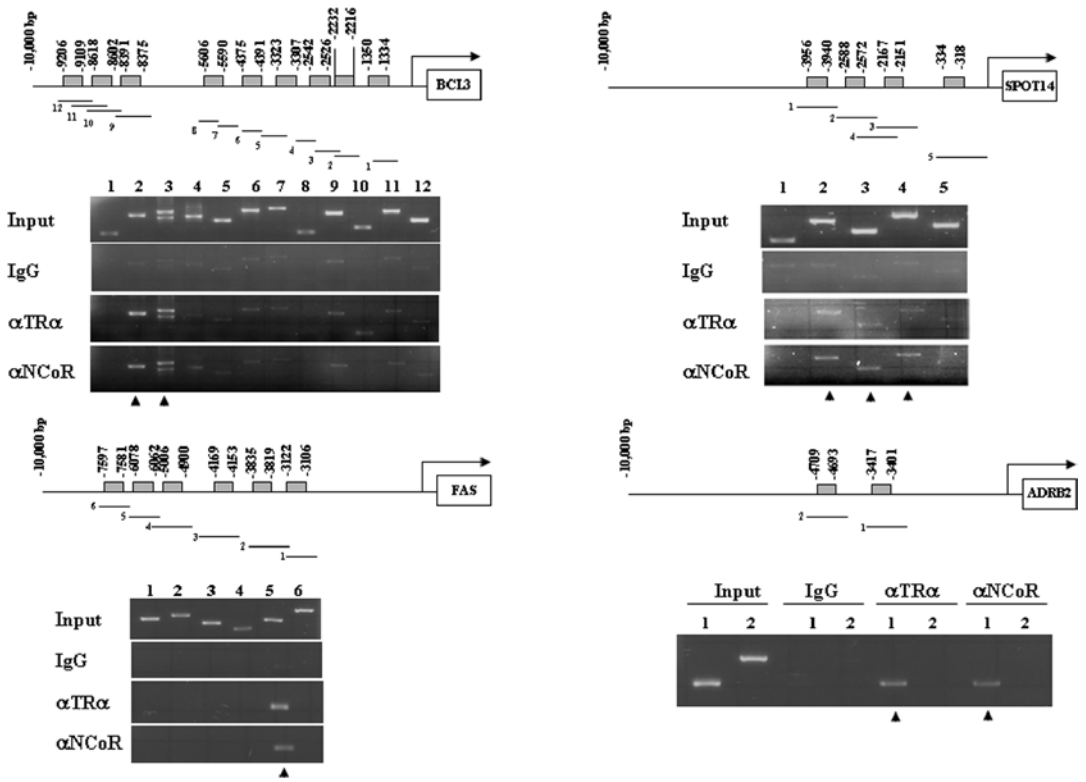

Figure 3 Experimental verification of TR binding sites in BCL3, Spot14, FAS and ADRB2

The position of each putative TRE identified by using the TRANSFAC program is indicated. Specific primers were designed to amplify a 150–300 bp fragment containing each putative TRE by ChIP assay. The identified TREs are marked by ▲.

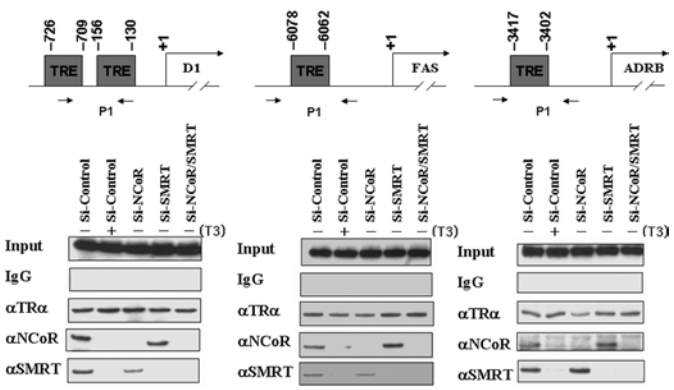

Figure 4 SMRT and N-CoR has an independent function in mediating the repression of multiple target genes by TR

HeLa α2 cells were treated with siRNA against SMRT and/or N-CoR to knockdown their expression individually or in combination. The recruitment of co-repressors to the genes *D1*, *FAS*, and *ADRB2* was then determined by ChIP assays using the antibodies indicated.

siRNA and siRNA for SMRT and N-CoR. Knocking down either SMRT or N-CoR increased the level of histone acetylation of histone H3 and H4 on the D1 promoter but not on the control GAPDH (Figure 5A). This result supported the idea that the co-repressor-free D1 promoter is associated with acetylated histones.

