Transcription, Chromatin, and Epigenetics:

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Function of Histone Deacetylase 6 as a Cofactor of Nuclear Receptor Coregulator LCoR*

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Ligand-dependent corepressor LCoR was identified as a protein that interacts with the estrogen receptor α (ERα) ligand binding domain in a hormone-dependent manner. LCoR also interacts directly with histone deacetylase 3 (HDAC3) and HDAC6. Notably, HDAC6 has emerged as a marker of breast cancer prognosis. However, although HDAC3 is nuclear, HDAC6 is cytoplasmic in many cells. We found that HDAC6 is partially nuclear in estrogen-responsive MCF7 cells, colocalizes with LCoR, represses transactivation of estrogen-inducible reporter genes, and augments corepression by LCoR. In contrast, no repression was observed upon HDAC6 expression in COS7 cells, where it is exclusively cytoplasmic. LCoR binds to HDAC6 *in vitro* via a central domain, and repression by LCoR mutants lacking this domain was attenuated. Kinetic chromatin immunoprecipitation assays revealed hormone-dependent recruitment of LCoR to promoters of ERα-induced target genes in synchrony with ERα. HDAC6 was also recruited to these promoters, and repeat chromatin immunoprecipitation experiments confirmed the corecruitment of LCoR with ERα and with HDAC6. Remarkably, however, although we find evidence for corecruitment of LCoR and ERα on genes repressed by the receptor, LCoR and HDAC6 failed to coimmunoprecipitate, suggesting that they are part of distinct complexes on these genes. Although small interfering RNA-mediated knockdown of LCoR or HDAC6 augmented expression of an estrogen-sensitive reporter gene in MCF7 cells, unexpectedly their ablation led to reduced expression of some endogenous estrogen target genes. Taken together, these data establish that HDAC6 can function as a cofactor of LCoR but suggest that they may act in enhance expressing some target genes.

Nuclear receptors are ligand-regulated transcription factors whose activities are controlled by a variety of lipophilic extracellular signals, including steroid and thyroid hormones, metabolites of vitamins A (retinoids) and D (1, 2). DNA-bound nuclear receptors regulate transcription by recruiting complexes of coregulatory proteins, classified as coactivators or corepressors depending on whether they act to stimulate or repress transcription (2–4). Many coactivators interact with receptors through signature L*XX*LL motifs, known as NR boxes, which are oriented within a hydrophobic pocket of agonist-bound receptor ligand binding domains (5). Several coactivators or their associated cofactors possess histone acetyltransferase activity, which essentially caps positively charged lysine residues and loosens their association with DNA, facilitating chromatin remodeling and subsequent access of the transcriptional machinery to promoters.

Nuclear receptor corepressors NCoR[7] and SMRT were isolated as factors that interacted with hormone-free but not hormone-bound thyroid and retinoid receptors (6, 7). They bind to receptor ligand binding domains through extended L*XXX*-I*XXX*(L/I) motifs known as CoRNR boxes (8, 9) and recruit multiprotein complexes implicated in transcriptional repression and histone deacetylation (2–4, 10–13). Hormone binding induces a conformational change in ligand binding domains that leads to dissociation of NCoR or SMRT. Both corepressors are components of several different complexes containing distinct combinations of ancillary proteins and class I or class II histone deacetylases (HDACs), suggesting that their function depends on cell type, combinations of transcription factors bound to specific promoters, and phase of the cell cycle.

We identified a ligand-dependent corepressor, LCoR, as an NR box-containing protein that interacted with the ligand binding domains of agonist-bound receptors and repressed hormone-dependent transactivation when overexpressed (14). Although LCoR interacts with nuclear receptors in essentially

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[7] The abbreviations used are: NCoR, nuclear receptor corepressor; ADORA1, adenosine A1 receptor; BMP7, bone morphogenetic protein 7; ChIP, chromatin immunoprecipitation; CYP26B1, cytochrome P450, family 26, subfamily b, polypeptide 1; E2, estradiol; ERα, estrogen receptor α; GREB1, gene regulated by estrogen in breast cancer protein; HDAC, histone deacetylase; IGFBP4, insulin-like growth factor binding protein 4; KRT4, keratin 4; LCoR, ligand-dependent corepressor; p300, E1A-binding protein p300; pS2, trefoil factor 1; SGK3, serum/glucocorticoid regulated kinase family, member 3; SMRT, silencing mediator for retinoid and thyroid-hormone receptors; TSA, trichostatin A; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HA, hemagglutinin; PBS, phosphate-buffered saline; ERE, estrogen response element; GST, glutathione *S*-transferase; NRIP1, nuclear receptor interacting protein 1.

the same manner as coactivators, it recruits both HDACs and C-terminal-binding proteins corepressors. LCoR interacts directly with class I HDAC3 and class II HDAC6 *in vitro* and coimmunoprecipitates with the two proteins from MCF7 cell extracts (14). Although HDAC3, like LCoR, is a nuclear protein, the interaction of LCoR with HDAC6 is remarkable as HDAC6 is cytoplasmic in many cells (15). Indeed, HDAC6 has been shown to function as a tubulin deacetylase (16, 17) through an association controlled by a tetradecapeptide motif (18). However, a portion of HDAC6 can be nuclear in some cells. Notably, experiments performed in breast cancer cells have revealed that HDAC6 is an estrogen target gene (19) and that HDAC6 protein is present in the nuclei of normal breast epithelial cells but is cytoplasmic in adjacent malignant cells (20, 21). Moreover, these studies found that HDAC6 expression levels correlate with better prognosis and response to endocrine therapy in breast cancer (19–21).

Based on the above, we examined the subcellular localization of HDAC6 in estrogen-responsive MCF7 breast cancer cells and its potential role as an LCoR cofactor. We find that HDAC6 is partially nuclear in MCF7 cells and that LCoR and HDAC6 are recruited together during ERα-dependent gene regulation in MCF7 cells. Remarkably, however, although ablation of LCoR or HDAC6 enhanced estrogen-dependent stimulation of a reporter gene, the effect was not reproduced on endogenous ERα target genes. Rather, the results suggested that the two proteins can act to enhance expression of specific estrogen-regulated genes.

EXPERIMENTAL PROCEDURES

Antibodies—A rabbit polyclonal antipeptide antibody was raised against LCoR amino acids 20–36 (QDPSQPNSTKNQSLPKA) fused to keyhole limpet hemocyanin and purified over a peptide affinity column (Bethyl Laboratories, Montgomery TX). Goat polyclonal HDAC3 (sc-8138), goat polyclonal HDAC6 (sc-5253), rabbit polyclonal HDAC6 (sc-11420), rabbit polyclonal ERα (sc-543), protein A-agarose (sc-2001), and protein G PLUS-agarose (sc-2002) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal p300 (ab3425) was from Abcam Inc. (Cambridge, MA). Cy3-donkey polyclonal α-goat (705-165-147) and Cy2-goat polyclonal α-rabbit (711-225-152), Cy3-donkey polyclonal α-rabbit (711-165-152), and Cy2-donkey polyclonal α-mouse (715-225-150) were purchased from Jackson ImmunoResearch (West Grove, PA). Mouse monoclonal α-FLAG M2 (F3165) and α-FLAG M2 horseradish peroxidase (HRP)-conjugate (A8592), monoclonal α-rabbit HRP conjugate (A2074), and rabbit polyclonal α-goat HRP conjugate (A5420) were from Sigma.

Recombinant Plasmids—PSG5/LCoR, FLAG-HDAC6/pcDNA3, HA-HDAC3/pCDNA3.1, and FLAG-LCoR/pcDNA3.1 have been described (14). FLAG-LCoRΔHDAC6/pcDNA3.1 was made using the QuikChange mutagenesis kit (200518, Stratagene, La Jolla, CA) as per the manufacturer's instructions. Primers were designed to delete amino acids 203–319 from LCoR. The new construct was sequenced to confirm proper deletion, and a Western blot was performed to show the equal level of expression when compared with wild-type LCoR.

