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# The N-CoR/Histone Deacetylase 3 Complex Is Required for Repression by Thyroid Hormone Receptor

Takahiro Ishizuka and Mitchell A. Lazar*

*Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine and Department of Genetics and The Penn Diabetes Center, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104*

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**Nuclear receptor corepressors (N-CoR) and silencing mediator for retinoid and thyroid receptors (SMRT) have both been implicated in thyroid hormone receptor (TR)-mediated repression. Here we show that endogenous N-CoR, TBL1, and histone deacetylase 3 (HDAC3), but not HDAC1, -2, or -4, are recruited to a stably integrated reporter gene repressed by unliganded TR as well as the orphan receptor RevErb. Unliganded TR also recruits this complex to a transiently transfected reporter, and transcriptional repression is associated with local histone deacetylation that is reversed by the presence of thyroid hormone. Knockdown of N-CoR using small interfering RNAs markedly reduces repression by the TR ligand binding domain in human 293T cells, whereas knockdown of SMRT has little effect. RevErb repression appears to involve both corepressors in this system. Knockdown of HDAC3 markedly reduces repression by both TR and RevErb, while knockdown of HDAC1 or 2 has more modest, partly nonspecific effects. Thus, HDAC3 is critical for repression by multiple nuclear receptors and the N-CoR HDAC3 complex plays a unique and necessary role in TR-mediated gene repression in human 293T cells.**

Thyroid hormone activates transcription by interacting with nuclear thyroid hormone receptors (TRs) (50). Hormone binding to the TR ligand binding domain (LBD) induces a conformation that recruits a number of transcriptional coactivators (45). In the absence of thyroid hormone, the TR assumes a conformation that stably interacts with the corepressor molecules nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid receptor (SMRT) (5, 17, 19). The corepressors do not serve completely redundant functions, as illustrated by the unique phenotype of mice in which the N-CoR gene is mutated (23). In vitro, TR prefers to bind to N-CoR due to the presence of a receptor interaction domain that is not present in SMRT (20, 38, 42). However, TR also binds strongly to SMRT under a variety of conditions (4, 34). The relative importance of N-CoR and SMRT in mammalian cells in which TR functions as a repressor is not clear. The orphan receptor RevErb is also a potent transcriptional repressor that interacts in vitro with N-CoR and SMRT (14, 48). RevErb strongly prefers to interact with N-CoR when bound to its cognate DNA binding site in vitro (47), but as for TR, it is not known whether this finding is functionally relevant in living cells.

In living cells, the ligand-induced exchange of coactivator and corepressor occurs in the context of chromatin (7). Nucleosomal histones are frequently hyperacetylated in transcriptionally active chromatin, and several coactivators implicated in hormone action (such as CBP/p300, SRC-1/2/3, and P/CAF) are histone acetyltransferase enzymes (22, 26, 35). Conversely, N-CoR and SMRT function in part by associating with histone deacetylase (HDAC) enzymes that reverse the acetylation reaction (9). Numerous HDACs, including HDAC1, -2, -3, -4, -5, -7, and -9, have been shown to interact with N-CoR or SMRT in one context or another (1, 6, 12, 16, 21, 24, 25, 28, 30, 39, 40, 44, 49).

HDAC3 is of particular interest for several reasons: HDAC3 is found in a tight complex with SMRT and N-CoR (12, 28, 44, 49), it must be presented to SMRT/N-CoR by the multiprotein TCP-1 ring complex (TRiC) chaperone (13), and its enzyme activity is completely dependent upon association with the deacetylase activating domain (DAD) of N-CoR or SMRT (11, 49). HDAC3 is recruited to reporter genes repressed by TR in *Xenopus* oocytes, although other HDACs are also involved (27). By contrast, recent studies indicate that HOS2, the *Saccharomyces cerevisiae* homolog of HDAC3, is recruited to regions of the genome that are actively being transcribed (41). Intriguingly, HOS2 is very similar to HDAC3 in many respects: it binds to yeast TRiC and also exists in a complex with Sif2 and Snt1 (33). Sif2 is homologous to mammalian TBL1, which is an integral component of the HDAC3-SMRT/N-CoR complex, and Snt1 has features in common with SMRT/N-CoR, including conservation of the SANT motif which is required for DAD function. This raises questions about the role of HDAC3 in mammalian repression in general and in nuclear receptor-mediated repression in particular.

Studies of repression by TR in mammalian systems have relied on in vitro interaction assays and on transient transfection of TRs and reporter genes into mammalian cells. Using RNA interference to knock down corepressor and HDAC expression and using chromatin immunoprecipitation (ChIP) to track TR-dependent recruitment of these factors to reporter genes that are either transiently transfected or stably integrated in human 293T cells, here we have assessed the importance of nuclear receptor corepressors and various HDACs. N-CoR and HDAC3 are both recruited by the DNA-bound TR and RevErb, and the results are similar regardless of whether the target gene is transiently transfected or stably incorporated

* Corresponding author. Mailing address: University of Pennsylvania School of Medicine, 611 CRB, 415 Curie Blvd., Philadelphia, PA 19104-6149. Phone: (215) 898-0198. Fax: (215) 898-5408. E-mail: lazar@mail.med.upenn.edu.

into the mammalian genome. We show that N-CoR but not SMRT is required for repression mediated by the TR LBD, while both N-CoR and SMRT contribute to repression by the RevErb LBD. HDAC3 is required for repression by both TR and RevErb. Our results clearly show that N-CoR and HDAC3 play important and unique roles in repression by TR LBD in human cells and that corepressor-HDAC3 complexes are also critical for repression by RevErb.

## MATERIALS AND METHODS

**Plasmids.** Gal4-TR (48), Gal4-HDAC1 (21), and Gal4-RevErb (14) constructs have been described previously. Others were created by using PCR or endonuclease restriction digestion or a combination of these techniques followed by ligation. Gal4-HDAC3 contains amino acids 1 to 428 of human HDAC3 cDNA. Gal4-Mad contains amino acids 1 to 47.