Next, we examined whether co-repressor-free, unliganded TR binds to acetylated histone tails. To do this, we transfected 20 nM of N-CoR siRNA into HeLa α2 cells and performed ChIP and re-ChIP assays using anti-TR and acetylated H3 antibodies. As shown in Figure 5(B), knockdown of N-CoR had no effect on the targeting of TR. However, an increased association between TR and acetylated histone H3 was observed, suggesting that the unliganded TR binding region in the D1 gene could be acetylated in the absence of a co-repressor. A series of studies from yeast [15] (in yeast TR constitutively activates transcription in the absence of T3) and a study from Rosenfeld and co-workers [16] showed that the unliganded receptor may exist in an equilibrium between active and repressive states. Next, therefore, we examined whether increased co-activator recruitment resulted in increased histone acetylation. For this purpose, HeLa α2 cells were treated with or without siRNA against SMRT or N-CoR, either individually or in combination. Three days later, the cells were initially processed for ChIP for TR and then re-ChIP assays were directed toward the co-activators SRC-1, -2 and -3, p300, and TRAP220. As shown in Figure 5(C), siRNA against N-CoR and/or SMRT led to a significant increase in the association of p300 to the ADRB2 promoter. The binding of TR was not affected by siRNA treatment. Interestingly, co-repressor knockdown did not appear to affect the recruitment of SRC-1, -2 and -3 to target genes. Taken together, these results support the idea that p300 binds to co-repressor-free, unliganded TRs and increases histone acetylation.

Both N-CoR and SMRT are functionally redundant in mediating repression

In this study, we found that N-CoR and SMRT were independently recruited to various TR target genes. Furthermore, the knockdown of either N-CoR or SMRT led to the de-repression of TR-mediated transcription. We next inquired whether overexpressed

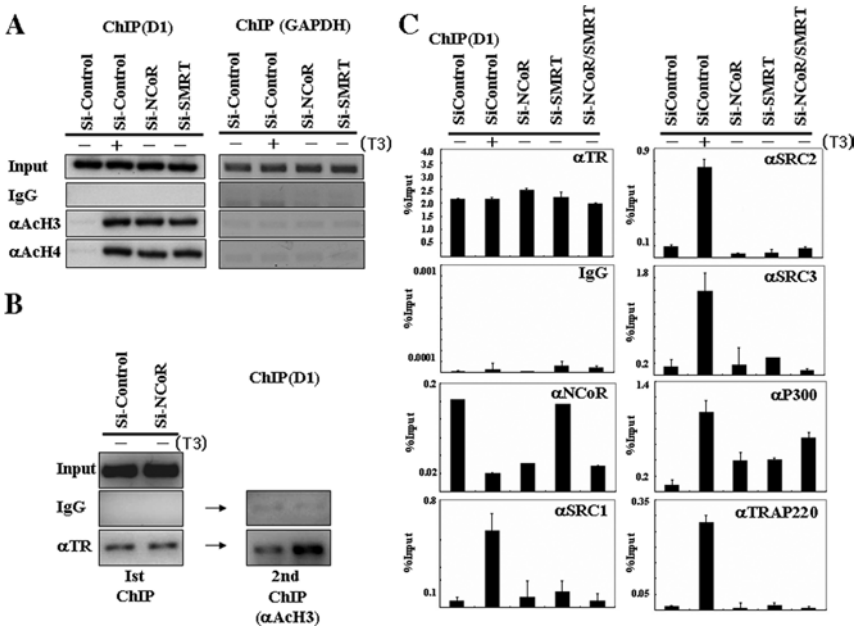

Figure 5 Unliganded, co-repressor-free TR partially activates transcription via contact with a co-activator