Cell Culture and Transfections—All cells were cultured under the recommended conditions. For immunocytochemistry, COS7 cells grown on collagen IV-treated microscope slides in 6-well plates in DMEM supplemented with 10% FBS were transfected in medium without serum with 12.5 μl of Lipofectamine 2000 (Invitrogen) containing 1 μg each of pSG5/LCoR and HA-FLAG-HDAC6/pcDNA3. Medium was replaced 24 h after transfection, and cells were prepared for immunocytochemistry after 48 h as described below. For analysis of the effects of HDAC3 or -6 on LCoR corepression, COS7 cells (60–70% confluent) grown in DMEM without phenol red supplemented with 10% FBS on 6-well plates were transfected in minimal medium without serum with Lipofectamine 2000 (5 μl) with 100 ng of ERα expression vector, 250 ng of ERE3-TATA-pXP2 reporter plasmid, and 250 ng of internal control vector pCMV-βgal. Quantities of expression vectors (LCoR/pSG5, HA-HDAC3/pCDNA3.1, FLAG-HDAC6/pcDNA3, FLAG-LCoR/pcDNA3.1, and FLAG-LCoRΔHDAC6/pcDNA3.1) used are indicated in the figures or corresponding figure legends. Medium was replaced 18 h after transfection with medium containing charcoal-stripped serum and estradiol (10 nM) for 30 h. MCF7 cells grown in 6-well plates were transfected similarly. MCF7 cells were also grown in 24-well plates and were transfected using a on-fifth scale. Trichostatin A (TSA) and trapoxin were added to 500 and 50 nM, respectively, as indicated. Cells were harvested in 250 μl of reporter lysis buffer (Promega). Note that the transfection conditions above were chosen because the amounts of HDAC and LCoR expression vectors used led to selective repression of ERα-dependent transactivation without affecting expression of the β-galactosidase internal control.

Immunocytochemistry—Cells were cultivated on collagen IV-treated microscope slides in 6-well plates, fixed with 2% paraformaldehyde for 15 min at room temperature, washed (3×) with 1× PBS, and permeabilized with 0.2% Triton X-100, 5% bovine serum albumin, 10% horse serum in PBS. MCF7 cells were then incubated with α-LCoR (1:500) and goat polyclonal antibodies against HDAC6 or Bmi1 (1:50) in buffer B (0.2% Triton X-100, 5% bovine serum albumin in PBS) for 1 h at room temperature. Cells were washed (3×) with PBS and incubated with goat anti-rabbit-Cy2 and donkey anti-goat Cy3 (1:300) in buffer B for 1 h at room temperature. Transiently transfected COS7 cells were incubated with α-LCoR (1:500) and anti-FLAG (1:300) to detect FLAG-HDAC6. Cells were washed (3×) with PBS and incubated with Cy3-donkey polyclonal α-rabbit (1:300) and Cy2-donkey polyclonal α-mouse (1:400) in buffer B for 1 h at room temperature. Slides were mounted with Immuno-Fluore mounting medium (ICN, Aurora, OH) and visualized using a Zeiss LSM 510 confocal microscope at 63× magnification.

Western Blotting—The following primary antibodies were used: LCoR (GenWay Biotech, 18-003-44018), and FLAG (Santa Cruz Biotechnology, sc-807). The following secondary antibody was purchased from Santa Cruz Biotechnology: goat anti-rabbit (sc-2004). A Western blot was performed as previously described (22) using MCF7 cells extracts. Cells were grown in 10 cm dishes (70% confluent) and transiently trans-

fected with 500 ng of FLAG-tagged LCoR. 30h later, cells were harvested.

Chromatin Immunoprecipitation (ChIP) and ReChIP Assays—ChIP and reChIP assays were performed as previously described (23) in MCF7 cells. Cells were grown in 10-cm dishes (70% confluent) and transiently transfected with 500 ng of FLAG-tagged LCoR. After the transfection, cells were starved for 2 days in DMEM-phenol free and FBS-free media and treated with 2.5 μM α-amanitin (Sigma, A2263) for 2 h before hormone treatment to properly synchronize cells. Cells were collected, and cofactor recruitment was evaluated on promoter regions containing EREs of estrogen target genes. Immunoprecipitations were performed with the following antibodies: ERα (sc-543), HDAC6 (Upstate, 07-732), FLAG (OctA-Probe, sc-807), and p300 (sc-8981). Protein A-agarose (sc-2001) was used for the immunoprecipitation, and normal rabbit IgG (sc-2027) was used for background control. Primer sequences used are were following: *pS2* promoter 5′-CTCTCACTATGAATCACTTCTGCAG-3′ (forward) and 5′-AGATAACATTTGCCtAAGGAGGCC-3′ (reverse), non-targeting *pS2* 5′-CAGCCCCCAAGAACTTCCAG-3′ (forward) and 5′-TGAGCAGGTTTGCAGCACACTT-3′ (reverse), *ADORA1* promoter 5′-CAGAAGCTCTGTTGGGCATG-3′ (forward) and 5′-ATCGGGCTTTGACGTGGT-3′ (reverse), *ADORA1* non-targeting 5′-TAGAATCCACTAGTCCACCTTCT-3′ (forward) and 5′-TCACTTGCTGCTTACTACTTACCCTTC-3′ (reverse), *IGFBP4* promoter 5′-CTTTCTTGCTGCAAAGTCCC-3′ (forward) and 5′-ATGGCCTTCCATGCTACAAG-3′ (reverse), *IGFBP4* non-targeting 5′-GCCAGGGACCGGTATAAAG-3′ (forward) and 5′-GACGTAGCGGGGGAAGTTAG-3′ (reverse), *NRIP1* promoter 5′-GATGCAGATTGGCTGACAGA-3′ (forward) and 5′-CCCACCCCCAATTTCTATCT-3′ (reverse), *NRIP1* non-targeting 5′-GCGAGGGGAGGGACTGGG-3′ (forward) and 5′-ATGTCTGCGAGGCTGACTTT-3′ (reverse), *BMP7* promoter 5′-TGCAGACGACGAAAAATCAG-3′ (forward) and 5′-AGGGGTGGGAGGTTTAGATG-3′ (reverse), and *BMP7* non-targeting 5′-CGCTATCAGTCACCCCATTT-3′ (forward) and 5′-CGAAAAGGCTTTGAGATTGC-3′ (reverse).

siRNA Knockdowns—siRNAs were purchased from Thermo Scientific Dharmacon (Lafayette, CO). The following ON-TARGETplus SMART pool siRNA were used: LCoR (L-026303-00), HDAC6 (L-003499-00), and non-targeting (D-001818-10). siRNAs were resuspended per the manufacturer's instructions. Transfections were done in 6-well plates as described previously. Lipofectamine 2000 (10 μl) was used as the transfection reagent. DMEM phenol-free with 10% stripped FBS was added 12 h after transfection. For Western blot analysis, cells were collected 48 h after transfection. *Luciferase* reporter assays after siRNA knockdowns were performed as follows. 100 ng of ERα expression vector and 250 ng of ERE3-TATA-pXP2 vector were transfected with the corresponding siRNA. DMEM phenol-free with 10% stripped FBS was added 12 h after transfection. Estradiol (10 nM) was added 36 h after transfection, and cells were collected 24 h later. *Luciferase* activity was measured as previously described.