**Mammalian cell culture and transfection.** 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (high glucose) supplemented with 10% fetal bovine serum and L-glutamine (all from GIBCO BRL). Cells were grown at 37°C in 5% $CO_2$. For stable transfection, cells were grown in 100-mm-diameter dishes and cotransfected with 10 μg of Gal4 upstream activation sequence (UAS) × 5-thymidine kinase (TK)-luciferase reporter containing five copies of the Gal4 17-mer binding site and 0.5 μg of pBabe-puro for 24 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were cultured in complete medium for 48 h prior to selection by puromycin (Sigma; 2 μg/ml) for 3 weeks. Of 10 clones tested for reporter activity, one clone was chosen for further study. For transient transfections, cells were grown in 12-well plates and 0.3 μg of (Gal4 UAS × 5)-simian virus 40 (SV40)-luciferase reporter, 0.3 μg of pCMX-Gal4 DNA binding domain (DBD) or pCMX-Gal4-fusion construct expression vector, and 0.1 μg of β-galactosidase expression vector were added to each well. At 24 h after transfection, the medium was changed to DMEM containing charcoal-dextran-stripped fetal bovine serum. Cells were incubated for an additional 24 h in the presence or absence of 10 nM T3 and harvested. A luciferase assay kit (Promega) was used to determine relative levels of the luciferase gene product. Light units were normalized to the cotransfected β-galactosidase expression plasmid. Severalfold repression was calculated relative to the Gal4 DBD, and results of duplicate samples were plotted.

**ChIP assay.** A ChIP assay was performed according to the protocols of Upstate Biotechnology with minor modifications. 293T cells were transfected with 10 μg of either Gal4 DBD or Gal4 fusion construct expression vector and 5 μg of (Gal4 × 5)-SV40-luciferase (except for stable transfected cells). After no treatment or treatment with 10 nM T3 (or a lower concentration for dose response assays), cells were cross-linked with 1% formaldehyde for 15 min at room temperature. Cells were collected by washing twice with ice-cold phosphate-buffered saline and centrifuged for 4 min at 1,000 × *g*. Cell pellets were resuspended in 250 μl of cell lysis buffer (50 mM Tris-HCl [pH 8.0], 85 mM KCl, 0.5% NP-40) with protease inhibitor (Roche Molecular Biochemicals), incubated on ice for 10 min, and centrifuged for 4 min at 2,000 × *g*. The pellets were resuspended in 250 μl of sodium dodecyl sulfate (SDS) lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with protease inhibitor and sonicated 4 times for 15 s each time followed by centrifugation at 14,000 × *g* for 10 min. Supernatants were collected and diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl) with protease inhibitor followed by preclearing with 2 μg of sheared salmon sperm DNA and protein A Sepharose (50 μl of a 50% slurry in 10 mM Tris-HCl [pH 8.1]–1 mM EDTA) for 2 h at 4°C. Immunoprecipitation with the following antibodies was performed at 4°C overnight: anti-N-CoR and anti-HDAC4 (21); anti-TBL1 (12); anti-Gal4, anti-HDAC1, anti-HDAC2, anti-HDAC3, and normal rabbit immunoglobulin G (Santa Cruz); and anti-HDAC3 and anti-acetylated histone H4 (Upstate Biotechnology). Immunoprecipitated complexes were collected with protein A Sepharose beads followed by sequential washes in low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and Tris-EDTA buffer. Precipitates were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$), and 5 M NaCl was added to reverse cross-links at 65°C for 6 h. DNA fragments were purified with a PCR purification kit (Qiagen). A total of 0.5 to 3 μl of purified sample was used in 23 to 28 cycles of PCR. Primers for the (Gal4 × 5)-TK promoter were 5′-AGGCTTTACACTTATGCTTCCG-3′ and 5′-CTTTATGTTTTTGGCGTCTTCCA-3′. Primers for the (Gal4 × 5)-SV40 promoter were 5′-TGTACTTTATGGTACTGTAACTG-3′ and 5′-CTTTATGTTTTTGGCGTCTTCCA-3′.

**Immunoblot analysis.** Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes with HMW transfer buffer (50 mM Tris, 380 mM glycine, 0.1% SDS, 20% methanol). Blots were probed with primary antibodies (listed below) in Tris-buffered saline containing 5% nonfat dry milk followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Boehringer Mannheim) at a 1:5,000 dilution. Blots were visualized with ECL reagent (Amersham) and the following antibodies and dilutions: anti-N-CoR antibody (21) at 1:4,000, anti-SMRT antibody (12) at 1:500, anti-HDAC1 antibody at 1:1,000, anti-HDAC2 antibody at 1:2,000, and anti-HDAC4 antibody at 1:400 (Santa Cruz), and anti-HDAC3 antibodies (Novus) at 1:1,000.

**RNA interference.** Vectors that express hairpin small interfering RNAs (SiRNA) under the control of the human H1 promoter were constructed by inserting pairs of annealed DNA oligonucleotides into pSilencer H1 3.0 vector (Ambion) between *Bam*HI and *Hin*dIII restriction sites according to the manufacturer's instructions. The target sequences were as follows: for NCoR, 5′-AAGAAGGATCCAGCATTCGGA-3′; for SMRT-A, 5′-AAGCTGAAGAAGAAGCAGCAA-3′; for SMRT-B, 5′-AAGGGTATCATCACCGCTGTG-3′; for HDAC1, 5′-AAGCAGATGCAGAGATTCAAC-3′; for HDAC2, 5′-AAGCATCAGGATTCTGTTACG-3′; and for HDAC3, 5′-AAGATGCTGAACCATGCACCT-3′. Cells in 12-well plates were transfected with 1.6 μg of pSilencer vector per well. At 48 h after transfection, cells in each well were divided into 6 wells of a 12-well plate. After additional incubation for 24 h, a second transfection was performed with 1.6 μg of pSilencer vector, 0.3 μg of (Gal4 × 5)-SV40 luciferase reporter, 0.1 μg of β-galactosidase expression vector, and a Gal4 DBD fusion construct. Twenty-four hours after the second transfection, the medium was changed to DMEM containing charcoal-dextran-stripped fetal bovine serum. Cells were incubated for an additional 48 h in the presence or absence of 10 nM of T3 and harvested for luciferase assays and immunoblot analysis.

