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Gene**

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J. Biol. Chem. published online January 25, 2012

Access the most updated version of this article at doi: [10.1074/jbc.M111.311605](https://doi.org/10.1074/jbc.M111.311605)

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Ligand-dependent Corepressor (LCoR) Recruitment by Krüppel-like Factor 6 (KLF6) Regulates
Expression of the Cyclin-dependent Kinase Inhibitor *CDKN1A* Gene*

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*Running title: *LCoR regulation of KLF6 target gene expression*

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Keywords: LCoR, KLF6, CDKN1A, transcriptional regulation

Background: LCoR was identified as a repressor of nuclear hormone receptors.

Results: A screen for new interacting factors revealed that LCoR acts with KLF6 to repress *CDKN1A* gene transcription.

Conclusion: KLF6 inhibits gene transcription by tethering a complex of proteins including LCoR to DNA.

Significance: Identification of a novel mechanism of action of LCoR, independent of nuclear receptors, with potential implications for prostate cancer.

ABSTRACT

The widely expressed transcriptional coregulator, ligand-dependent corepressor (LCoR), initially characterized as a regulator of nuclear receptor-mediated transactivation, functions through recruitment of C-terminal Binding Proteins (CtBPs) and histone deacetylases (HDACs) to its N-terminal and central domains, respectively. We performed a yeast two-hybrid screen for novel cofactors, and identified an interaction between the C-terminal domain of LCoR and the transcription

factor Krüppel-like Factor 6 (KLF6), a putative tumor suppressor in prostate cancer. Subsequent experiments have revealed LCoR regulation of several KLF6 target genes notably p21^{WAF1/CIP1} (*CDKN1A*) and to a lesser extent E-cadherin (*CDH1*), indicating that LCoR regulates gene transcription through multiple classes of transcription factors. In multiple cancer cells, LCoR and KLF6 bind together on the promoters of the genes encoding *CDKN1A* and *CDH1*. LCoR contributes to KLF6-mediated transcriptional repression in a promoter- and cell type-dependent manner. Its inhibition of reporter constructs driven by the *CDKN1A* and *CDH1* promoters in PC-3 prostate carcinoma cells is sensitive to treatment with the HDAC inhibitor trichostatin A (TSA). Additionally, the LCoR cofactor CtBP1 bound the same promoters and augmented the LCoR-dependent repression in PC-3 cells. Consistent with their inferred roles in transcriptional repression, siRNA-mediated knockdown of KLF6, LCoR or CtBP1 in PC-3 cells induced expression of *CDKN1A* and *CDH1* and additional KLF6 target genes. We propose

a novel model of LCoR function in which promoter-bound KLF6 inhibits transcription of the *CDKN1A* gene and other genes as well by tethering a transcriptional corepressor complex containing LCoR, with specific contributions by CtBP1 and HDACs.

Aberrant gene regulation by transcription factors and associated regulatory proteins can disrupt the proper function of biological processes such as proliferative signaling, cell death, and energy metabolism, and result in oncogenic transformation (1,2). The transcription factor, KLF6, which contains a highly conserved C₂H₂ zinc finger DNA-binding motif that interacts with “GC-boxes” within responsive promoters (3), is frequently inactivated by loss of heterozygosity, somatic mutation, and/or decreased expression in human cancers (3). Evidence in prostate cancer (PCa), suggests that KLF6 acts as a tumor suppressor through transcriptional control of specific genes (4-7). For example, KLF6 regulates expression of *CDKN1A*, the gene encoding the cell cycle regulator p21^{WAF1/CIP1}, providing a potential mechanistic explanation for its tumor suppressor function and its control of cellular proliferation (4,8,9). Indeed, numerous studies exist that demonstrate the growth suppressive capabilities of p21^{WAF1/CIP1} in cancer (10-14). However, overexpression of this gene has been demonstrated in an androgen-independent prostate carcinoma model derived from LNCaP cells (15), and shown to be associated with a worse clinical outcome in PCa before and after androgen deprivation therapy (16-18). Thus, it would appear that p21^{WAF1/CIP1} has both tumor suppressive and oncogenic properties depending on the cellular contexts in which it is expressed (19). In addition, in an ovarian cancer cell line, KLF6 regulates expression of E-cadherin (*CDH1*) (7), a cell adhesion molecule. E-cadherin has putative tumor suppressor functions in multiple cancers, and *CDH1* silencing has been shown to promote epithelial-to-mesenchymal transition, which culminates in metastasis (20).

Ligand-dependent corepressor (LCoR), initially identified as a factor that interacts with class I and II agonist-bound nuclear receptors (NR), is expressed in a wide variety of fetal and adult human tissues (21). LCoR is recruited to

ligand-bound NRs through a single LXXLL motif/NR box, and suppresses target gene transcription by recruiting corepressors carboxy (C)-terminal-binding protein (CtBP) 1 and 2 by tandem N-terminal extended PXDLS motifs (21,22), and histone deacetylases (HDAC) 3 and 6 (21,23) through central domains. A recent study has revealed that LCoR binds to and suppresses transactivation by the androgen receptor and inhibits PCa growth *in vivo* (24). LCoR is also present in a CtBP corepressor complex (25), and interacts with lysine (K)-specific demethylase 1 (LSD1), a pivotal member of the ZEB1-CtBP-CoREST-LSD1 repressive complex (26). Surprisingly, in MCF-7 breast adenocarcinoma cells, knockdown of LCoR had either no effect or decreased expression of estrogen receptor α (ER α)-regulated genes (22,23,27). Hence, LCoR may have promoter-specific effects on transcriptional regulation, similar to another member of the ligand-dependent repressor family, RIP140/NRIP1 (28,29).

Here, using a yeast two-hybrid screen with the open reading frame of LCoR as bait, we identified Krüppel-like factor 6 (KLF6) as a novel LCoR-interacting transcription factor. We find that the two proteins interact directly *in vitro*, and on the *CDKN1A* and *CDH1* promoters in human prostate adenocarcinoma PC-3 cells. LCoR functions as a corepressor of *CDKN1A* and other KLF6 target genes in PCa and breast cancer cells. Recruitment of CtBP1 and HDACs, previously identified LCoR cofactors, was required for LCoR corepression in PC-3 cells. LCoR recruitment to the proximal *CDKN1A* promoter, and corepression of reporter gene transcription driven by a *CDKN1A* promoter fragment, were dependent on endogenous KLF6, consistent with elevated expression of *CDKN1A* and other target genes in KLF6-deficient cells. These data suggest that KLF6 acts as a repressor of *CDKN1A* transcription and other genes as well through recruitment of LCoR and associated cofactors, and identify a novel function for LCoR independent of NRs.

EXPERIMENTAL PROCEDURES

Isolation of KLF6 cDNA sequence- A yeast two-hybrid screen (9.8 x 10⁵ transformants; BD Biosciences human fetal brain MATCHMAKER cDNA library HL4028AH; Mountain View, CA)

with an ORF of LCoR yielded 20 His⁺/LacZ⁺ colonies. Two colonies contained approximately 1.0 kb inserts corresponding to KLF6. Homologies to human genomic sequences were found using BLASTN, and multiple alignments of the different cDNA clones were performed using ClustalW employing standard parameters.

Antibodies and Reagents- A rabbit polyclonal antibody was raised against LCoR amino acids 20-36 (QDPSQPNSTKNQSLPKA) fused to keyhole limpet hemocyanin and purified on a peptide affinity column (Bethyl Laboratories, Montgomery, TX). An additional LCoR (sc-134674) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) as well as antibodies for KLF6 (SC-7158) and β -actin (sc-47778). An additional anti-KLF6 antibody was purchased from Invitrogen (37-8400). Antibody for FLAG (F1804) was purchased from Sigma-Aldrich (St-Louis, MO). The antibody for CtBP1 (07-306) was purchased from Millipore (Billerica, MA).