RNA Isolation, cDNA Synthesis, and Quantitative Real-time-PCR—Cells were grown in 100-mm dishes. Medium was replaced with charcoal-stripped medium containing ligand. Total RNA was extracted with TRIZOL reagent. cDNA synthesis was performed with iScript cDNA synthesis kit (Bio-Rad) according to the instructions of the manufacturer. A Mini-Opticon real-time PCR system with iQ SYBR Green Supermix (Bio-Rad) were used for quantitative real-time-PCR expression analysis of target genes. The program was: 1) incubation at 94 °C for 60 s, 2) incubation at 95 °C for 20 s, 3) incubation at 60 °C for 30 s (decreasing temperature by 1° per cycle), 4) incubation at 72 °C for 30 s, 5) plate reading, 6) repetition from step 2 five more times, 7) incubation at 95 °C for 20 s, 8) incubation at 57.5 °C for 30 s, 9) incubation at 72 °C for 30 s, 10) plate reading, 11) repetition from step 7 thirty-five more times, 12) melting curve formed, and end. Results were normalized to β-actin mRNA expression. The following primers were used: *pS2* 5′-ACCATGGAGACAAGGTGAT-3′ (forward) and *pS2* 5′-AAATTCACACTCCTCTTCTG-3′ (reverse), *GREB1* 5′-CCACAAAGGGTGGTCTCCAGAA-3′ (forward) and *GREB1* 5′-CACTGGCTTGGCCTTGCATATT-3′ (reverse), *SGK3* 5′-CAAAAGAAGATTCCACCACCA-3′ (forward) and *SGK3* 5′-TGTCAAAGTTTCTGATATCATCTC-3′ (reverse), *CYP26B1* 5′-ACATCCACCGCAACAAGC-3′ (forward) and *CYP26B1* 5′-GGATCTTGGGCAGGTAACTCT-3′ (reverse), *BMP7* 5′-GGTCATGAGCTTCGTCAACC-3′ (forward) and *BMP7* 5′-GCAGGAAGAGATCCGATTCC-3′ (reverse), *KRT4* 5′-GCCGACAATGACTTTGTGGT-3′ (forward) and *KRT4* 5′-CCTCCAACTCCACCTTGTTC-3′ (reverse), and *β-actin* 5′-GGCATGGGTCAGAAGGATTCC-3′ (forward) and *β-actin* 5′-GCTGGGGTGTTGAAGGTCTC-3′ (reverse), *ADORA1* 5′-GACCTACTTCCACACCTGCCTCA-3′ (forward) and *ADORA1* 5′-CCAGCCAAACATAGGGGTCAGT-3′ (reverse), *IGFBP4* 5′-GGGGGCAAGATGAAGGTCAAT-3′ (forward) and *IGFBP4* 5′-CGGTCCACACACCAGCACTT-3′ (reverse) and *NRIP1* 5′-GTGATTCCAGGATGGTTTGG-3′ (forward) and *NRIP1* 5′-ATGGTTTTAATAAAGGTTAAGGATGC-3′ (reverse).

RESULTS

Colocalization of HDAC6 with LCoR in MCF7 Cells—Our previous results showed that endogenous LCoR coimmunoprecipitated with endogenous HDAC3 and -6 from extracts of MCF7 cells (14). However, as HDAC6 is cytoplasmic in many cells, we further investigated the colocalization of LCoR and HDAC6 in MCF7 cells by immunocytochemistry. As expected (14), LCoR was almost exclusively nuclear, as was HDAC3, and there was a marked colocalization of the two proteins (Fig. 1*A*). Moreover, a substantial portion of HDAC6 was nuclear in MCF7 cells, and there was a clear colocalization of nuclear HDAC6 with LCoR (Fig. 1*A*), substantiating the possibility that the two proteins function together.

Cell-specific Repression of Hormone-dependent Transactivation by HDAC6—The capacity of HDAC6 to function as a (cell-specific) cofactor in LCoR-dependent corepression of estrogen-dependent transactivation was further analyzed in transiently transfected COS7 and MCF7 cells. COS7 cells were chosen for comparison because HDAC6 remains cytoplasmic even when overexpressed in transient expression experiments (Fig. 1*B*). Coexpression of HDAC6 with LCoR in COS7 cells had no effect on LCoR-dependent corepression (Fig. 1*C*). As a control for the repressive effects of