## RESULTS

**The N-CoR/HDAC3 complex is recruited to a target gene repressed by unliganded TR LBD.** Since the repressive role of histone deacetylation is related to changes in chromatin structure, we first studied 293T cells stably transfected with a reporter gene containing five Gal4 DBD binding sites upstream of the TK promoter driving the luciferase reporter gene. In this system, Gal4-TR repressed transcription of the integrated luciferase reporter gene and this was reversed by the addition of thyroid hormone (Fig. 1a). ChIP was employed to determine the TR- and T3-dependent recruitment of factors to the integrated target gene. As expected, both the Gal4 DBD alone and Gal4-TR bound to the gene both in the presence and absence of the thyroid hormone (Fig. 1b). Notably, in the absence of T3, TR recruited N-CoR, HDAC3, and a third component of the core N-CoR complex, TBL1 (12, 28, 44, 49), when the reporter gene was actively repressed (Fig. 1b). Recruitment of each of these factors was reversed by the addition of T3. By contrast, association of HDAC1, -2, and -4 with the reporter gene did not change as a function of TR binding or ligand (Fig. 1b). The HDAC1 antibody was able to detect Gal4-HDAC1 by ChIP with effectiveness comparable to that of the HDAC3 antibody with Gal4-HDAC3 (Fig. 1c). Thus, the failure to identify HDAC1 along with N-CoR and HDAC3 is indicative of its lack of recruitment by Gal4-TR. Repression by the TR LBD in human cells is associated with the specific recruitment of the N-CoR/HDAC3/TBL1 complex to the TR target gene without significant recruitment of HDAC1 or -2 or the class II HDAC4.

**The N-CoR/HDAC3 complex is recruited to a target gene repressed by the RevErb LBD.** We next tested whether Gal4-RevErb specifically recruits the N-CoR/HDAC3/TBL1 com-

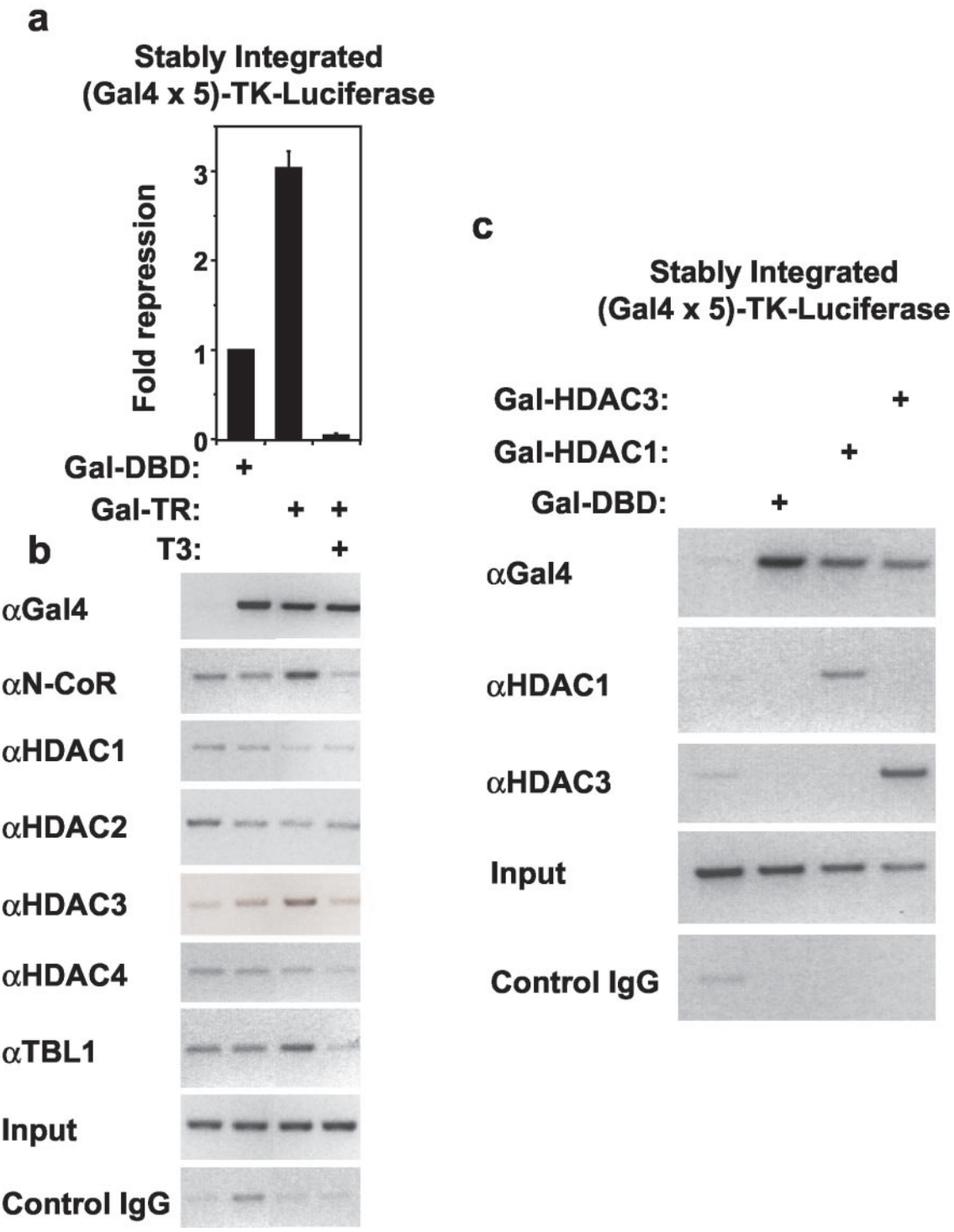


FIG. 1. Unliganded TR specifically recruits N-CoR, HDAC3, and TBL1 to a repressed, stably integrated target gene. (a) Transcription assay. A (Gal4 × 5)-TK-luciferase plasmid was stably transfected into 293T cells as described in Materials and Methods. Effects of transfected Gal4 DBD or DBD fusion to the TRβ LBD (in the presence or absence of T3) are shown. Results are plotted as repression levels. (b) ChIP analysis of the experiment shown in panel a. (c) ChIP analysis of cells with stably integrated reporters transfected with a Gal4 DBD alone or fused to HDAC1 or HDAC3. Control IgG, control immunoglobulin G.

plex to the integrated reporter gene. Like unliganded Gal4-TR, the RevErb LBD repressed transcription from this reporter (Fig. 2a). This was associated with recruitment of N-CoR, HDAC3, and TBL1 to the gene (Fig. 2b). By contrast, HDAC1, -2, and -4 were not recruited. Thus, the unique role of HDAC3 in nuclear receptor repression extends to RevErb as well, at least in the context of this reporter gene in this human cell line.