Recombinant plasmids- pSG5-LCoR-433, pcDNA3.1LCoR-M1M2, pcDNA3.1-FLAG-LCoR-433/M1M2, and pcDNA3-CtBP1 have been previously described (21). pCMV-LCoR-406 and pCMV-KLF6 were kind gifts from Dr. Y Barak (Magee Womens Institute, Pittsburgh, PA). *CDKN1A*-225bp-luc and *CDH1*-230bp-luc have been previously described (4,7) as well as *CDKN1A* - 2.5kbp-luc (30).

Cell Culture and Transfections- PC-3 and MCF-7 cells were obtained from the American Type Culture Collection (ATCC), and cultured in DMEM (319-005-CL, Multicell) supplemented with 10% FBS. CaCo-2 cells were obtained from ATCC, and cultured in α -MEM (12571-063, GIBCO) supplemented with 10% FBS. All experiments were carried out between passage numbers 5 and 25.

GST Pull-down Assays and Immunoprecipitation- Glutathione S-transferase pull-downs were performed using the MagneGSTTM Pull-Down System (Promega, USA) as per the manufacturer's instructions. Pull-down eluates were subsequently analyzed by western blot using standard protocols and specified antibodies. All the GST pull-down conditions were run on the same gel, however, the input control was loaded on the far left, and it was

moved to the right purely for aesthetic reasons. The GST pull-downs were repeated at least three times with similar results, and 1 representative experiment was chosen for figure production. For immunoprecipitation of endogenous proteins, CaCo-2/PC-3 cells in 100 mm dishes were lysed for 10 min on ice in 500 μ l of lysis buffer (20mM Tris pH 7.5, 100mM NaCl, 0.5% NP-40, 0.5mM EDTA, protease inhibitor cocktail; Roche, Indianapolis, USA). Cell debris were pelleted by centrifugation (10,000 RPM 10 min at 4°C), and 300ug of protein were pre-cleared with α -IgG and protein A/G agarose beads for 1 hr at 4°C. Samples were immunoprecipitated with α -IgG, α -KLF6 (37-8400, SC-7158), or α -FLAG (F1804), and protein-A/G agarose beads overnight at 4°C. Beads were washed in lysis buffer (4X), boiled in Laemmli buffer and Western blotted as previously described (31).

Luciferase Assays- For analysis of the effects of specific proteins on transactivation of the *CDKN1A/CDH1* promoters, PC-3/MCF-7 cells were grown (80-90% confluent) in six-well plates. Two hours prior to transfection, the media was replaced with fresh media. After this period, cells were transfected in OPTIMEM (GIBCO) with 10 μ l Lipofectamine 2000 (Invitrogen), 100ng of pCMV- β -gal, 250ng of *CDKN1A/CDH1*-luc, and the corresponding amounts of specified plasmids for six hours. Transfected DNA quantity was kept constant in all conditions by adding a corresponding amount of empty vector. Then, the transfection media was replaced, and cells were allowed to recover for 24h, after which cells were harvested in 200 μ l of reporter lysis buffer (Promega). For TSA treatments the corresponding amount of TSA was added to the media for 24h, after which samples were collected. Three independent biological experiments were performed in triplicate, and 1 representative experiment was chosen for figure production. All experiments were normalized to β -gal activity. Values reported are means + SD.

Chromatin Immunoprecipitation (ChIP) and ReChIP Assays- ChIP and reChIP assays were essentially performed as previously described in PC-3, CaCo-2, and MCF-7 cells (23). Immunoprecipitations were performed with corresponding antibodies (SC-7158, F1804, sc-134674) and 2 sets of specific and 1 set of non-

specific (NS) primer sequences were used to validate protein binding. For the *CDKN1A* promoter, the primer sequences used were 5'-GCTGGGCAGCCAGGAGCCTG-3' (forward) and 5'-CTGCTCACACCTCAGCTGGC-3'(reverse), 5'-ATGTGTCCAGCGCACCAAC-3' (forward), and 5'-GCGGCCCTGATATACAACC-3' (reverse), and NS 5'-TGGGGTTATCTCTGTGTTAGGG-3' (forward), and 5'-TAGCCTCTACTGCCACCATCTT-3' (reverse). For the *CDH1* promoter, the primers sequences used were 5'CAGCTACTAGAGAGGCTGGGGCCAG-3' (forward), and 5'-CGTACCGCTGATTGGCTGAGGGTTC-3' (reverse), and 5'-GTCTTAGTGAGCCACCGGCGG-3' (forward), and 5'- GTTCACCTGCCGGCCACAGCC-3' (reverse), and NS 5'-TTCTCCCTTCTAAGCTCCTGTG-3' (forward), and 5'-CCACCCTCCTAACGTTCTAGTG-3' (reverse). Three independent biological experiments were performed with similar results, and 1 representative experiment was chosen for figure production. Values reported are means + SD.

siRNA Knockdowns- siRNA for LCoR (HSC.RNAL.N001170765.12.1/3) and KLF6 (HSC.RNAL.N001300.12.1/2) were purchased from IDT Integrated DNA Technologies. siRNA for CtBP1 (ON-TARGETplus -J-008609-08) was purchased from Thermo Scientific Dharmacon (Lafayette, CO). Briefly, PC-3 or MCF-7 cells were plated in 6-wells plates, and grown to about 50-60% confluence. Two hours prior to transfection, the media was replaced with fresh media, and then the cells were transfected in OPTIMEM (GIBCO) with corresponding siRNA for 5h (MCF-7) or 6h (PC-3), and 10 µl of Lipofectamine 2000 (Invitrogen, for PC-3 cells) or Lipofectamine RNAI Max (Invitrogen, for MCF-7 cells). After this incubation, media was added, and cells were allowed to recover. For protein knockdown and mRNA target gene analysis, cells were collected 48h and 72h after transfection with siCtBP1 and siLCoR/siKLF6 respectively. mRNA expression analysis was carried out by qPCR. Three independent biological experiments were

performed in triplicate, and 1 representative experiment was chosen for figure production. Values reported are means + SD.

siRNA Knockdown and ChIP- PC-3 cells were grown in 100mm dishes and the knockdowns and ChIP assays were essentially performed as described in the previous sections.

siRNA Knockdown and Reporter gene experiments- PC-3 cells were grown (50-60% confluent) in six-well plates, and plasmid DNA and siRNA were co-transfected in PC-3 cells as described in the Luciferase Assays section. After a 72h incubation period, cells were harvested in 200 ul of reporter lysis buffer (Promega). Three independent biological experiments were performed in triplicate, and pooled for figure production. All experiments were normalized to β-gal activity. Values reported are means + SEM.

RNA isolation, cDNA synthesis, and quantitative Real-Time PCR- RNA isolation, cDNA synthesis and quantitative real time PCR (RT-qPCR) were performed as previously described (23), with the following modifications to qPCR: the reagent used was SsoFast EvaGreen (172-5211, BioRad), and the machine used was an Illumina Eco. Results were normalized to β-actin mRNA expression. The primers that were used are detailed in Table S1.

KLF6 overexpression experiments- PC-3 cells were grown (80-90% confluent) in six-well plates. Two hours prior to transfection, the media was replaced with fresh media. After this period, cells were transfected in OPTIMEM (GIBCO) with 10 ul Lipofectamine 2000 (Invitrogen), 500ng of pCMV-KLF6 or empty vector for six hours. Cells were allowed to recover for 48h, and then collected for protein analysis or RNA extraction.

Statistical Analysis - Statistical analysis was carried out with the program SYSTAT10.1 by performing Student's two-sample t-test or one way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons as indicated. Probability values are as follows: * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001.