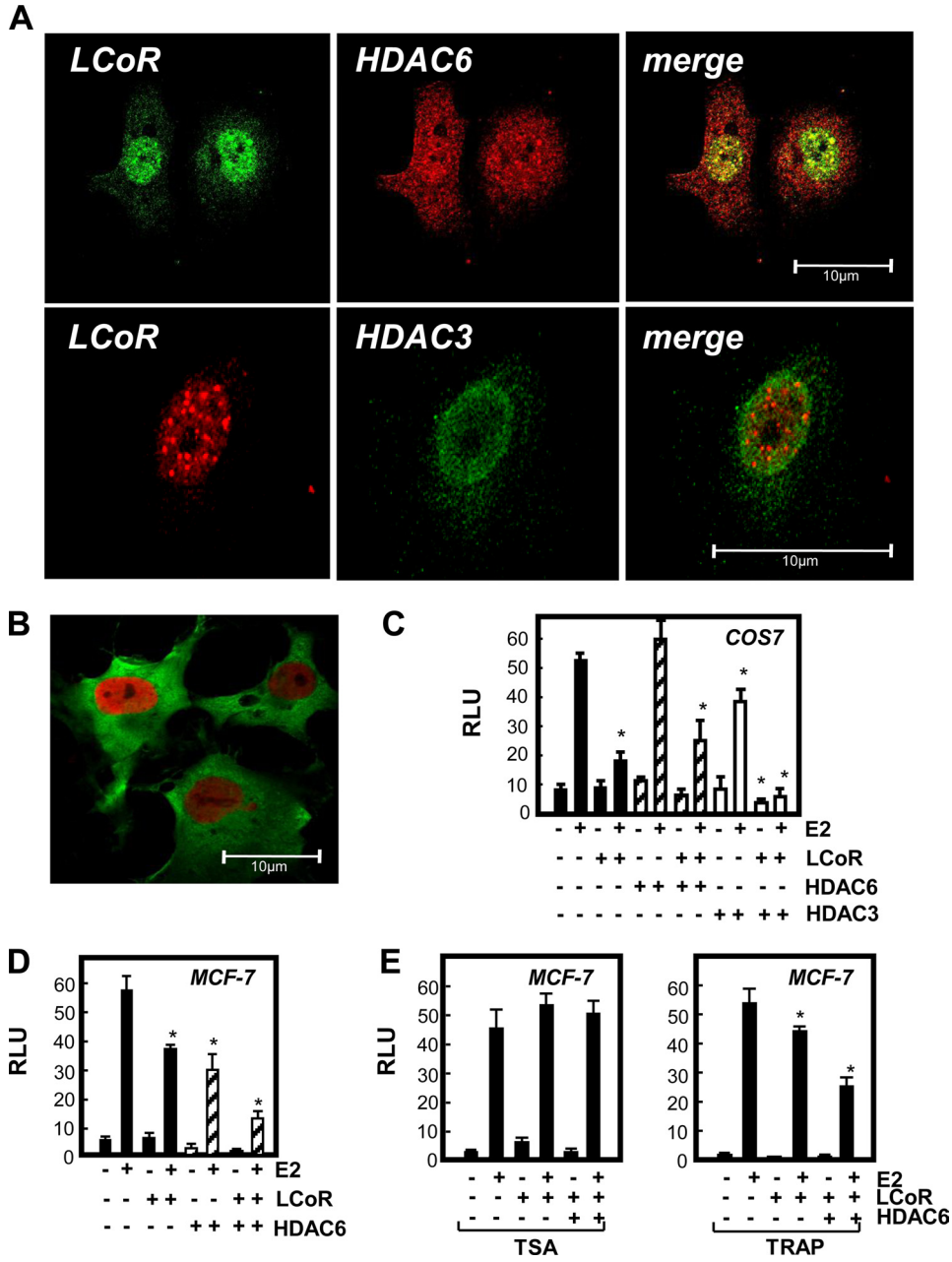

FIGURE 1. *A*, shown is the colocalization of endogenous HDAC3 and endogenous LCoR (*first row*) or endogenous HDAC6 and endogenous LCoR (*second row*) by confocal microscopy (see "Experimental Procedures" for details). *B*, shown is colocalization of transiently expressed HDAC6 and LCoR in COS7 cells by confocal microscopy. Overexpressed HDAC6 is exclusively cytoplasmic in COS7 cells. LCoR (nuclear) was detected with Cy3-conjugated antibody and HA-FLAG-HDAC6 (cytoplasmic) with Cy2-conjugated antibody. *C*, shown is the contribution of HDAC6 to LCoR corepression in COS7 cells. COS7 cells were transfected with expression vectors for ERα, LCoR, and HDAC6, as indicated (*E2*, 10 nM). Coexpression of HDAC3 with LCoR and ERα was used as a positive control for HDAC-specific repression. *D*, shown is the contribution of HDAC6 to LCoR corepression in MCF7 cells. MCF7 cells were transfected with expression vectors for ERα, LCoR, and HDAC6, as indicated (*E2*, 10 nM). *E*, shown is the effect of HDAC inhibitor TSA (500 nM) or trapoxin (*TRAP*; 50 nM) on repression by LCoR and HDAC6 in MCF7 cells. For *C–E*, data are the averages of three or more independent experiments, and *error bars* represent the S.E.; *, $p < 0.05$ for results of corresponding overexpression (LCoR, HDAC3, or HDAC6) *versus* empty vector control. *RLU*, relative *Luciferase* units.

HDAC cotransfection in COS7, we performed a similar coexpression experiment with HDAC3, which unlike HDAC6, is nuclear in a wide variety of cell types (10, 11). HDAC3 repressed ERα-dependent *Luciferase* expression in COS7 cells on its own (but not that of the internal control) and enhanced transcriptional repression by LCoR (Fig. 1*C*).

In contrast to the above, HDAC6 partially repressed ERα-dependent transactivation in MCF7 cells on its own and enhanced corepression by LCoR (Fig. 1*D*). Note that these transfections were performed with limiting amounts of LCoR and HDAC6 under conditions that repressed estrogen-dependent reporter gene activity without affecting expression from the internal control plasmid. Although corepression was apparently further enhanced when cells were cotransfected with larger combined amounts of LCoR and HDAC6 expression vectors, these conditions also affected expression of the β-galactosidase internal control (data not shown).

Consistent with our previous findings that LCoR corepression of ERα-dependent transcription is sensitive to the HDAC inhibitor TSA (14), corepression by both LCoR and HDAC6 of ERα transactivation was fully abolished by TSA (Fig. 1*E*). In contrast, treatment of cotransfected cells with HDAC inhibitor trapoxin only partially abolished corepression (*right-hand panel*; Fig. 1*E*), consistent with the resistance of HDAC6 activity to trapoxin (17, 18). Taken together, these data strongly support the idea that HDAC6 can function as a nuclear cofactor of LCoR in MCF7 cells.

Delineation of an HDAC6-interacting Domain of LCoR—The domain of interaction of HDAC6 with LCoR was determined by generating a series of GST fusions of C- and N-terminal deletion mutants of LCoR (Fig. 2*A*) and analyzing the capacity of these mutants to pull down *in vitro* translated HDAC6. All deletion mutants were well expressed in bacteria (Fig. 2*A*). GST pulldown experiments performed with these mutants showed that residues lying between amino acids 203 and 319 in the central portion of LCoR were required for interaction with HDAC6 *in vitro* (Figs. 2, *B–D*).

The role of the HDAC6 interaction domain in corepression by LCoR was analyzed in transfection experiments in MCF7 cells by expression of FLAG-tagged wild-type LCoR and the FLAG-tagged mutant form lacking amino acids 203–319.

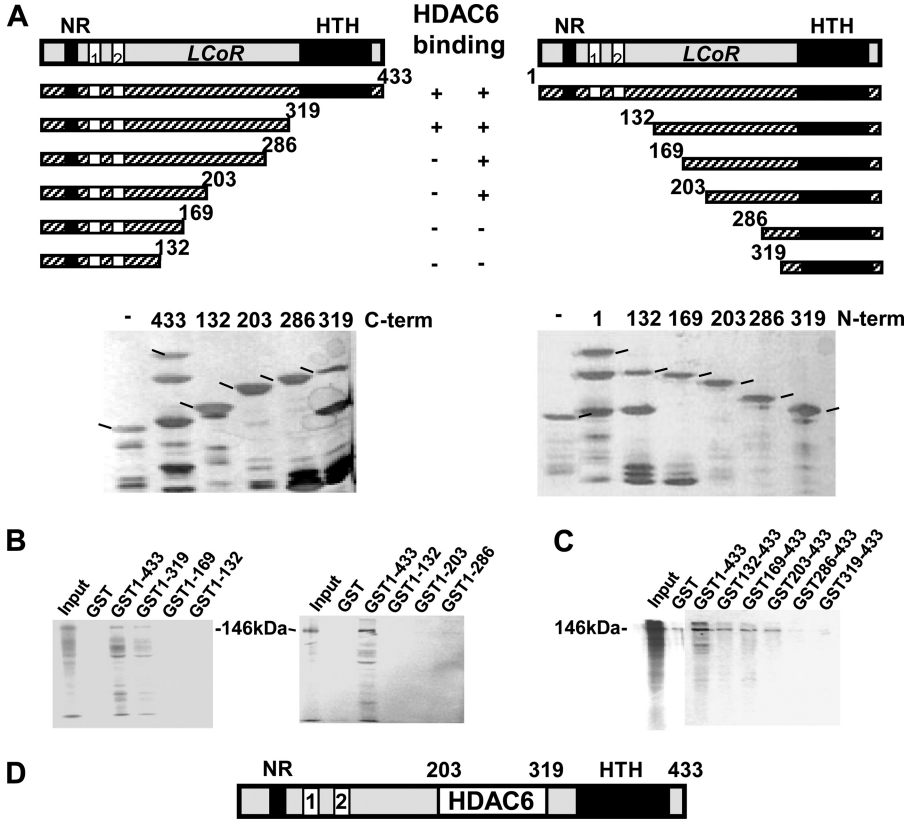

FIGURE 2. **Delineation of the domain of interaction of LCoR with HDAC6.** *A*, shown are the C- and N-terminal mutants of LCoR fused to GST used in this study. The results of GST pulldown assays with *in vitro*-translated HDAC6 are summarized in the middle with SDS-PAGE analyses confirming the expression of GST-LCoR fusions. Note that the C-terminal deletion mutant truncated at 169 was expressed at similar levels to other mutants (not shown). *B* and *C*, shown are GST pulldown analyses of the interaction of *in vitro*-translated HDAC6 with C- and N-terminal LCoR mutants presented in *A*. The 146-kDa band corresponding to HDAC6 is indicated. *D*, shown is a schematic representation of the domain structure of LCoR based on present results and those of Fernandes *et al.* (14). The NR box (L*XX*LL motif) required for interaction with nuclear receptors is indicated as are the two motifs (*1* and *2*) required for interaction with C-terminal-binding protein corepressors. The central domain required for interaction with HDAC6 is indicated as is the C-terminal helix-turn-helix (*HTH*) domain.

Reporter gene experiments showed that corepression by the wild-type and mutant forms of LCoR was similar at low concentrations. However, the mutant exhibited no dose-dependent increase in corepression (Fig. 3*A*). Western analysis with an anti-FLAG antibody showed that the tagged proteins were expressed at similar levels (Fig. 3*B*). Moreover, the deletion mutant could be detected with an antibody against LCoR (Fig. 3*B*). To verify that the LCoR mutant lacking the HDAC6 domain is still an active protein, a dominant-negative experiment was performed where constant levels of LCoR were cotransfected with greater amounts of the mutant form (Fig. 3*C*). The coexpression of the mutant LCoR reduced the repression observed with the wild-type protein, hence showing competition between the two forms of LCoR.