(**A**) ChIP assays to determine the level of histone acetylation and methylation on the D1 and GAPDH promoters. (**B**) HeLa α2 cells were treated with a control siRNA or N-CoR siRNA for 3 days. After that, ChIP and reChIP assays were performed as follows. Samples were divided into two groups, one was precipitated with a TR antibody and the other with an IgG as a control. The precipitated chromatin fragments were extensively washed, eluted and re-assayed with antibodies against acetylated H3. (**C**) Chromatin samples were first precipitated with a TR antibody. The precipitated chromatin fragments were extensively washed, eluted and re-assayed with antibodies as indicated. The results were analysed by real-time PCR and were shown as the percentage of input. The results are shown as mean $\pm$ S.D. calculated from three independent experiments.

N-CoR could restore repression after knocking down SMRT. For this experiment, HeLa α2 cells were treated with siRNA against SMRT. Two days later, myc-N-CoR expression plasmid was transfected into siRNA treated HeLa α2 cells. As expected, knockdown of SMRT led to the de-repression of the *D1* gene by unliganded TR. Interestingly, as the amount of N-CoR protein increased, transcriptional repression was enhanced but had little effect on GAPDH (Figure 6A). These results suggest that N-CoR can restore the loss of repression induced by knocking down SMRT. To further substantiate this result, we next explored the basis of restoration of repression by overexpressed N-CoR in the setting of knockdown. We analysed the recruitment of overexpressed N-CoR with a knockdown of SMRT over both the D1 and ADRB2 promoters using the ChIP assay. As shown in Figure 6(B), the recruitment of N-CoR over both the D1 and ADRB2 promoters in the setting of SMRT knockdown was significantly increased, indicating the limited amounts of NCoR and SMRT in α2 cells. Together, these results demonstrate that both SMRT and N-CoR are functionally redundant, and the limited pool of SMRT and N-CoR explain the de-repression observed after knocking down either of them in the HeLa α2 cells.

DISCUSSION

A common theme in transcriptional regulation by NRs is the involvement of a highly related protein family. For instance, highly related SMRT and N-CoR function as co-repressors, whereas three members of the SRC protein family (SRC-1, SRC-2 and SRC-3) are important co-activators. Whether SMRT and N-CoR have a redundant or distinct function in mediating repression by unliganded TR has not been clearly addressed. We have previously shown that knocking down either N-CoR or SMRT led to de-repression of the *D1* gene in HeLa α2 cells [7]. Furthermore, an early study from Rosenfeld and co-workers showed that injection of neutralizing antibodies against N-CoR abolished repression by TR [16]. In this study, we focused on the functional relationship between the co-repressor N-CoR and SMRT in mediating repression by TRα. We also investigated whether reducing the expression of N-CoR and/or SMRT would allow de-repression by unliganded TR and how co-repressor-free, unliganded TR de-represses target gene expression.

Our previous studies demonstrate that both N-CoR and SMRT were essential for transcriptional repression of the D1 gene, which is mediated by unliganded TR [7,10]. This result is somewhat surprising since N-CoR and SMRT would be expected to be functionally redundant. Thus to substantiate whether this result was gene-specific or a general mechanism, we identified additional TR target genes in HeLa α2 cells. As shown in Figure 2, semi-quantitative RT–PCR analysis revealed that, similar to the *D1* gene, these four genes were de-repressed to various extents after SMRT siRNA or N-CoR siRNA treatment. For ADRB2, FAS and Spot14, combined N-CoR siRNA and SMRT siRNA treatment appeared to have a stronger de-repression than that of N-CoR siRNA or SMRT siRNA alone. A mild effect of SMRT siRNA on Spot14 is probably due to experimental variation, since combined siRNAs had a stronger effect than did N-CoR siRNA alone. Quantitative real-time PCR clearly showed that these genes are repressed by unliganded TR and that this repression requires SMRT and N-CoR. This suggests that both N-CoR and SMRT are required for the efficient repression of multiple TR target genes.