RESULTS

LCoR interacts with the transcription factor KLF6- The early and widespread expression of LCoR (21) and its presence in multi-protein complexes (25) suggest that in addition to nuclear

receptors, it may regulate other classes of transcription factors. Given the robust expression of LCoR in human fetal brain (21), we utilized a human fetal brain cDNA library (Clontech) to perform a yeast two-hybrid screen to identify new molecular partners. Two independent approximately 1.0kb-long clones, encoding nearly the entire open reading frame of KLF6, were isolated from this screen (Fig. 1A). To confirm binding, KLF6 and FLAG-tagged LCoR were transfected into PC-3 cells, verified for FLAG-LCoR expression (Fig. 1B, upper left), immunoprecipitated with either anti-KLF6 or -FLAG antibody, and probed for FLAG or KLF6, respectively. Reciprocal arms of this experiment confirmed LCoR-KLF6 interaction in PC-3 cells (Fig. 1B). Immunoprecipitation of KLF6 from undifferentiated colorectal adenocarcinoma CaCo-2 cells also revealed an association between endogenous KLF6 and LCoR (Fig. 1B, lower right). In order to confirm the interaction between the two proteins and to delineate the KLF6-interacting domain of LCoR, we carried out GST pull-downs with full-length and truncated mutants of LCoR. These experiments localized KLF6-binding to the C-terminal domain encoded by amino acids (aa) 302 to 433 of LCoR (Fig. 1C).

KLF6 and LCoR bind to the CDKN1A and CDH1 promoters- Chromatin immunoprecipitation (ChIP) assays, followed by semi-quantitative PCR or qPCR in PC-3, MCF-7, and CaCo-2 cells, confirmed that KLF6 binds to GC-boxes situated within the *CDKN1A* and *CDH1* promoters, as previously reported (Fig. 2A, D and data not shown) (7,9). As a control for the binding specificity of the proteins studied, qPCR reactions with primers targeting a non-specific (NS) region within the body of the gene were carried out. This control was performed for all subsequent ChIP assays, and revealed that no binding was detected within this non-specific area studied (data not shown).

In the absence of suitable anti-LCoR antibodies capable of immunoprecipitation, initial ChIP assays were performed in cells transfected with FLAG-tagged LCoR, as described previously (22,23), revealing an association of FLAG-LCoR with regions of the *CDKN1A* and *CDH1* promoters bound by KLF6 in CaCo-2 cells (Fig. S1A, and data not shown). The subsequent

availability of IP-compatible anti-LCoR antibody facilitated ChIP analyses, which confirmed the presence of endogenous LCoR on the same KLF6 binding regions in PC-3 cells (Fig. 2B, E). In contrast, while association of endogenous LCoR with the *CDKN1A* promoter was readily detected by ChIP in MCF-7 cells, no binding to the *CDH1* promoter was observed (Fig. 2E), consistent with deficient KLF6 binding to this promoter (Fig. 2D). This data suggests that the *CDH1* proximal promoter region in MCF-7 cells is neither bound by KLF6 nor LCoR. Furthermore, a ChIP for KLF6 followed by re-ChIP for endogenous LCoR or FLAG-LCoR showed that the two proteins are co-recruited to the *CDKN1A* promoter in PC-3 or CaCo-2 cells (Fig. 2C, S1B), and the *CDH1* promoter in PC-3 cells (Fig. 2F).

To further verify that LCoR is recruited to the promoter region of the *CDKN1A* gene by endogenous KLF6, we knocked down its expression (see Fig. 7, below, for controls for knockdowns), and performed a ChIP for LCoR. Results of this assay revealed that recruitment of LCoR to the proximal promoter region of the *CDKN1A* gene was abolished in KLF6-deficient cells (Fig. 2G), strongly suggesting that KLF6 recruits LCoR to the GC-box located near the transcription start site.

The potential regulation of *CDKN1A* and *CDH1* expression by LCoR is intriguing, as its cofactor CtBP1 has also been shown to regulate both promoters (25,32,33). As CtBP1 augments corepression by LCoR in transient expression experiments (22), we probed CtBP1 binding to KLF6 binding regions of both promoters by ChIP assay of extracts of PC-3 cells. These assays revealed that CtBP1 is present on both promoters (Fig. 2H), suggesting that LCoR may regulate KLF6 transcription of target genes through recruitment of CtBP1.

KLF6 and LCoR regulate transcription of a CDKN1A or CDH1 promoter-driven reporter gene- The National Center for Biotechnology Information (NCBI) curated genomic databases (<http://www.ncbi.nlm.nih.gov/gene/84458>) describe the existence of two LCoR isoforms; isoform 1 (LCoR-433) contains 433 amino acids, and isoform 2 (LCoR-406) differs in its 5' UTR, 3' UTR, and coding region relative to isoform 1, but maintains the reading frame, which results in a

similar protein that lacks 27 C-terminal amino acids. KLF6 mediates transactivation of the *CDKN1A* promoter (4), and so we performed reporter gene assays with different *CDKN1A* promoter constructs to determine the effects of the different LCoR isoforms as well as a previously described LCoR deletion mutant that lacks CtBP1 binding sites (LCoR-M1M2) (22). Previous reports found that KLF6 binds to and transactivates the *CDKN1A* promoter through two GC boxes located between -70bp and the transcriptional start site (4,8). We tested two fragments of the proximal promoter, one stretching from -225nt to +1 (225bp), and a longer fragment running from -2500 to +1 (2.5kbp) of the transcription start site.

As shown previously (4,9), KLF6 transactivates the 225bp promoter fragment of the *CDKN1A* promoter in PC-3 cells (Fig. S2A). However, KLF6 consistently suppressed expression of a reporter gene driven by the 2.5kbp fragment in both PC-3 and MCF-7 cells (Fig. S2B). Additionally, as a control to test for function of the 2.5kbp *CDKN1A* fragment, we confirmed its transactivation by transiently expressing p53, as previously reported (34) (Fig. S2C).

Transient expression experiments revealed that all three variants of LCoR were strong repressors of either the 225bp or 2.5kbp *CDKN1A* promoters in PC-3 cells (Fig. 3A, B, D). Both LCoR isoforms also corepressed promoter activity in MCF-7 cells, whereas the LCoR-M1M2 mutant was ineffective (Fig. S2D). When co-transfected along with KLF6 into PC-3 and MCF-7 cells, all three LCoR variants reversed KLF6-mediated transactivation of the 225bp, and augmented KLF6-mediated repression of the 2.5kbp *CDKN1A* promoter fragment (Fig. 3C and 3E).

Given the discrepancy between the responses of the two different promoter lengths used when transiently expressing KLF6 alone, we performed an additional transient expression of KLF6, followed by qRT-PCR to determine endogenous *CDKN1A* levels. Results from this experiment revealed that endogenous *CDKN1A* expression decreases when KLF6 is overexpressed (Fig. S2E). This suggests that the longer *CDKN1A* promoter fragment reflects endogenous regulation by KLF6, and that KLF6 and LCoR may act together to suppress expression of the endogenous *CDKN1A*

gene. To ensure that the weaker repression by the M1M2 mutant was not due to differential expression, we performed an additional reporter gene experiment with FLAG-tagged vectors, whose products could be distinguished from endogenous protein. This revealed comparable expression of tagged LCoR-433 and LCoR-M1M2. Moreover, the relative corepression by the tagged -433 and -M1M2 variants was essentially identical to that of their un-tagged counterparts (compare Fig.S3 and Fig. 3D).