Hormone-dependent Association of LCoR and HDAC6 with Estrogen-responsive Promoters in Vivo—To further substantiate the role of HDAC6 as a cofactor of LCoR in transcriptional regulation in MCF7 cells, we performed chromatin immunoprecipitation (ChIP) assays to analyze the recruitment of LCoR and HDAC6 to ER binding regions of estrogen-inducible promoters of the pS2, insulin-like growth factor-binding protein 4 (IGFBP4), adenosine A1 receptor (ADORA1), and nuclear receptor interacting protein 1 (NRIP1) genes *in vivo*. As we lack an antibody that reliably immunoprecipitates endogenous LCoR, we analyzed recruitment of transiently expressed tagged LCoR to the pS2 promoter with an anti-FLAG antibody. Rapid (15 min) estradiol-dependent recruitment of ERα was observed to the ERE region of the pS2 promoter but not to non-target sequences (Fig. 4*A*). The kinetics of ERα recruitment under these conditions is entirely consistent with data reported by other groups (24, 25). The anti-FLAG antibody consistently detected recruitment of tagged LCoR by 30 min of estradiol treatment to the region of the pS2 ERE but not non-target DNA (Fig. 4*A*).

The recruitment of HDAC6 to the pS2 promoter followed a similar, but not identical pattern to that of LCoR; for example, unlike LCoR, HDAC6 did not dissociate from the pS2 promoter after 60 min of estradiol treatment (Fig. 4*A*). As others have shown that HDAC6 can function as a regulator of the histone acetyltransferase p300 (26), we analyzed p300 recruitment to pS2 and found that it was recruited rapidly but lacked the clear cyclical pattern of LCoR. Overlapping patterns of corecruitment of ERα, LCoR, and HDAC6 were also observed to ER binding regions of promoters of genes encoding IGFBP4, ADORA, and NRIP1 (25) (Figs. 4, *B–D*). Note that we consistently observed binding of ERα and NRIP1 promoters in the absence of estradiol (Figs. 4, *C* and *D*), a phenomenon that has been observed by others on estrogen-inducible promoters (25).

We also analyzed binding of ERα, LCoR, and HDAC6 to regulatory regions of the gene encoding bone morphogenic protein 7 (BMP7), previously identified as being down-regulated by estrogen (27). A peak of LCoR recruitment to the BMP7 promoter occurred 30 min after the addition of estradiol (Fig. 5*A*), similar to the kinetics of recruitment to estrogen-inducible genes. However, we observed a largely estrogen-independent association of HDAC6 with the BMP7 promoter.

The binding of p300 to promoters complicates interpretation of experiments, as HDAC6 could be recruited to the promoters in association with either p300 or LCoR. Therefore, to determine whether LCoR and HDAC6 are corecruited to promoters *in vivo*, we performed a series of reChIP experiments on all promoters analyzed. Experiments were performed from extracts of MCF7 cells treated with estradiol for 30 min, a time corresponding to peak LCoR recruitment to all promoters analyzed. Extracts were immunoprecipitated with an anti-FLAG antibody followed by a second round of immunoprecipitation

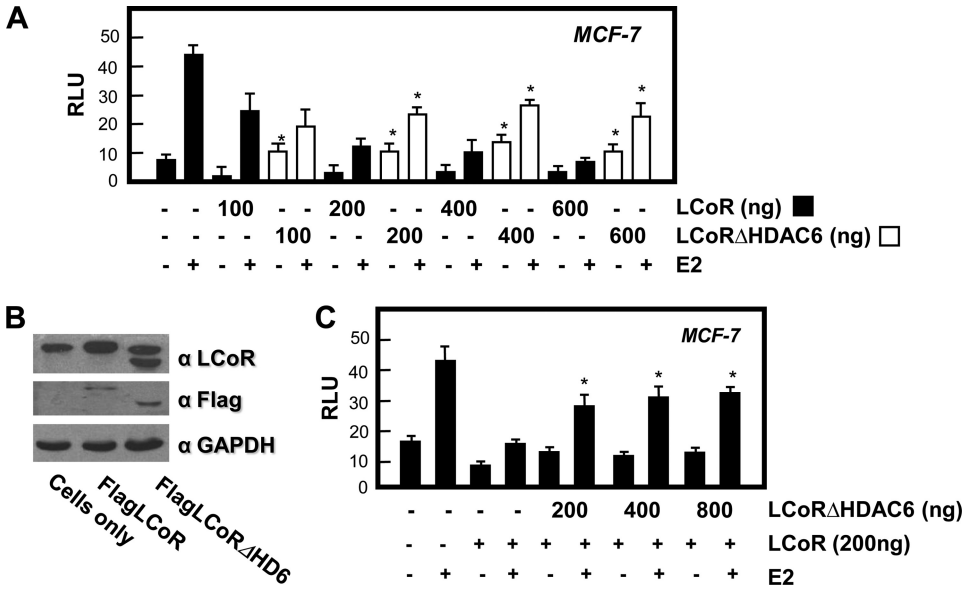

FIGURE 3. **Analysis of the function of the HDAC6-interacting domain of LCoR.** *A*, shown are dose-response curves of FLAG-LCoR or FLAG-LCoRΔHDAC6 in MCF7 cells treated with E2 (10 nM). MCF7 cells were transfected with expression vectors for ERα and FLAG-LCoR or FLAG-LCoRΔHDAC6 (as indicated). Data are shown as relative *Luciferase* units (*RLU*). *B*, Western blot of MCF7 extracts expressing FLAG-LCoR or FLAG-LCoRΔHDAC6 probed for LCoR (*first row*) or FLAG (*second row*). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the loading control (*third row*). *C*, shown is a dominant negative experiment in MCF7 cells treated with E2 (10 nM). Cells were transfected with expression vectors for ERα and FLAG-LCoR alone (200 ng) or ERα and FLAG-LCoR (200 ng) along with increasing amounts of FLAG-LCoRΔHDAC6 (200, 400, and 800 ng) as indicated. Data are shown as relative *Luciferase* units (*RLU*). Data are the averages three or more independent experiments; *error bars* represent the S.E.; *, $p < 0.05$ for results of corresponding expression (FLAG-LCoR or FLAG-LCoRΔHDAC6) *versus* empty vector control.

with either anti-ERα or anti-HDAC6 antibodies. The observed coimmunoprecipitation of LCoR with ERα or HDAC6 confirms their coassociation with the ERE regions of estrogen-inducible pS2, ADORA1, IGFBP4, and NRIP1 promoters (Fig. 5*B*). Remarkably, however, although ERα and LCoR were recruited together on the BMP7 promoter, we found no evidence for corecruitment of LCoR and HDAC6 (Fig. 5*B*), a result that was reproduced several times. Note that all reChIP experiments presented in Fig. 5 were performed on the same sets of extracts. Thus, although LCoR and HDAC6 are present on The BMP7 promoter, they appear to be associated with distinct complexes.

Effects of Ablation of LCoR or HDAC6 Expression in MCF7 Cells on Estrogen-regulated Gene Transcription—To determine the functional significance of association of LCoR and HDAC6 with ERα target genes, we performed knockdown experiments with siRNAs targeting LCoR or HDAC6 (Fig. 6*A*). Knockdown of LCoR or HDAC6 augmented both basal and hormone-stimulated expression from an estrogen-sensitive reporter gene. Essentially identical results were obtained in several independent sets of siRNA transfections. Note that the elevated *Luciferase* expression seen in the absence of estradiol is consistent with the dose-dependent inhibition of basal expression from estrogen-sensitive promoter/reporters observed upon LCoR overexpression (14). These data suggest that LCoR and HDAC6 can function as attenuators of (hormone-regulated) expression of estrogen target genes.