We also identified TR binding site(s) in these genes by the combination of sequence mining and physical association as determined by ChIP assays. In the case of BCL3 and Spot14, we cannot rule out the possibility that only one of the two TREs is actually functional, because the distance between the two putative TREs in both cases is within the limit of the ChIP

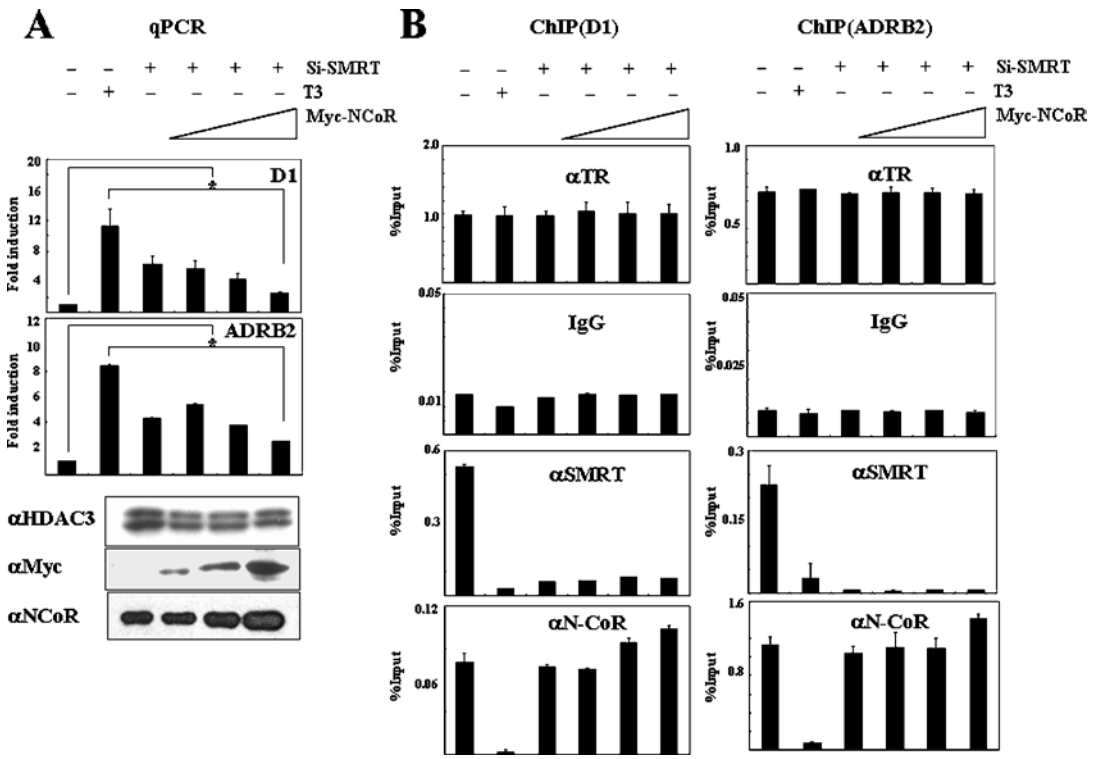

Figure 6 Expression of N-CoR can restore repression after knockdown by SMRT

(**A**) HeLa α2 cells were treated with SMRT siRNA first, then transfected with myc-N-CoR expression construct. Total RNA was then prepared from each sample and the levels of D1 and ADRB2 were analysed by real-time PCR. (**B**) The results from ChIP assay were analysed by real-time PCR and were shown as the percentage of input. The results are shown as mean $\pm$ S.D. calculated from three independent experiments. $^*P < 0.05$.