To further substantiate that the repressive effect of LCoR on the *CDKN1A* promoter was dependent on the presence of endogenous KLF6 we knocked down its expression in PC-3 cells (Fig. 3F) to determine whether corepression was attenuated in deficient cells (see Fig. 7, below, for controls for knockdowns). In a reporter gene assay of the 2.5kbp *CDKN1A* promoter, ablation of KLF6 attenuated LCoR-mediated repression, consistent with a functional interaction of LCoR and KLF6 on this promoter. However, the only partial dependence of LCoR corepression on KLF6 expression suggests that LCoR may interact directly or indirectly with other transcription factors bound to other regions of the promoter.

To extend these observations to additional KLF6 target genes, we analysed luciferase expression driven by a -230 to +1 fragment of the *CDH1* promoter containing a responsive GC-box (7). Expression of transcription factor AP-2 transactivated the *CDH1* promoter fragment, as previously described (35), confirming its functional integrity (Fig. S4). Whereas KLF6 was a strong dose-dependent inhibitor of the 2.5kbp *CDKN1A* promoter (Fig. 3), it modestly inhibited reporter expression driven the *CDH1* promoter in PC-3 cells (Fig. 4A). However, prior to proceeding with the LCoR reporter gene assays, we transiently overexpressed KLF6, and performed qRT-PCR to confirm that this construct accurately reflects endogenous *CDH1* regulation by KLF6. KLF6 overexpression (see Fig S2E for controls for overexpression) attenuated *CDH1* mRNA levels confirming that that this promoter construct reflects endogenous regulation by KLF6 (Fig. 4A).

The LCoR isoform variants (433, 406) suppressed expression of the *CDH1* promoter-driven reporter gene differently from the LCoR mutant variant (M1M2) in PC-3 cells (Fig. 4B, C).

In contrast, the LCoR-433 variant did not repress *CDHI* expression in MCF-7 cells (Fig. 4E), consistent with the absence of interaction of endogenous LCoR with the promoter as assessed by ChIP assay (Fig. 2). The weak corepression by LCoR-M1M2 in PC-3 cells is consistent with previous reports of inhibition of *CDHI* expression by CtBP1 (25). Co-expression of KLF6 did not significantly enhance suppression of the *CDHI* promoter by LCoR isoforms, with the exception of LCoR-M1M2 (Fig. 4D). Taken together, these results suggest that corepression of the *CDHI* promoter by LCoR is cell-specific, and that, in PC-3 cells, its function is largely independent of KLF6 expression.

LCoR corepresses KLF6 target genes through CtBP1- and HDAC-dependent mechanisms- Our ChIP assays showing CtBP1 recruitment to the *CDKN1A* and *CDHI* promoters (Fig. 2), and the attenuated corepression by LCoR-M1M2 mutants (Figs. 3 and 4), are consistent with CtBP1 functioning as an LCoR cofactor on KLF6 target genes. Additionally, CtBP1 moderately suppressed expression of a reporter gene driven by fragments of the *CDKN1A* and *CDHI* promoters (Fig. S5A, S5B). To determine whether the two proteins collaborate in repression of luciferase expression driven by either the 225bp or 2.5kb fragments of *CDKN1A*, we co-expressed LCoR-433 or its M1M2 mutant and CtBP1 (Fig. 5A). While expression of CtBP1 on its own had little or no effect on reporter gene expression, it clearly augmented the repressive effect of LCoR, an effect that was attenuated but not eliminated in the presence of the LCoR-M1M2 mutant (Fig. 5A). A similar trend was obtained with the *CDHI* promoter fragment, although the effect of CtBP1 on LCoR-mediated repression did not reach statistical significance (Fig. 5B). These results suggest that LCoR and CtBP1 collaborate to repress *CDKN1A* and *CDHI* promoter activity. However, the observation that the combined effect of LCoR and CtBP1 on the *CDKN1A* promoter are only partially attenuated in the presence of the M1M2 mutant, suggests that CtBP1 can interact with other factors on the promoter in addition to LCoR.

Previous reports have shown that HDAC inhibitors such as trichostatin A (TSA) increase both *CDKN1A* and *CDHI* expression (36-38).

Therefore, to ascertain if LCoR represses *CDKN1A* or *CDHI*-promoter-driven reporter expression through an HDAC-mediated mechanism, we treated transiently transfected cells with TSA at two concentrations. We decided to use a low (60nM) and high dose (2µM) of TSA as effective TSA concentrations reported vary from one study to the next (21,25,36). Both TSA concentrations attenuated LCoR-driven corepression (Figs. 6A, B), with the effect being more pronounced at the higher concentration. This indicates that HDACs contribute substantially to repression of both promoters.

LCoR and CtBP1 repress endogenous KLF6 target gene expression- To substantiate the results of reporter gene assays, we performed siRNA-mediated knockdown of KLF6, and CtBP1 in PC-3 cells, and LCoR in PC-3 and MCF-7 cells (Fig. 7A) and tested the effects on expression of several KLF6 target genes. Ablation of expression of LCoR, KLF6, or CtBP1 in PC-3 cells increased the expression of *CDKN1A* and *CDHI* between 1.5- and 2-fold (Fig.7B), substantiating findings above that these proteins are repressors of the *CDKN1A* and *CDHI* promoters. Consistent with the apparent lack of association of LCoR with the *CDHI* gene observed in ChIP assays above, and the weak or absent corepression of the promoter in reporter gene assays, ablation of LCoR in MCF-7 cells only increased the expression of *CDKN1A*, while the expression of *CDHI* remained stable (Fig. 7B). In addition to *CDKN1A* and *CDHI*, KLF6 has been shown to regulate the expression of the growth/survival genes encoding Activating Transcription Factor 3 (*ATF3*) and Tissue Factor Pathway Inhibitor 2 (*TFPI2*) (39,40), and genes encoding proteins associated with tumour cell migration matrix metalloproteinase-9 (*MMP9*) and urokinase plasminogen activator (*PLAU*) (41,42). LCoR ablation increased the expression of the *TFPI2* gene about 2-fold, whereas it slightly reduced *ATF3* levels in PC-3 cells and had no significant effect on *MMP9* or *PLAU* expression (Fig. 7C). In MCF-7 cells, *TFPI2* expression increased again around 2-fold after LCoR ablation whereas *ATF3* and *PLAU* expression increased around 3-fold (Fig. 7D).

SiRNA-mediated knockdown of KLF6 in PC-3 cells also increased mRNA expression of *ATF3*, and *TFPI2*, with effects around 1.5 fold

(Fig. 7E). This is consistent with KLF6 being a transcriptional repressor of these genes in PC-3 cells. siRNA-mediated knockdown of CtBP1 in PC-3 cells had no significant effect on any of the other genes tested (Fig. 7F).

To further validate the siRNA knockdown study, ablation of LCoR and KLF6 was performed with a second independent siRNA, generating an essentially identical series of results confirming that LCoR is a corepressor of *CDKN1A* and *TFPI2* (Fig. S6), while transcriptional regulation of *ATF3* and *CDH1* by LCoR is cell-specific. As LCoR ablation only moderately alters *ATF3* mRNA expression in PC-3 cells, we performed a ChIP assay to determine whether LCoR was associated with the *ATF3* promoter region bound by KLF6 (39), to rule out non-specific effects. This revealed that LCoR binds a region that contains a KLF6 responsive GC-box (Fig. S7), suggesting that LCoR can regulate *ATF3* gene expression through recruitment by KLF6.