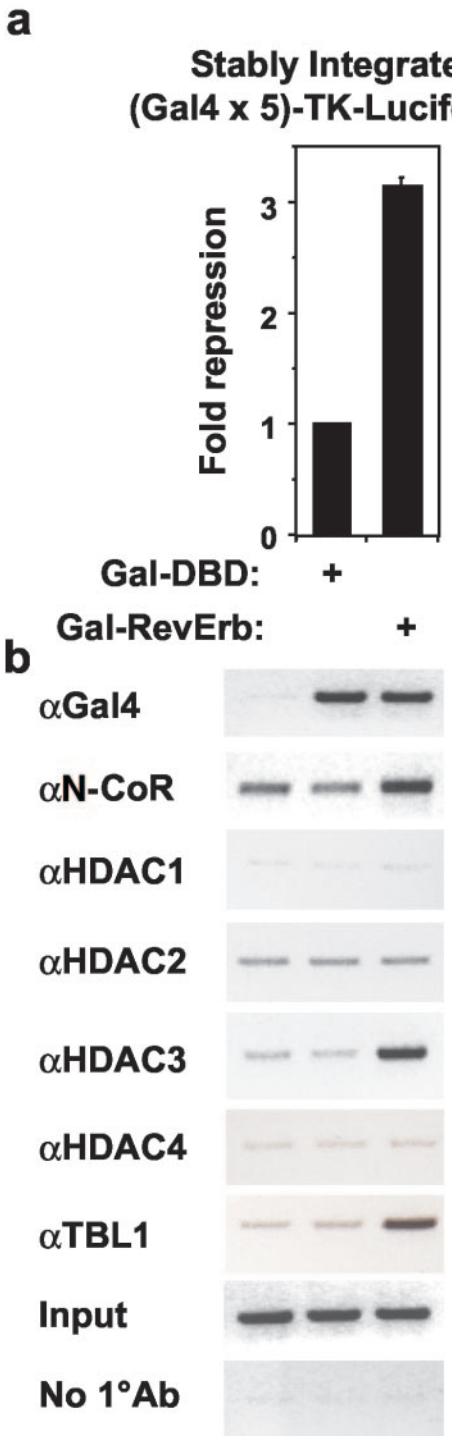


FIG. 2. RevErb specifically recruits N-CoR, HDAC3, and TBL1 to a repressed, stably integrated target gene. (a) Transcription assay. The stable 293T cell line containing the (Gal4 × 5)-TK-luciferase reporter was transfected with Gal4 DBDs either alone or fused to RevErb LBD. Results are plotted as repression levels. (b) ChIP analysis of the experiment shown in panel a.

**Repression and N-CoR/HDAC3 complex recruitment by the unliganded TR LBD on a transiently transfected reporter gene.** Studies of transcription have relied heavily on transient transfection of reporter genes. The recognition that chromatin context and structure are major determinants of gene transcription has raised questions about the validity of the transient transfection model in which gene reporter is not integrated into chromosomal DNA (43). Nevertheless, transfected templates are chromatinized (43). Here we compared the results obtained with the stably integrated reporter with those in a transient system. The transiently transfected reporter was repressed by the presence of unliganded TR and activated when T3 was added (Fig. 3a). ChIP analysis revealed recruitment of N-CoR and HDAC3, and this was reversed by T3 (Fig. 3b). Recruitment of the N-CoR/HDAC3 complex was paralleled by a dramatic reduction in acetylation of histone H4 at this promoter (Fig. 3b). HDAC3 recruitment and reduced histone acetylation in response to the presence of Gal4-TR are reversed by T3 in a dose-responsive manner, with a 50% effective concentration between 0.1 and 1 nM (Fig. 3c), consistent with the $K_d$ of the TR for T3. Thus, the assay for transient transfection faithfully reproduces the key features of TR repression: (i) repression by unliganded TR, (ii) activation by liganded TR, (iii) association of Gal4-TR LBD with the promoter in the presence or absence of ligand, (iv) recruitment of N-CoR and HDAC3 by unliganded TR, (v) local histone deacetylation in the context of transcriptional repression, and (vi) dissociation of HDAC3 and increase in histone acetylation at physiological concentrations of T3.

**Knockdown of N-CoR but not SMRT inhibits repression by TR.** We next addressed the requirement for N-CoR and SMRT for TR repression. SiRNA were used to specifically knock down expression of N-CoR or SMRT in human 293T cells. Transfection of N-CoR SiRNA reduced N-CoR protein expression nearly 75% without detectable change in SMRT expression, and SiRNA directed to SMRT nearly abolished SMRT expression specifically (Fig. 4a). Remarkably, knockdown of N-CoR markedly impaired the repression function of TR (Fig. 4b). By contrast, TR repression was not reduced by the knockdown of SMRT; in fact, the SMRT SiRNA repressed reporter gene activity by ~2-fold in the presence of either Gal4 DBD or Gal4-TR (Fig. 4b). The effect of N-CoR knockdown was specific in that it had little effect on the repressive activity of Mad (Fig. 4b), whose repressive activity is mediated by the Sin3/HDAC1 complex (2, 36), although a role for N-CoR in Mad repression has been reported in another system (23). The effects of knocking down N-CoR and SMRT were similar for TRα and TRβ, although TRβ had higher levels of inherent repressive activity. These studies clearly show that in human 293T cells, repression by TR relies on N-CoR but is independent of SMRT.