assay resolution (average size of chromatin fragments is about 500 bp). It is noteworthy that the TREs defined by our approach correlate well with the result from the study by Mariash and co-workers, which mapped two major TREs in a region between -2700 to -2000 of the Spot 14 gene [18], thus in part validating our approach. Our approach may not identify all of the functional TREs that these genes contain, given the complex nature of TREs and the possibility that additional TREs downstream of the transcriptional start sites may exist. However, for the purpose of our study, it is not necessary to identify all the functional TRE(s) for each gene. Moreover, it is also not necessary to identify the exact sequence of TREs. For this reason, we choose not to propose further characterization of the functional TREs in this study unless it is deemed necessary. We demonstrated that the knockdown of SMRT had no effect on the recruitment of N-CoR and also that the recruitment of SMRT to various TR target genes was not altered in the absence of N-CoR, suggesting that SMRT and N-CoR are independently recruited to various TR target genes.

Previous studies that examined the functional role of N-CoR and SMRT suggested that unliganded TR could partially activate transcription if it was not associated with co-repressor proteins, as suggested by siRNA experiments against N-CoR or SMRT [7]. However, whether the effect of knocking down N-CoR and SMRT on transcription is due to the combinatorial effect of 'relief' from repression or 'activation' by co-repressor-free, unliganded TR remains relatively unexplored. One explanation is that co-repressor-free, unliganded TR may not repress transcription but may actively activate transcription. This idea is supported by a series of studies with yeast [15], where TR constitutively activates transcription in the absence of T3, and by a study from Rosenfeld and co-workers showing that the liganded receptor may exist in an equilibrium between active and repressive states [16]. Our ChIP and re-ChIP analysis indicated that knocking down N-CoR had no effect on the targeting of TR. However, an increased association between TR and acetylated histone H3 was observed, suggesting that the unliganded TR binding region could be acetylated in the absence of a co-repressor. Furthermore, siRNA against N-CoR and/or SMRT led to a significant increase in p300 recruitment to the D1 promoter. Taken together, these results support the idea that the co-activator p300 binds to co-repressor-free and unliganded TRα, leading to hyper-acetylation of histone tails, and finally resulting in transcriptional de-repression.

Our quantitative real time PCR data in Figure 2 supports that knockdown of N-CoR or SMRT indeed appears to partially de-repress the *D1* gene, since knockdown of both SMRT and N-CoR had a stronger (possibly additive) de-repression than knockdown of either SMRT or N-CoR alone. Therefore, we hypothesized that the de-repression is due to a shortage of co-repressor proteins. In order to know whether overexpressed N-CoR can restore repression after knocking down SMRT, myc-N-CoR was overexpressed in HeLa α2 cells after knocking down SMRT. Knocking down SMRT led to transcriptional de-repression by unliganded TR. Interestingly, as the amount of N-CoR protein increased, the transcriptional repression was also increased. Moreover, the recruitment of N-CoR in the setting of SMRT knockdown over was significantly increased. It is possible that the knockdown of SMRT frees up new sites for overexpressed N-CoR to bind. This result suggested that both SMRT and N-CoR are functionally redundant and a limited pool of SMRT and N-CoR is the explanation for de-repression after knocking down either one of them in the HeLa α2 cells.

In summary, we demonstrated in this study that the functional relationship between N-CoR and SMRT in mediating transcriptional repression by unliganded-TRα. We also demonstrated that SMRT and N-CoR, to a large extent, are functionally redundant for repression by TRα but a limited pool is involved in mediating repression. Our study suggests that, in addition to its ability to repress transcription through the co-repressor complex, unliganded, co-repressor-free TRα is defective in repression and interacts with the co-activator p300, resulting in hyperacetylation. The present study addressed the functional relationship between SMRT and N-CoR in repression by unliganded TRα. Previous studies suggested that TRβ interacts preferentially with N-CoR *in vitro* and that repression by TRβ in HEK-293T cells [human embryonic kidney-293 cells expressing the large T-antigen of SV40 (simian virus 40)] is primarily dependent on N-CoR but not SMRT [6]. Because HeLa α2 cells are not considered as an ideal physiological system, further investigation using a more physiologically relevant cell line, such as GH4C1 or GH3 [19], will provide new insight into the functional relationship between SMRT and N-CoR in mediating transcriptional repression by TRα and TRβ.

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