DISCUSSION

In this study, we identified a novel functional interaction between the transcription factor KLF6 and corepressor LCoR. These results reveal that the corepressor functions of LCoR are not specific to members of the nuclear receptor family of transcription factors. Moreover, we delineated the C-terminal region in LCoR between a.a. 302 and 433 that is required for the interaction with KLF6, which lies at the opposite end of the molecule from the NR box essential for nuclear receptor interactions, underlining the fact that LCoR is a multi-domain protein (Fig. 8A). This region contains a helix-turn-helix motif, which previous studies have found to be important for LCoR-mediated corepression (22,23). ChIP, reporter gene assays, and siRNA-mediated knockdowns revealed that KLF6, LCoR, and CtBP1 interact on the *CDKN1A* and *CDH1* promoters, and corepress expression of *CDKN1A*, whereas repression of *CDH1* is cell-specific. Indeed, *CDH1* expression was not affected by LCoR ablation in MCF-7 cells. Moreover, knockdown of KLF6 did not completely abrogate LCoR repression of the long (2.5kb) *CDKN1A* promoter suggesting that LCoR can interact either directly or indirectly with other transcription factors on other regions of this promoter.

Our reporter gene experiments supported previous reports of KLF6-mediated induction of *CDKN1A* expression in assays with a short 225bp promoter fragment (4,8,9). However, we observed inhibition by KLF6 of expression driven by a 2.5kb fragment of the *CDKN1A* promoter in two different cell lines, as well as the *CDH1* promoter in transiently transfected PC-3 cells, which is consistent with the results of either the siRNA-mediated knockdowns with two independent KLF6 siRNAs that led to elevated expression of endogenous *CDKN1A* and *CDH1*, or the overexpression of KLF6 which decreased endogenous mRNA levels of *CDKN1A* and *CDH1* in PC-3 cells. This suggests that the short *CDKN1A* promoter lacks additional promoter interactions required to reflect endogenous KLF6 gene regulation in these cells.

LCoR-mediated repression of *CDKN1A* in PC-3 cells was only partially blocked by TSA, and was not reduced significantly by mutation of its CtBP1 interaction sites, suggesting that other recruited cofactors may contribute to repression. For example, LCoR has been shown to be a component of a complex containing LSD1, a histone demethylase (25,26). LSD1 has been found to repress *CDKN1A* expression in other cell types (43,44), and hence could act through KLF6-LCoR recruitment. Other studies have shown that CtBP1 regulates *CDKN1A* expression via interactions with PARP1 and BRCA1 (33,45). Additionally, LSD1 has also been shown to function in a complex with CtBP1 (25,26), suggesting that KLF6 may associate with a large multi-protein transcriptional regulator complex to regulate *CDKN1A* expression.

Based on these considerations, we propose a novel mechanism for LCoR function in which KLF6, alone or in concert with other transcription factors (TF) provides the tethering point on the proximal *CDKN1A* promoter for a repressive complex containing LCoR, along with specific contributions from CtBP1 and HDACs (Fig. 8B). Inactivation of KLF6 through loss of heterozygosity, somatic mutation, and/or decreased expression in PCa, as described in many studies [for a review; (3)], would result in deficient recruitment of the corepressor complex containing LCoR on the proximal *CDKN1A* promoter, and the subsequent de-repression and

accumulation of *CDKN1A* (Fig. 8B). This mechanism would bridge the observations that exist between different expression studies that show KLF6 absence/inactivation in PCa (3), yet find p21^{WAF1/CIP1} overexpression in PCa (16-18). This model of transcriptional repression could also be applied to other genes. For example, ATF3 overexpression has also been found in PCa (46), and therefore a similar mechanism could be at play. Regulation of *TFPI2* expression could also occur through this mechanism with promoter-specific contributions by CtBP1.

Given that transcription factors and coregulators can play opposing transcriptional roles in different physiological contexts (26,28,29,47), the opposing results concerning KLF6 or LCoR regulation of target genes in different cell lines is not surprising as the original studies that identified these genes as KLF6 targets were carried out in various cell lines from liver to vascular endothelial cells (40,41). Furthermore, the exact role of a transcriptional regulator depends on the additional interactions that occur on the promoter, which might differ in different tissues as was found for LSD1 whose role in transcriptional regulation is complex constituent-specific, and cell type-dependent (26). For instance, the KLF6-mediated repression of *CDHI* expression, albeit modest, is in apparent contrast to studies in SKOV-3 ovarian cancer cells, in which targeted reduction of KLF6 led to a decrease in *CDHI* expression and transient expression of KLF6 stimulated expression of the same *CDHI* promoter-reporter recombinant used above (7). This is consistent with KLF6 regulating promoters in a cell-specific manner. Additionally, our studies suggest that KLF6 plays only a secondary role in LCoR-mediated repression of the *CDHI* promoter. LCoR may regulate transcription of this gene through the action of other Krüppel-like transcription factors. For example, the Krüppel-like transcription factor ZEB1, a well-established repressor of *CDHI*

(48,49), has been identified in a complex containing LCoR (26). Therefore, LCoR could potentially regulate transcription of *CDHI* through ZEB1.

Considering the cell-specific effects of KLF6 on target gene expression observed here, and the literature that exists concerning differential tissue-specific transcriptional regulation, we cannot rule out the possibility that KLF6 or LCoR may function in either activation or repression of a given gene under different physiological or pathophysiological conditions. Our previous studies in LCoR-replete and -deficient MCF-7 cells have suggested that LCoR may contribute to estrogen-induced expression of some genes (23). Given the identification of a complex containing LCoR and LSD1 (26), this suggests a possible mechanism that would underlie tissue-specific activation/repression transcriptional roles. Remarkably, in addition to a transcriptional role being complex-constituent specific, a study identified a phosphorylation mark on a histone threonine that regulates methylation of different lysine residues by LSD1, thereby preventing LSD1 repression and promoting LSD1-androgen receptor-mediated gene expression in LNCaP cells (50). This suggests that LCoR may participate in both repression and activation with a histone mark-specific transcriptional role. Thus, the transcriptional outcome of the KLF6-LCoR interactions may not always result in repression, but may be dependent on the additional molecular constituents of the complex and the histone-marks associated with it.

In conclusion, our results present evidence for a novel functional interaction between the transcription factor KLF6 and the transcriptional regulator LCoR, highlighting a new role in transcriptional regulation for LCoR independent of NRs. These proteins corepress several KLF6 target genes, but notably *CDKN1A* and *CDHI* in a cell type-dependent manner, with promoter-specific contributions from CtBP1 and HDACs.

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Acknowledgements – We thank Drs. Yaacov Barak and Manuella Bouttier for critical reading of the manuscript.

FOOTNOTES

*This work was supported by Canadian Institutes of Health Research Operating Grant to J.H.W.

¹Supported by a studentship from the Montreal Centre for Experimental Therapeutics in Cancer.

FIGURE LEGENDS

FIGURE 1. Identification and confirmation of interaction between LCoR and KLF6. A. Top: Schematic representation of the two KLF6 cDNA clones identified with the yeast two-hybrid. Bottom: LCoR truncation mutants used in this study. B. Upper left panel: PC-3 cells were transfected with appropriate plasmids (pcDNA3.1FLAG-LCoR and pCMV-KLF6), and a western blot was performed to confirm expression of transiently transfected FLAG-LCoR. Lower left panel: KLF6 was immunoprecipitated from PC-3 cells (SC-7158) and immunoprecipitates were probed for FLAG (F1804). Upper right panel: Coimmunoprecipitation of KLF6 and FLAG-LCoR in PC-3 cells. FLAG was immunoprecipitated with specific antibody (F1804) followed by a western blot for KLF6 (SC-7158). Due to the intensity of the signal from the IP, two different exposures had to be performed for the input (high) and IP (low). Lower right panel: Coimmunoprecipitation of endogenous LCoR and KLF6 in CaCo-2 cells. KLF6 was immunoprecipitated with specific antibody (37-8400) followed by a western blot for LCoR (Bethyl). C, Results of a KLF6 (SC-7158) WB of pull-downs with GST control or fusions with full-length LCoR or truncated mutants and *in vitro* bacterially translated KLF6.