**Both N-CoR and SMRT contribute to repression by RevErb LBD.** We next examined the effect of N-CoR and SMRT knockdown on repression by RevErb. N-CoR knockdown reproducibly reduced RevErb repression, although the effect was more modest than seen with the LBDs of TRβ and TRα (Fig. 5a). SMRT knockdown did not reduce repression by RevErb, but unlike what was observed for TR, the SMRT SiRNA did not further increase repression despite its effect on the Gal4 DBD (Fig. 5a). Moreover, unlike TR, combined knockdown of N-CoR and SMRT reduced RevErb repression to a greater degree than that of either alone (Fig. 5a). Because SMRT knockdown with this SiRNA had a receptor-independent repressive effect on the reporter, we utilized a second SMRT-

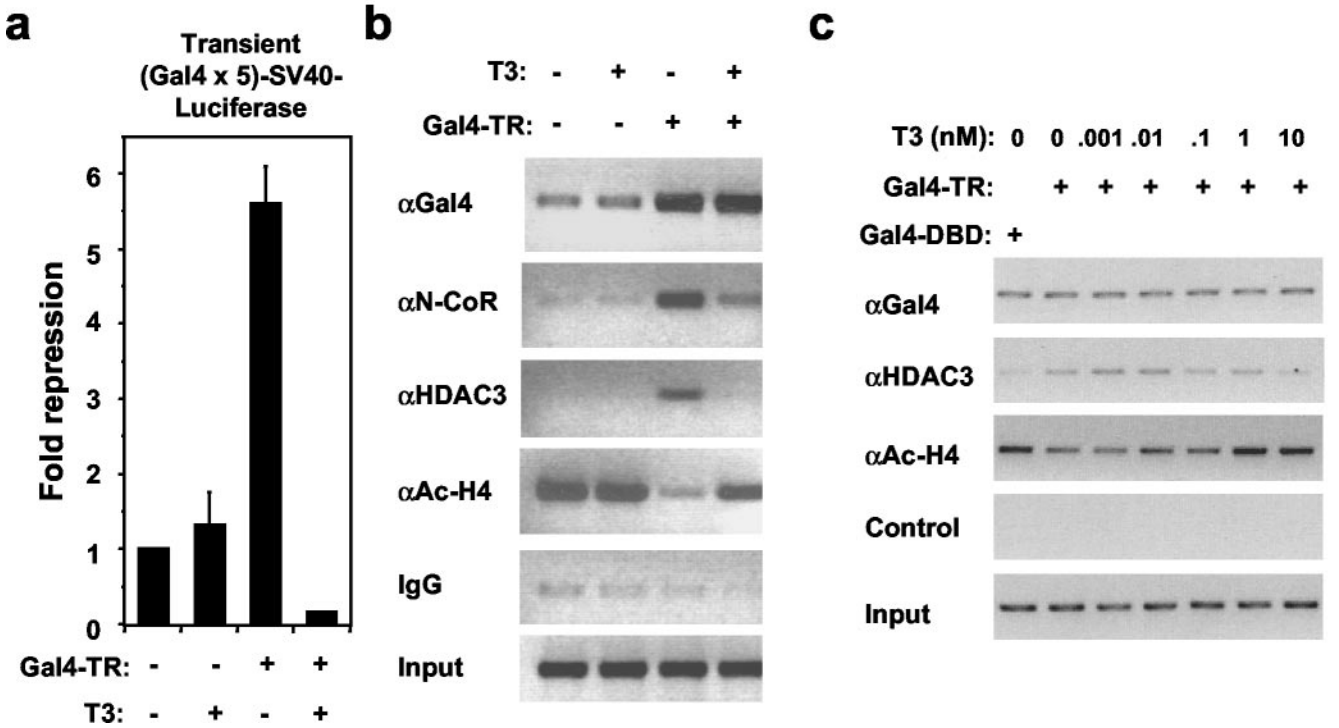


FIG. 3. Unliganded TR specifically recruits N-CoR and HDAC3 to a repressed, transiently transfected reporter gene. (a) Transcription assay. The (Gal4 × 5)-TK-luciferase reporter was transiently transfected with pCMX or pCMX-Gal4-TR LBDs into 293T cells. (b) ChIP analysis of the experiment shown in panel a. IgG, immunoglobulin G. (c) T3 dose response.

directed interfering hairpin RNA based on a reagent described by Yoon and colleagues while this paper was under review (46). This SiRNA (SMRT-B) reduced SMRT protein expression to a level comparable to that of the initial SiRNA (SMRT-A) (Fig. 5b). However, unlike SMRT-A, this SiRNA did not alter the basal repression of the reporter gene by Gal4-DBD. The SMRT-B SiRNA also had no effect on repression by TRβ (Fig. 5c) or TRα (data not shown) in consistency with the earlier conclusion that the TR LBDs were most affected by N-CoR knockdown. By contrast, the SMRT-B SiRNA reduced Gal4-RevErb repression to a degree similar to that of N-CoR knockdown (Fig. 5c). Thus, it appears that whereas N-CoR but not SMRT contributes to repression by Gal4-TR LBDs, both N-CoR and SMRT contribute to repression by Gal4-RevErb LBDs.

**HDAC3 contributes to repression by TR and RevErb.** We next used SiRNA to specifically knock down HDAC1, -2, and -3; in all cases, knockdown was approximately 75% (Fig. 6a). Knockdown of HDAC3 markedly inhibited repression by both TR and RevErb (Fig. 6b). By contrast, knockdown of HDAC1 and -2 had only modest effects on TR repression and only HDAC1 knockdown reduced RevErb repression. The HDAC1 SiRNA activated expression of the reporter (manifest as reduced repression) in the absence of Gal4-TR or Gal4-RevErb (Fig. 6b). This has previously been noted for another TR-responsive reporter (27), and thus, the effect of HDAC1 knockdown appears to be general and not receptor specific. By contrast, HDAC3 plays a specific and critical role in repression by both TR and RevErb. Coupled with the ChIP studies, we conclude that the N-CoR/HDAC3/TBL1 complex is required for repression by TR LBD and that other HDACs play minor (at most) roles in human 293T cells. Repression by RevErb LBD also utilizes HDAC3, most likely in conjunction with SMRT and N-CoR.