The effects of LCoR and HDAC6 ablation in MCF7 cells on regulation of endogenous estrogen target genes were also examined (Fig. 7). Genes analyzed included those tested in ChIP assays in Fig. 5 along with several other direct target genes identified in recent microarray studies in MCF7 cells (28). Knockdowns generally led to unexpected and gene-specific changes in gene expression. In contrast to data obtained in repeated experiments with an estrogen-sensitive reporter gene (Fig. 6), ablation of either LCoR or HDAC6 expression did not augment estrogen-stimulated expression of any of the genes tested (Fig. 7). Rather, knockdown of LCoR either did not affect expression (pS2, NRIP1, GREB1, SGK3; Figs. 7, *A*, *D*, *F*, *G*) or markedly reduced estrogen-induced transcription of the IGFBP4, ADORA1, and CYP26B1 genes (Figs. 7, *B*, *C*, and *E*). In addition, combined knockdown of LCoR and HDAC6 led to reduced estrogen-dependent stimulation of the GREB1 gene even though individual knockdowns had no substantial effect on hormone-regulated expression of these genes (Figs. 7*B*). A similar effect of double knockdown was observed on the SGK3 gene (Fig. 7*C*), although it did not quite reach statistical significance. These effects, seen in multiple biological replicates, are in striking contrast to the enhanced reporter gene expression seen above after LCoR or HDAC6 knockdown and are not consistent with the two proteins serving corepressor functions on the genes affected. These results suggest an unexpected role of LCoR in activation of a subset of estrogen-stimulated genes. In contrast, combined ablation of LCoR and HDAC6 augmented estrogen-stimulated expression of the NRIP1 gene (Fig. 7*D*).

Consistent with previous reports (29–33) estrogen repressed expression of the BMP7 gene (Fig. 7*H*), and the gene encoding the ras-associated protein keratin 4 (Fig. 7*I*). Although ablation of LCoR had no substantial effect on BMP7 mRNA levels, knockdown of HDAC6 or together with LCoR reduced BMP7 expression in the absence of estrogen (Fig. 7*H*). In addition, knockdown of LCoR or HDAC6 individually or together markedly reduced basal expression of KRT4 but had no substantial effects on estrogen-repressed expression of the gene.

Taken together, the colocalization, direct association, and corecruitment of LCoR and HDAC6 along with results of knockdown of LCoR or HDAC6 on expression of an estrogen-sensitive reporter gene are consistent with HDAC6 functioning as a cofactor of LCoR in transcriptional corepression. However, analysis of the effects of knockdowns on endogenous estrogen target genes suggest that the two proteins function independently on some genes and reveal a potential roles of both LCoR and HDAC6 in enhancing expression of specific genes.

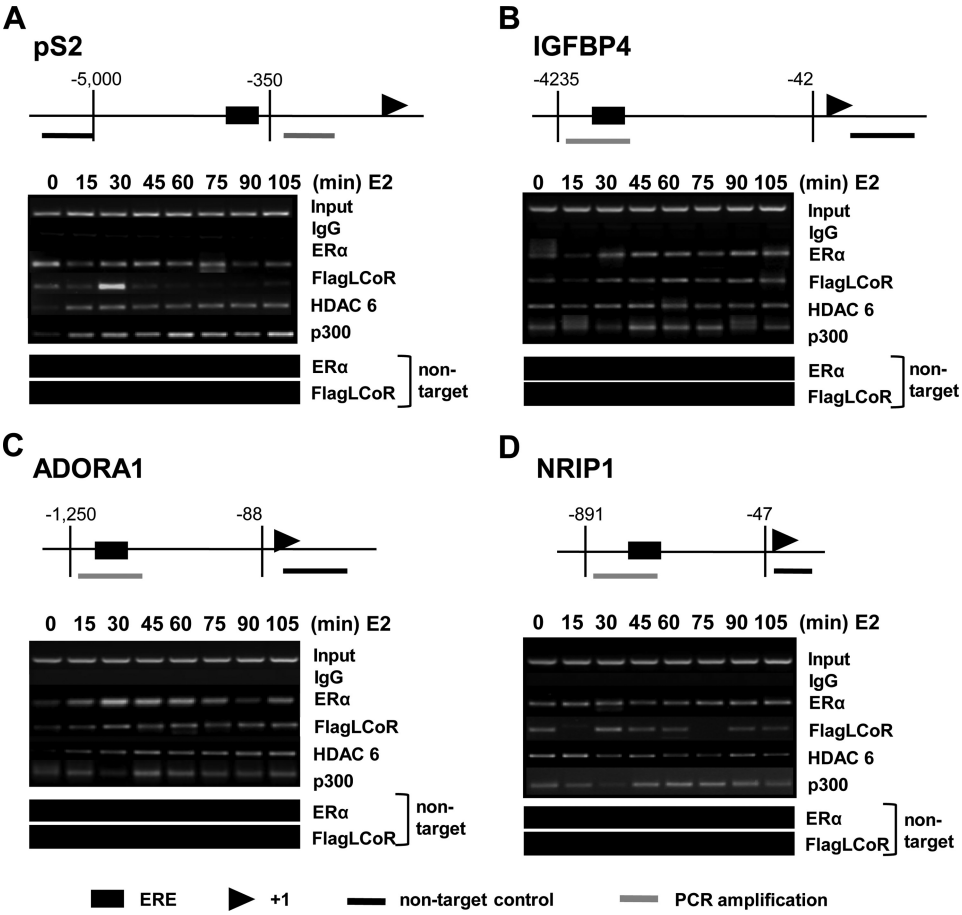

FIGURE 4. **Kinetic ChIP assays of estrogen-induced target genes.** MCF7 cells synchronized for 2 h with α-amanitin (2.5 μM) and treated with E2 (10 nM) were collected at 15-min increments and immunoprecipitated with IgG or antibodies against ERα, FLAG, HDAC6, or p300 as indicated. *A–D*, shown are kinetic ChIP assays on the pS2, IGFBP4, ADORA1, and NRIP1 promoters. Graphical representations of promoters indicate the location of the ERE sequence, the ER binding region amplified by PCR, and non-target sequences analyzed. Note that the region of the pS2 promoter amplified lies immediately adjacent to the ERE and is identical to that amplified by others (24, 28) in analysis of estrogen regulation of the promoter.

DISCUSSION

This study has analyzed the recruitment of corepressor LCoR and associated HDAC6 to estrogen-regulated genes in MCF7 cells. Both proteins are widely expressed in adult organisms; HDAC6 is present in mouse oocytes and zygotes (34), and LCoR is expressed as early as the two-cell stage of mouse embryonic development (14). HDAC6 likely plays numerous biochemical roles during development and in the adult. Cytoplasmic HDAC6 is best known for its function as a tubulin deacetylase (16). Remarkably, however, HDAC6 knock-out mice are viable and exhibit hyperacetylated tubulin in most tissues while demonstrating apparently normal development (35), suggesting that other HDACs can substitute for some cytoplasmic and nuclear functions of HDAC6.