FIGURE 2. Analysis of KLF6, LCoR, and CtBP1 binding to the *CDKN1A* and *CDH1* promoters by ChIP assay. Top and Middle: Schematic representations of the *CDKN1A* and *CDH1* promoter sections analysed by ChIP. Regions amplified encompassing the GC-boxes and non-specific (NS) control sequences are marked. A and D. ChIP assays were performed in PC-3, MCF-7, and CaCo-2 cells with KLF6 (SC-7158) antibody followed by qPCR with specific primers for the *CDKN1A* (A) or *CDH1* (D) promoter. Two-sample t-tests were performed to ascertain statistical significance on all assays. B and E. ChIP assays were performed in PC-3 and MCF-7 cells with anti-LCoR (sc-134674) antibody followed by qPCR with specific primers for the *CDKN1A* (B) or *CDH1* (E) promoters. C and F. Results of re-ChIP assays are shown, in which a first round of ChIP for KLF6 (SC-7158) followed by a second round of immunoprecipitation for LCoR (sc-134674) was performed in PC-3 cells, followed by qPCR with specific primers for the *CDKN1A* (C) or *CDH1* (F) promoters. G. Results of qPCR with specific primers for the *CDKN1A* promoter from ChIP assays for LCoR (sc-134674) following knockdown with a non-specific control (scr) or KLF6-specific siRNA. H. ChIP assays were performed in PC-3 cells with an anti-CtBP1 (07-306) antibody followed by qPCR with specific primers for the *CDKN1A/CDH1* promoters.

FIGURE 3. KLF6 & LCoR co-regulate the expression of a reporter gene driven by the *CDKN1A* promoter. Top: Schematic representation of the *CDKN1A* promoter and the promoter recombinants used. A. Luciferase reporter gene assays with a short fragment (225bp) of the *CDKN1A* promoter were performed in PC-3 cells with different LCoR variants. One way ANOVAs followed by the Tukey test for multiple comparisons were performed to establish statistical significance on all assays. B. A luciferase reporter gene assay with a short fragment of the *CDKN1A* promoter was performed in PC-3 cells with 500ng of the different LCoR variants. C. Co-expression luciferase reporter gene assay with a short *CDKN1A* promoter was performed in PC-3 and MCF-7 cells with 250ng of wtKLF6 and 500ng of different LCoR variants. D. A luciferase reporter gene assay with a long fragment (2.5kbp) of the *CDKN1A* promoter was performed in PC-3 cells with 500ng of the different LCoR variants. E. A co-expression luciferase reporter gene assay with a long (2.5kb) *CDKN1A* promoter was performed with 250ng of wtKLF6 and 500ng of different LCoR variants in PC-3 and MCF-7 cells. F. Luciferase reporter gene assays of the *CDKN1A* promoter (2.5kbp) were performed in control (scr) and KLF6-deficient PC-3 cells coupled with transient expression of LCoR (500ng).

FIGURE 4. Analysis of KLF6 and LCoR regulation of the expression of a reporter gene driven by a *CDH1* promoter fragment. Top: Schematic representation of the *CDH1* promoter and the promoter

recombinant used. A. Left: Luciferase reporter gene assays with a short fragment (230bp) of the *CDHI* promoter were performed in PC-3 cells with wild type KLF6 (wtKLF6). One way ANOVAs followed by the Tukey test for multiple comparisons were performed to examine statistical significance in all assays. Right: RT-qPCR with *CDHI* primers of PC-3 extracts following transfection with a control or KLF6 expression vector. B. Luciferase reporter gene assays with a short fragment (230bp) of the *CDHI* promoter were performed in PC-3 cells with different LCoR variants. C. A luciferase reporter gene assay with the same *CDHI* promoter was performed in PC-3 cells with 500ng of the different LCoR variants. D. A luciferase reporter gene assay with a short *CDHI* promoter was performed in PC-3 cells cotransfected with 250ng of wtKLF6 and 500ng of different LCoR variants. E. A luciferase reporter gene assay with a short fragment of the *CDHI* promoter and LCoR-433 was performed in MCF-7 cells.

FIGURE 5. LCoR represses a reporter gene driven by the *CDKN1A* or *CDHI* promoters through CtBP1-dependent mechanisms. Co-expression luciferase reporter gene assays with the 225bp (Left) or 2.5kbp (Right) fragment of the *CDKN1A* promoter was performed in PC-3 cells with 500ng of CtBP1 and 500ng of LCoR/M1M2. One way ANOVAs followed by the Tukey test for multiple comparisons were performed to establish statistical significance in all assays. B. A co-expression luciferase reporter gene assay with a fragment of the *CDHI* promoter was performed in PC-3 cells with 500ng of CtBP1 and 500ng of LCoR/M1M2.

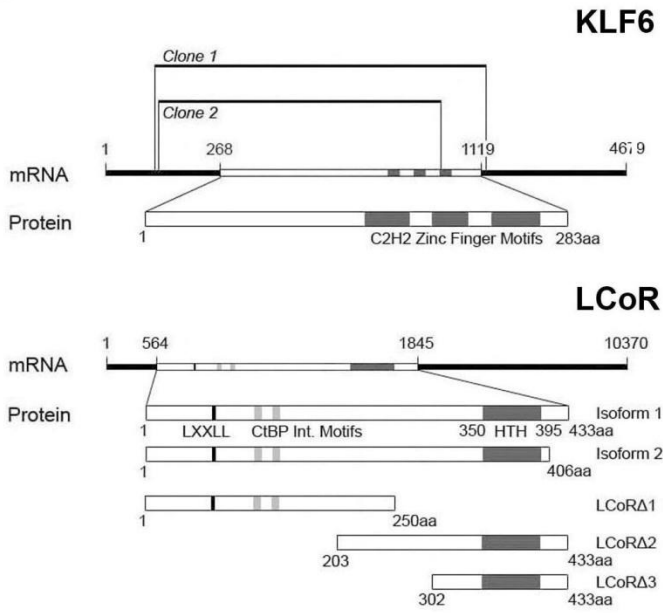
FIGURE 6. LCoR represses a reporter gene driven by the *CDKN1A* or *CDHI* promoters through HDAC-dependent mechanisms. A. Luciferase reporter gene assays with a long fragment of the *CDKN1A* promoter were performed in PC-3 cells with 500ng of the different LCoR variants and the indicated amounts of vehicle DMSO (-TSA) or TSA (+TSA). B. Luciferase reporter gene assays with a fragment of the *CDHI* promoter were performed in PC-3 cells with 500ng of the different LCoR variants and the indicated amounts of vehicle DMSO (-TSA) or TSA (+TSA). One way ANOVAs followed by the Tukey test for multiple comparisons were performed to establish statistical significance.

FIGURE 7. Gene knockdowns confirm regulation of *CDKN1A* and *CDHI* and additional target genes by KLF6 and LCoR. A. (Left panel) mRNA levels of the indicated genes were measured by qPCR after siRNA-mediated gene knockdown in PC-3 cells. (Right panel) Western blots of PC-3/MCF-7 cell extracts were performed for the specified proteins after siRNA-mediated gene knockdowns. B. mRNA levels of the *CDKN1A* or *CDHI* genes were measured by RT-qPCR after siRNA-mediated knockdown of LCoR, KLF6, and CtBP1 in PC-3 cells and LCoR in MCF-7 cells. C and D. mRNA levels of the indicated KLF6 target genes were measured by RT-qPCR after siRNA-mediated LCoR knockdown in PC-3 (C) and MCF-7 (D) cells. E. mRNA levels of the indicated KLF6 target genes were measured by RT-qPCR after siRNA-mediated KLF6 knockdown in PC-3 cells. F. mRNA levels of the indicated KLF6 target genes were measured by RT-qPCR after siRNA-mediated CtBP1 knockdown in PC-3 cells. Two-sample t-tests were performed to determine statistical significance in all cases.