## DISCUSSION

We have shown that the N-CoR/HDAC3 complex is recruited to repressed genes and required for repression by TR. N-CoR is uniquely involved in repression by the TR LBD, whereas both SMRT and N-CoR contribute to repression by RevErb. HDAC3 is critically important for repression by both nuclear receptors. This work has several important implications related to the mechanism of thyroid hormone action, functions of N-CoR and SMRT, specificity of HDAC function, and the role of HDAC3 in repression.

The phenomenon of repression by unliganded TR was originally observed in the context of transient transfection of reporter genes into mammalian cells (3, 8) and has been mainly studied in this situation over the last decade. The realization that corepressors induce chromatin modifications has raised the question of whether repression of transiently transfected reporter genes utilizes alternative mechanisms. The present work shows that the mechanism of repression by TR is similar for transient and stably integrated reporter genes and clearly demonstrates modulation of histone acetylation correlating with transcriptional activity of a transiently transfected reporter. Moreover, recruitment of HDAC3 is required for repression of this reporter. Thus, we conclude that chromatin modification plays a major role in TR-targeted repression of transiently transfected as well as stably integrated genes.

The N-CoRs N-CoR and SMRT were discovered on the

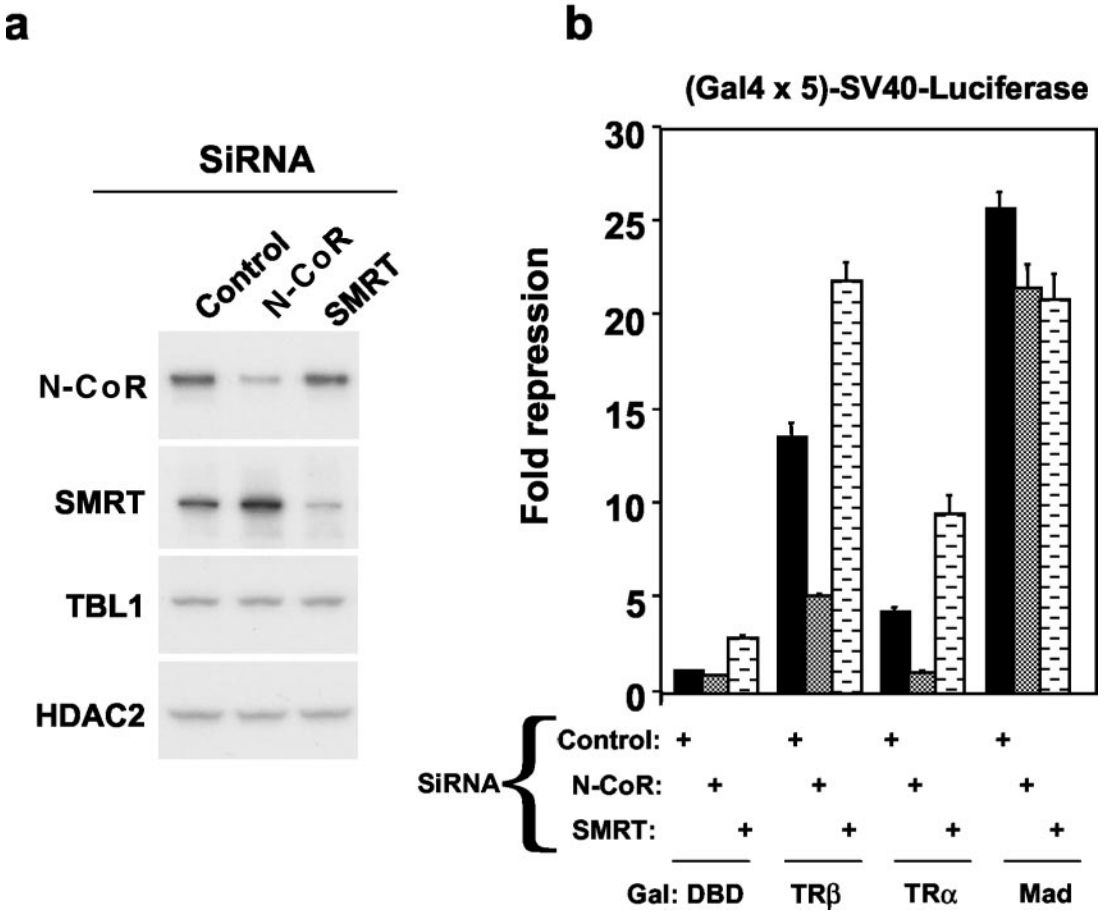


FIG. 4. Knockdown of N-CoR but not SMRT reduces repression by TR. (a) Knockdown using SiRNA for N-CoR and SMRT. Immunoblot for N-CoR, SMRT, and HDAC2 (Control) after transfecting 293T cells with SiRNA for N-CoR or SMRT. (b) Transcription assay. The (Gal4 × 5)-SV40-luciferase reporter was transiently transfected along with the Gal4 DBD, Gal4-TRα, Gal4-TRβ, or Gal4-Mad into 293T cells treated with SiRNA for N-CoR or SMRT as in panel a.

basis of protein-protein interactions with unliganded TR and other nuclear receptors (5, 17, 34, 37, 48). Both N-CoR and SMRT contain multiple domains capable of repression and interact with multiple unliganded nuclear receptors. Genetic deficiency of N-CoR is lethal, indicating that the functions of N-CoR and SMRT are not completely redundant (23). N-CoR and SMRT both contain short CoRNR polypeptide motifs that specifically recognize unliganded nuclear receptors (18, 31, 32). These CoRNR peptides are better conserved within a given corepressor across species than between N-CoR and SMRT in the same species. Indeed, biochemical and mammalian two-hybrid studies have revealed that TR prefers N-CoR to SMRT, possibly reflecting the presence of a unique CoRNR motif found in N-CoR but not SMRT (20, 38, 42). Here we have shown that N-CoR was specifically recruited to TR-repressed target genes in living mammalian cells along with TBL1 and HDAC3, which have been found in tight complex with N-CoR as well as SMRT (12, 28, 49). A critical role for this complex in repression is suggested by its dissociation in the setting of derepression by thyroid hormones. The present study proves that the preference of TR for N-CoR over SMRT is biologically relevant, as knockdown of SMRT had virtually no effect whereas N-CoR knockdown greatly attenuated repression by the TR LBD in human 293T cells. While this work was under review, Yoon and colleagues reported that knockdown of either N-CoR or SMRT completely abolished TR repression in HeLa cells (46). It will be of interest to determine whether this difference is cell type or assay system related.