Evidence accumulated to date has suggested that HDAC6 can either suppress or promote tumorigenesis and that its precise function may depend on its subcellular localization. Immunohistochemical analyses showed that a portion of HDAC6 was nuclear in normal breast epithelial cells but entirely cytoplasmic in adjacent breast tumor cells, suggesting that nuclear localization of HDAC6 is at least partly dependent on the state of differentiation of cells (20). This notion is supported by the observation that transfer of a portion of HDAC6 from the cytoplasm to the nucleus accompanied the induced differentiation and cell cycle arrest of the mouse B16 melanoma line (36). MCF7 cells express both ERα and the progesterone receptor and are estrogen-dependent for growth, consistent with a relatively well differentiated phenotype. HDAC6 expression is induced by estradiol in MCF7 and other breast cancer cells, and its level of expression correlates with a better prognosis and response to endocrine therapy (19–21). In addition, patients with ER-positive breast tumors who received tamoxifen as adjuvant therapy for two years have a better prognosis and survival rate when tumors expressed HDAC6 (19). Moreover, inhibition of HDAC6 enhanced heat shock protein 90-mediated maturation of matrix metalloproteinase-2, which was associated with increased breast cancer cell invasion in an *in vitro* model (34). However, other studies have shown that cytoplasmic HDAC6 may enhance cell motility and, thus, metastases and that inhibition of the tubulin acetylation activity of HDAC6 in multiple myeloma may have therapeutic potential (37, 38).

Our previous work showed that LCoR interacted with HDAC6 *in vitro* and coimmunoprecipitated with HDAC6 from MCF7 breast cancer cell extracts (14). However, given several studies showing the cytoplasmic location and function of HDAC6 (39) as well as its emergence as a prognostic marker of breast cancer, we were interested in examining its potential function as an LCoR cofactor more closely. We found that a substantial portion of HDAC6 was nuclear in MCF7 cells. Its function as an LCoR cofactor was supported by the finding that its coexpression with ERα repressed estradiol-dependent transactivation in reporter gene assays and that it augmented the repressive effect of coexpressed LCoR. This effect was cell-specific as HDAC6 was entirely cytoplasmic when expressed in COS7 cells and did not enhance corepression by overexpressed LCoR. In contrast, HDAC3, which is a class I HDAC and a nuclear protein, strongly repressed hormone-dependent transcription in COS7 cells. Given its estrogen-dependent expression (19–21), our results raise the possibility that HDAC6 may function with LCoR on some genes as part of a feedback loop to regulate estrogen-dependent gene regulation in breast cancer cells.

The notion that HDAC6 can function in transcriptional repression is supported by studies showing that HDAC6 con-

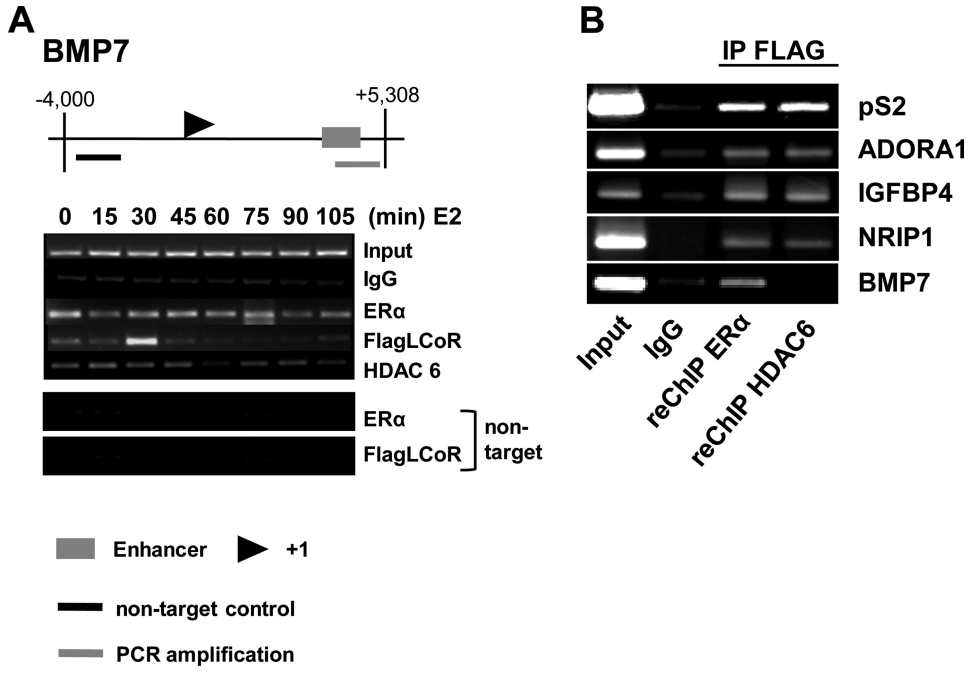

FIGURE 5. **Chromatin immunoprecipitation assays of estrogen-repressed target gene BMP7.** *A*, MCF7 cells synchronized for 2 h with α-amanitin (2.5 μM) and treated with E2 (10 nM) were collected at 15-min increments and immunoprecipitated (*IP*) with IgG or antibodies against ERα, FLAG, or HDAC6 as indicated. Graphical representations of promoter indicate the location of enhancer sequences, the region amplified by PCR, and the non-target sequences amplified. *B*, shown are reChIP assays. MCF7 cells were treated with E2 (10 nM) for 30 min and immunoprecipitated with FLAG. A second round of immunoprecipitations with IgG, ERα, or HDAC6 was performed as indicated. The promoters of the pS2, ADORA1, IGFBP4, NRIP1, and BMP7 genes were investigated.

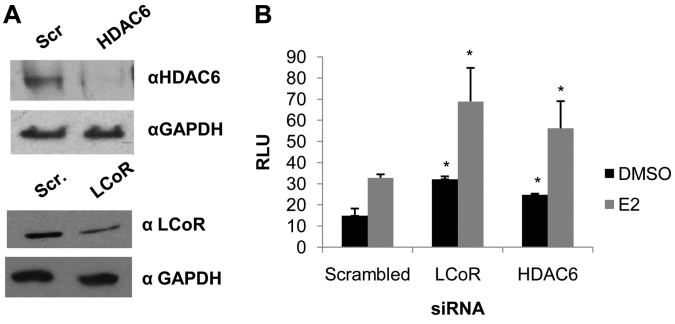

FIGURE 6. **siRNA knockdown of LCoR and HDAC6 expression.** *A*, shown is a Western blot of MCF7 extracts. Cells were transfected with corresponding siRNA (scrambled, LCoR, or HDAC6) for 48 h, and cells were harvested. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression was used as a control. *B*, shown is a *Luciferase* assay analyzing the effects of knockdowns on estrogen-regulated reporter expression. An ERα expression vector and ERE3-TATA-pXP2 reporter plasmid were transfected along with scrambled, LCoR, or HDAC6 siRNA. After 24 h of treatment with DMSO or E2 (10 nM), cells were harvested, and *Luciferase* activity was measured. Data are shown as relative *Luciferase* units (*RLU*). Data are the averages of three or more independent experiments; *error bars* represent the S.E.; *, $p < 0.05$ for results of specific knockdown (LCoR and HDAC6) *versus* results with scrambled siRNA.

tributed to SUMO (small ubiquitin-related modifier)-dependent repression of p300 histone acetyltransferase activity (26). p300 is a component of histone acetyltransferase complexes recruited by nuclear receptors, including ERα, during transcriptional activation (3, 4, 24). A role for HDAC6 in transcriptional repression is also supported by studies showing that it can act as a cofactor of the repressor Runx2 in osteoblastic cells (40). However, HDAC6 is also associated with the promoter of the c-*jun* gene, whose transcription is inhibited by treatment with TSA, suggesting that HDAC6 may contribute to activation of c-*jun* expression (41).

Kinetic ChIP assays investigating the association of cofactors with estrogen target promoters have shown recruitment to be dynamic, following a specific sequential order (42, 43). We found above that estrogen-induced recruitment of LCoR to the well characterized pS2 promoter peaked at 30–45 min. Notably in this regard, others have found in MCF7 cells that estrogen-dependent recruitment of NR box-containing corepressor NRIP1 (RIP140) to the pS2 promoter also peaked at 30–45 min (44), raising the possibility of functional redundancy between the two corepressors. Like LCoR, overexpression of NRIP1 represses estrogen-dependent gene expression in transient expression studies (45). Similar to NRIP1 and LCoR, recruitment of corepressors NCoR and SMRT in the presence of estrogen was also observed on the pS2 promoter (44, 47). Moreover, the association of HDACs with the pS2 promoter in the presence of estrogen has been documented (24). These studies demonstrated estrogen-dependent recruitment of HDAC1 and -7, which appeared to act redundantly.