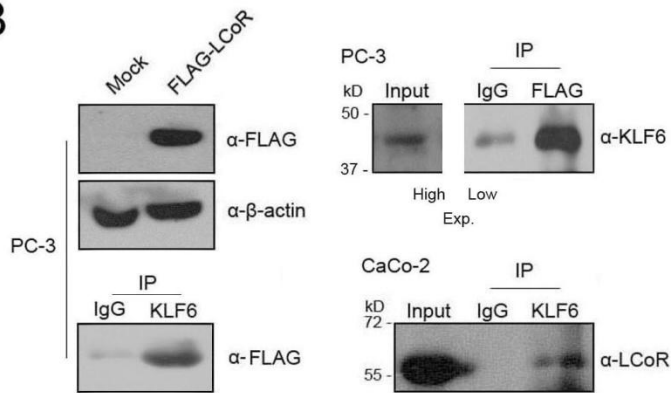
FIGURE 8. Model of LCoR-mediated corepression of the *CDKN1A* promoter. A. Schematic representation of the domain structure of LCoR. B. A corepressor complex containing LCoR and associated co-factors is recruited to the promoter of the *CDKN1A* gene through the action of the DNA-binding protein KLF6. TF refers to other potential interactions of the corepressor complex with transcription factors bound to other regions of the promoter.