Our results suggest that N-CoR and SMRT both contribute to repression by RevErb. This is consistent with previous in vitro studies showing that RevErb interacts with both N-CoR and SMRT, although the regions of the RevErb LBD required for interaction differ for N-CoR and SMRT (48). However, full-length RevErb bound to its homodimeric binding site selectively interacted with the receptor interaction domain of N-CoR but not SMRT (48). This result underscores the importance of cell type, target gene, and LBD context in the regulation of repression. Unfortunately, at the time of the present study our SMRT antibodies were inadequate for ChIP in this system. Future studies will be needed to determine whether endogenous RevErb preferentially recruits full-length, endogenous N-CoR and/or SMRT to endogenous genes.

Numerous labs investigating the mechanism of repression by N-CoR and SMRT have implicated recruitment of HDAC activity. However, the precise HDAC(s) involved remains somewhat controversial. Initial studies suggested that the Sin3/

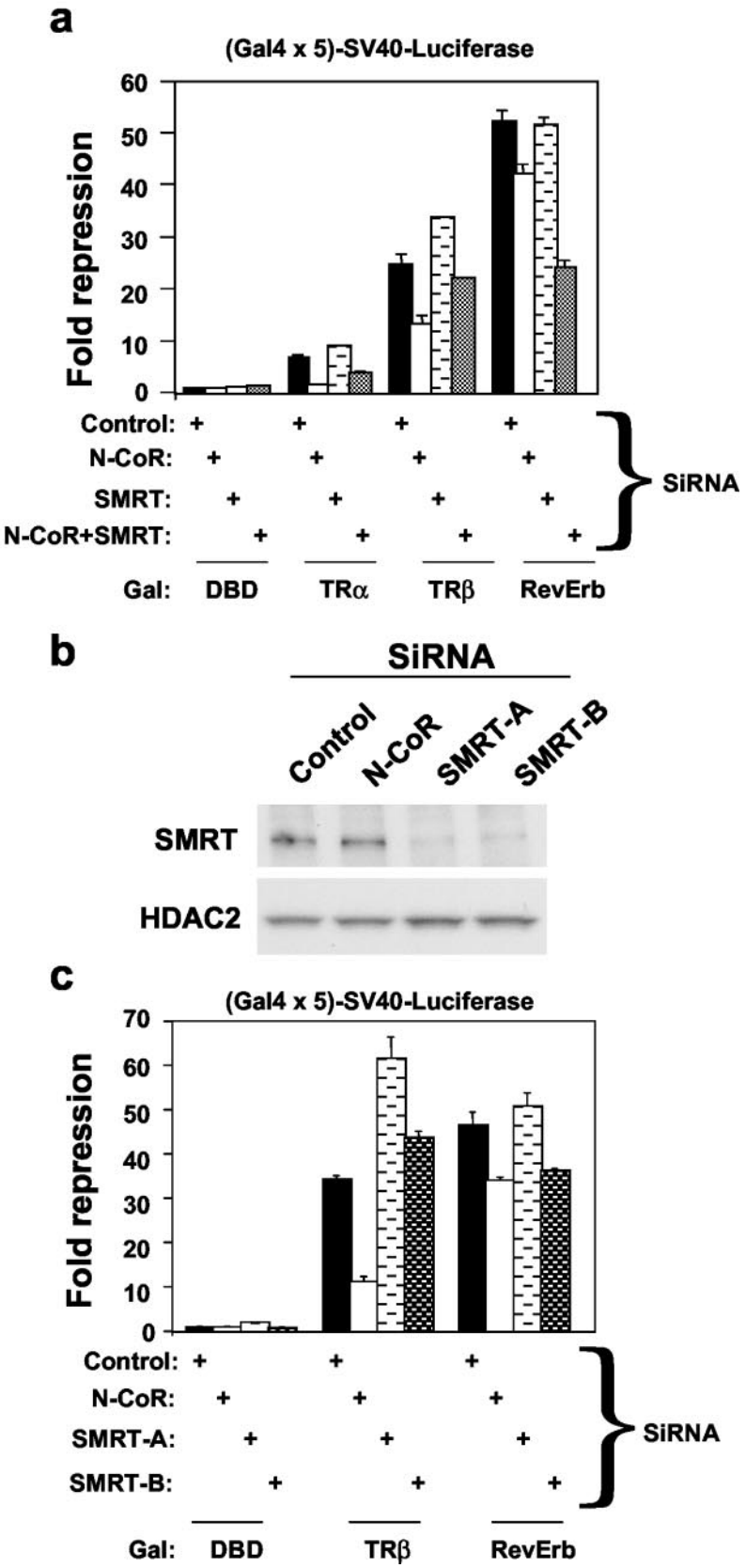


FIG. 5. SMRT contributes to repression by RevErb. (a) The (Gal4 × 5)-SV40-luciferase reporter was transiently transfected along with the Gal4 DBD, Gal4-TRα, Gal4-TRβ, or Gal4-RevErb into 293T cells treated with SiRNA for N-CoR or SMRT or both. (b) Knockdown of SMRT. (c) SMRT-B SiRNA attenuates repression by RevErb but not TR.