Knockdown of LCoR or HDAC6 expression in MCF7 cells augmented basal and estrogen-stimulated expression of an ERE-containing reporter gene, consistent with the results of our transient expression studies and supporting their potential roles as attenuators of ERα-dependent transactivation. However, the effects of ablation of LCoR or HDAC6 on endogenous ERα target genes were distinct and gene-specific. Loss of LCoR and/or HDAC6 had no effect on estrogen-regulated expression of the pS2 gene, for example. We speculate that the loss of LCoR and associated cofactor function in regulation of estrogen target genes in MCF7 cells can be compensated for by other corepressors recruited in the presence of hormone such as NRIP1 or ZNF366 (48). It is important to note that although knockdown of NRIP1 in MCF7 cells augmented estrogen-stimulated expression of a transiently expressed reporter plasmid (49), NRIP1 ablation had no effect on regulation of a number of endogenous estrogen target genes in another study (50).

Unexpectedly, the results of LCoR or HDAC6 ablation provide evidence for potential roles of these proteins in the maintenance of gene expression. LCoR knockdown abolished or reduced expression of IGFBP4, ADORA1, and CYP26B1 genes stimulated by estradiol, and ablation of LCoR or HDAC6 diminished basal expression of the KRT4 and BMP7 genes. Although these findings point to roles for LCoR and/or HDAC6 in control of (estrogen-regulated) gene expression, none is con-

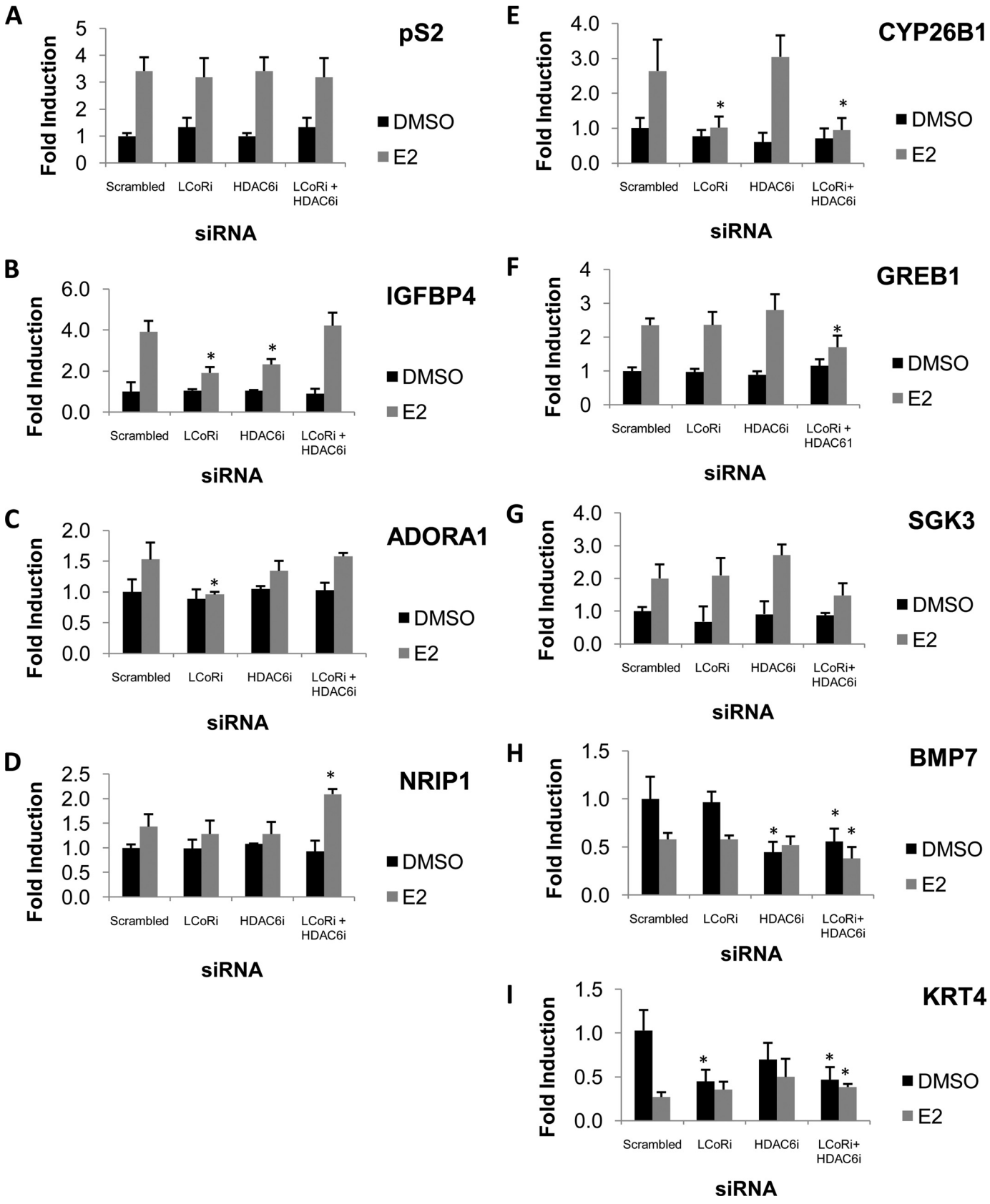

FIGURE 7. **Effects of LCoR and HDAC6 ablation in MCF7 cells on regulation of endogenous estrogen target genes.** Cells were transfected with corresponding siRNA (scrambled, LCoR, HDAC6, or LCoR and HDAC6) for 36 h, then treated with vehicle (DMSO) or E2 (10 nM) for 24 h. Quantitative real-time-PCR was performed on pS2 (*A*) IGFBP4 (*B*), ADORA1 (*C*), NRIP1 (*D*), CYP26B1 (*E*), and GREB1 (*F*), SGK3 (*G*), BMP7 (*H*), KRT4 (*I*) genes. β-Actin was used as internal control. Results are shown as -fold induction. Data are the averages three or more independent experiments; error bars represent the S.E.; *, $p < 0.05$ for results of specific knockdown (LCoR, HDAC6, or LCoR and HDAC6) *versus* results with scrambled siRNA.

sistent with their function as corepressors on the genes affected. The effects of LCoR ablation on endogenous estrogen-regulated gene expression are also in contrast to observations in the accompanying manuscript that LCoR knockdown generally augmented progesterone receptor-stimulated expression of endogenous target genes (51).

It is important to note that, although the nature of the effects of LCoR or HDAC6 ablation on endogenous gene regulation was unexpected, the results are consistent with data in the literature on roles in gene activation of factors generally associated with gene repression (52). For example, knockdown of NRIP1 in human embryonal carcinoma cells diminished ligand-dependent activation of a subset of retinoic acid-inducible target genes (46). In addition, a number of studies have shown that pharmacological inhibition of HDAC activity leads to activation and repression of roughly equal numbers of genes, providing evidence for a role of HDACs in both gene activation and repression (52). Recruitment of LCoR and HDAC6 to some estrogen-regulated promoters may be necessary for direct or indirect regulation of post-translational modifications of non-histone proteins associated with the dynamics of gene activation (52).

In summary, our results provide evidence that HDAC6 can function as a cofactor of LCoR and show that LCoR and HDAC6 are corecruited to promoters regulated by estradiol. Although transient expression experiments suggest that LCoR and HDAC6 can function as corepressors, results of gene knockdown experiments indicate that the proteins individually or together are required for maintenance of expression of a subset of estrogen target genes.

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