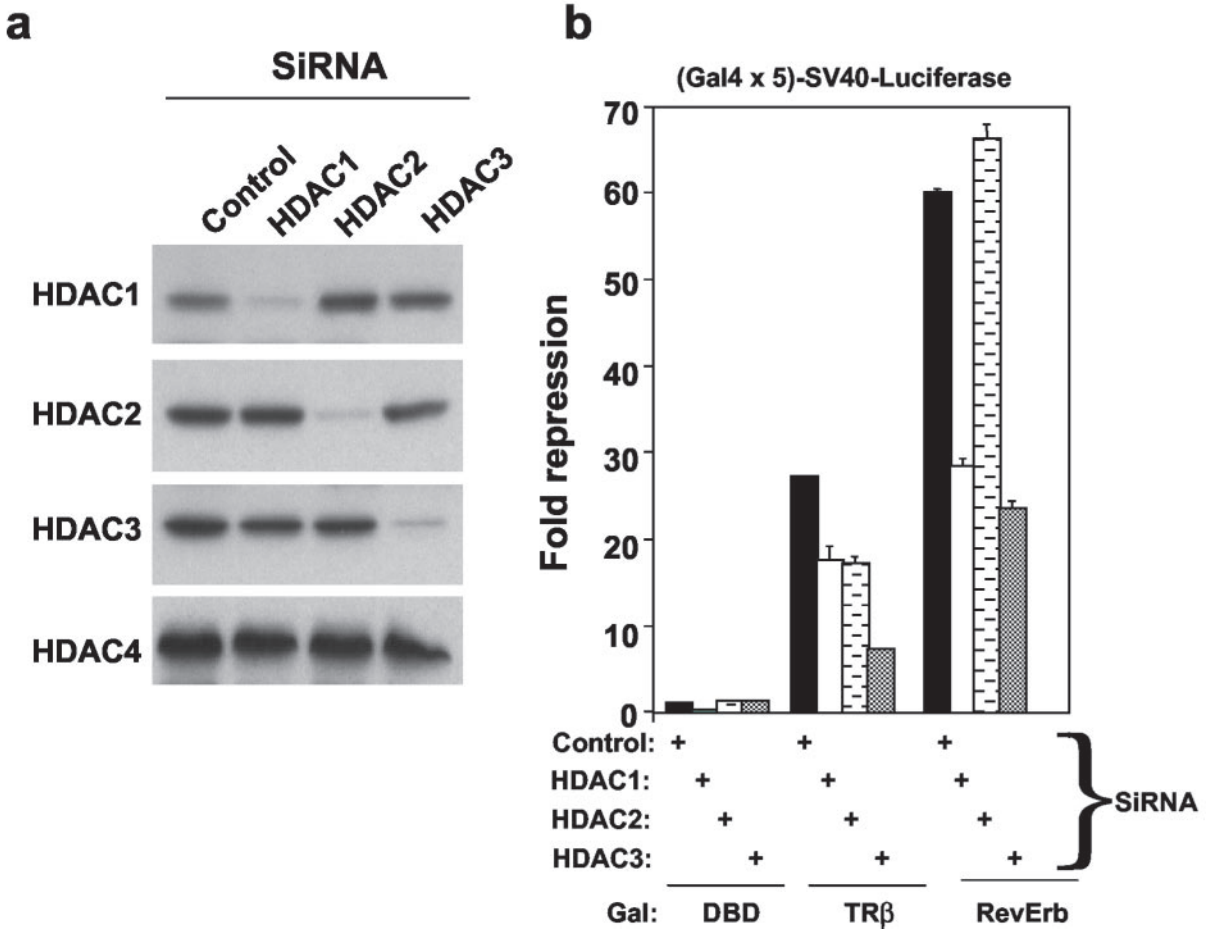


FIG. 6. Knockdown of HDAC3 markedly reduces repression by TR and RevErb. (a) Knockdown using SiRNA for HDAC1, -2, and -3. Shown are immunoblots for HDAC1 to -3 and HDAC4 (Control) after transfecting 293T cells with SiRNA for HDAC1, -2, and -3. (b) Transcription assay. The (Gal4 × 5)-TK-luciferase reporter was transiently transfected along with Gal4 DBD, Gal4-TRβ, or Gal4-RevErb into 293T cells treated with SiRNA for HDAC1 to -3 as described for panel a.

HDAC1 corepressor complex was involved and demonstrated in vitro interaction between Sin3 and N-CoR/SMRT (1, 16, 30). However, biochemical analysis of HDAC1 and Sin3 complexes did not reveal stable association with N-CoR/SMRT (15, 51, 52). Conversely, several purifications of N-CoR and SMRT complexes did not copurify HDAC1 (12, 28, 49), although others did find evidence for an N-CoR complex containing HDAC1 (24, 39). Class II HDAC4, -5, and -7 have also been identified as N-CoR/SMRT-interacting proteins (6, 21, 25). In addition, several groups have shown that HDAC3 copurifies with N-CoR/SMRT (12, 28, 49) and N-CoR has been shown to be a component of HDAC3 complexes (12, 28, 44, 49). Here we show that HDAC3, but not other HDACs, was recruited along with N-CoR to the target gene repressed by unliganded TR as well as by RevErb. Moreover, knockdown of HDAC3 dramatically reduces repression in human 293T cells, whereas knockdown of HDAC1 (as well as HDAC2) had modest effects that, in the case of HDAC1, were related to the reporter gene but not the receptor construct. These data strongly suggest a unique role of HDAC3 in repression by TR and RevErb in this human system. It is possible that other HDACs contribute to TR repression in other cell types or on other genes. For example, CaM kinase-directed nuclear export of class II HDACs may prevent their interaction with TR under certain cellular conditions (10, 29).

The involvement of HDAC3 in repression by TR and RevErb has evolutionary implications as well. The frog homolog of HDAC3 has been shown to be recruited by unliganded TR to target genes in *Xenopus laevis* (27). Yeast also possess an HDAC3 homolog called HOS2. Intriguingly, HOS2 exists in a complex with the yeast TBL1 homolog as well as a protein that contains a SANT motif homologous to the DAD of SMRT/N-CoR (33). The similarity extends to the interaction of yeast HDAC3 with TRiC (33), whose mammalian homolog is involved in assembling the stable HDAC3-SMRT complex in mammalian cells (13). Interestingly, a recent genomic study of HDAC3 in yeast implicated HOS2 recruitment in gene activation rather than repression (41). Our study clearly shows that HDAC3 is recruited to genes repressed by nuclear receptors in mammalian cells and required for transcriptional repression in this context. Thus, despite conservation of their enzymatically active deacetylase complexes, there is a fundamental difference between the transcriptional roles of mammalian HDAC3 and its yeast homolog.

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