Fig.1

A



B



C

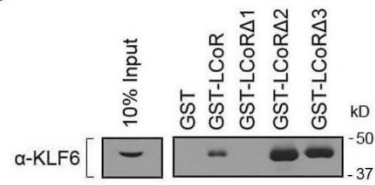


Fig.2

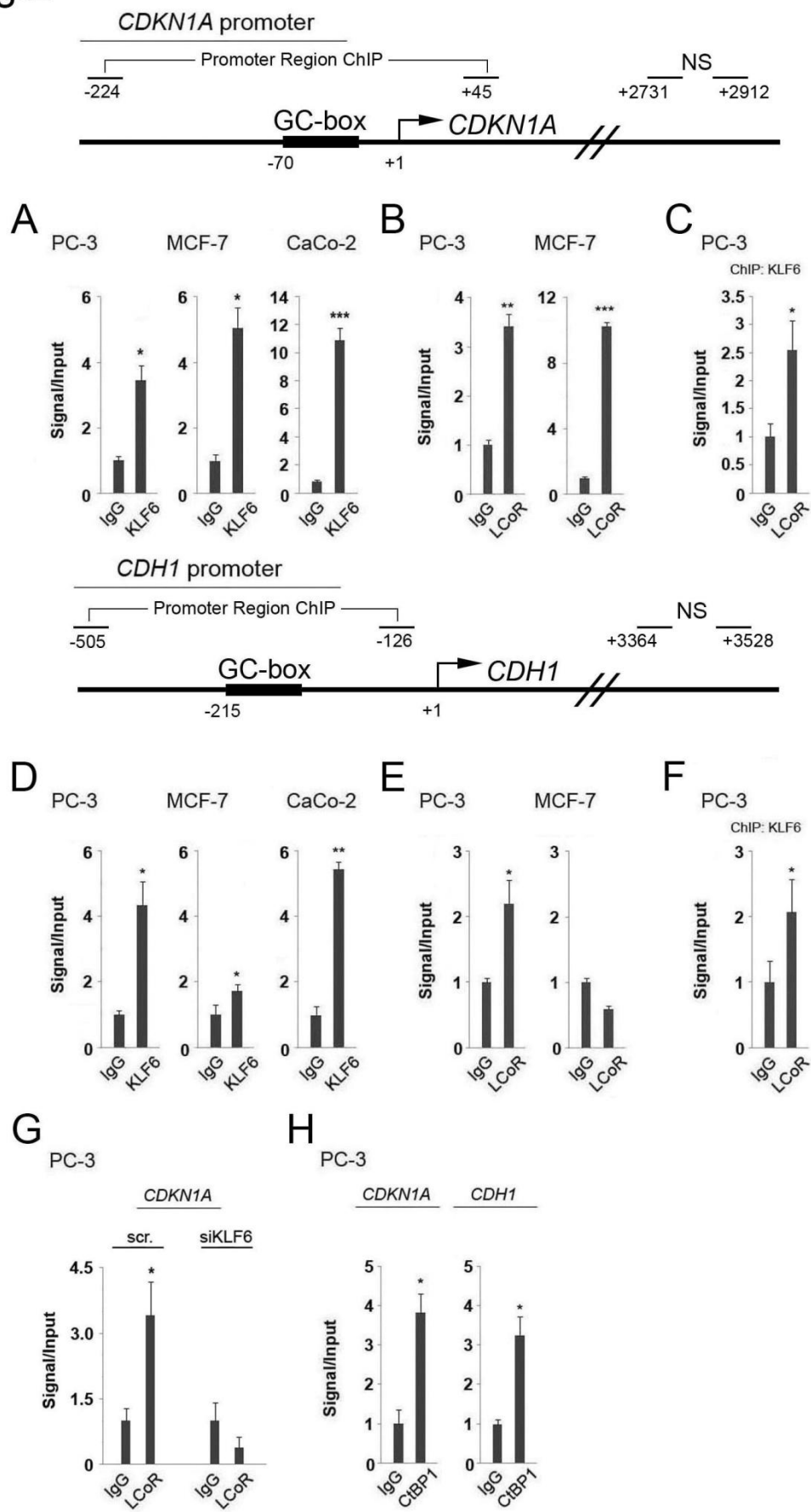


Fig. 3

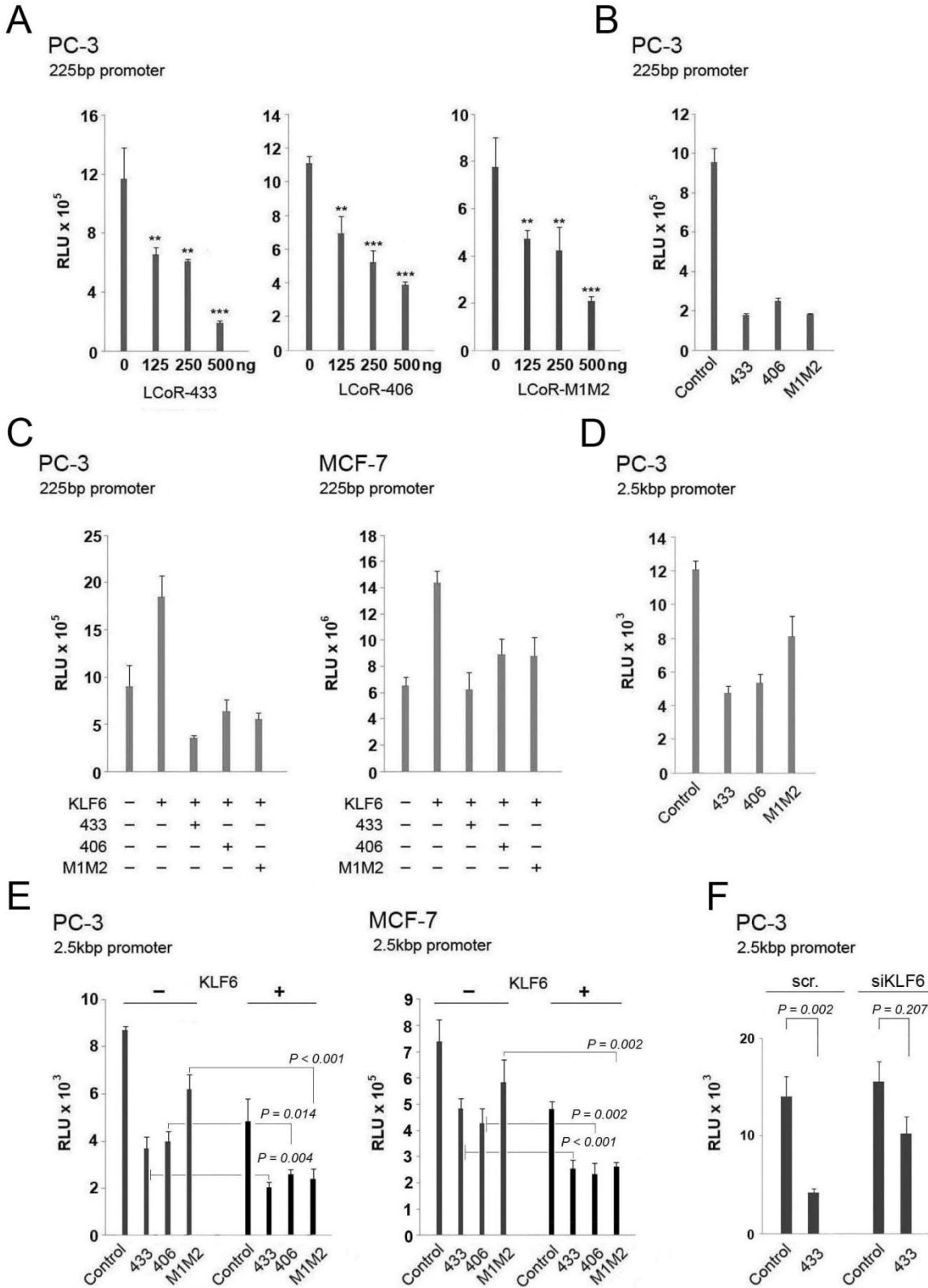
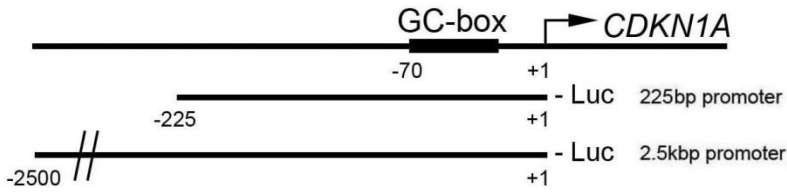


Fig.4

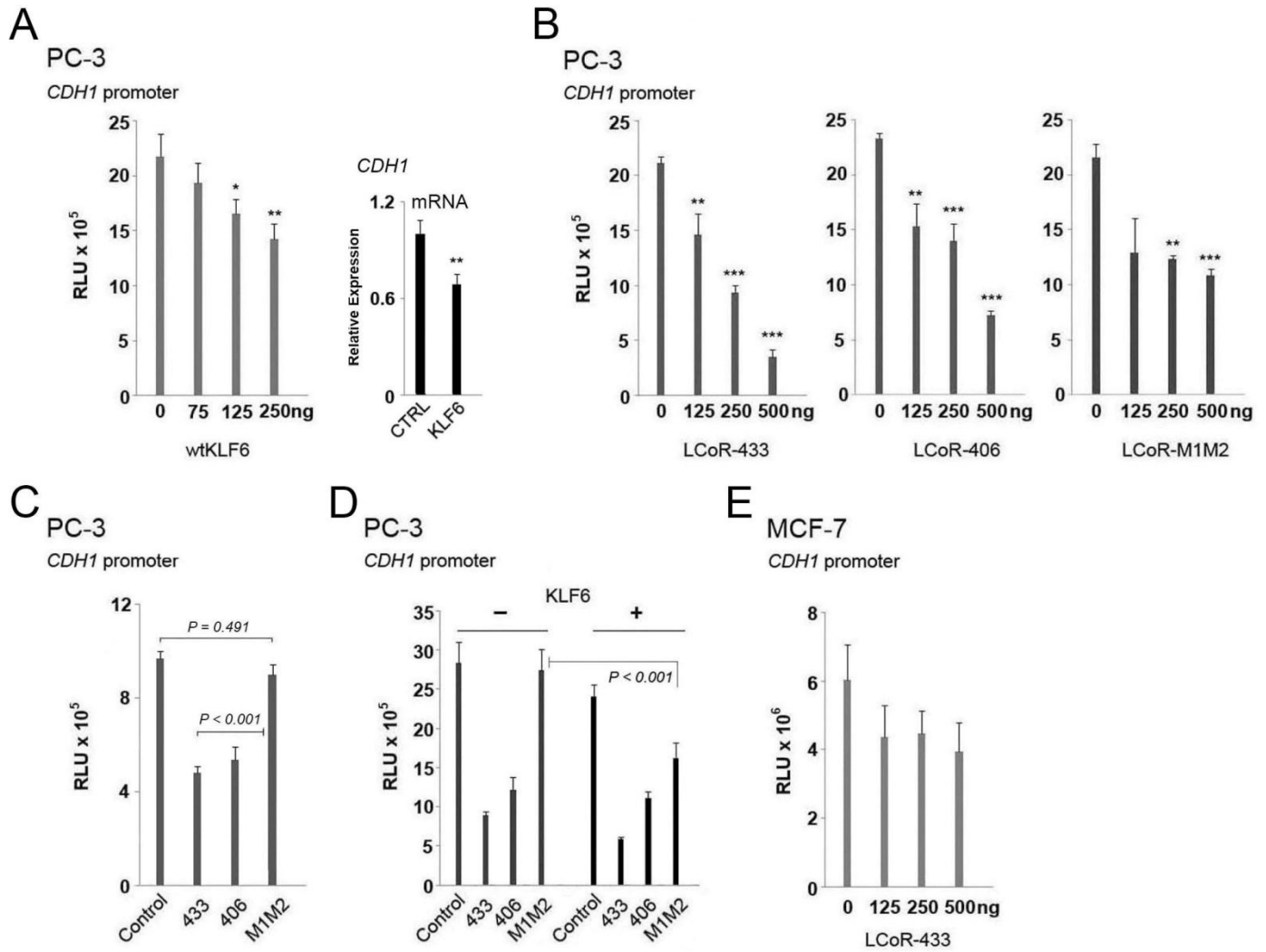
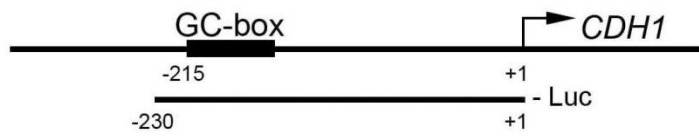
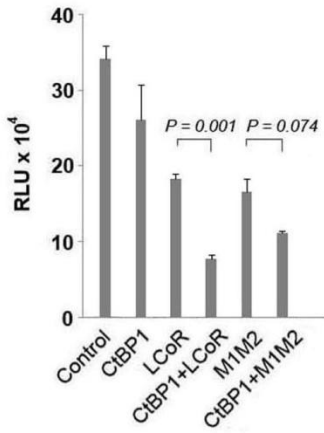


Fig.5

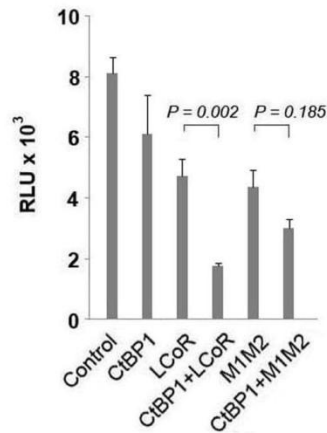
A

CDKN1A - 225bp



Co-Expression Comparison	P-value
LCoR vs M1M2	0.913
CtBP1+LCoR vs CtBP1+M1M2	0.426

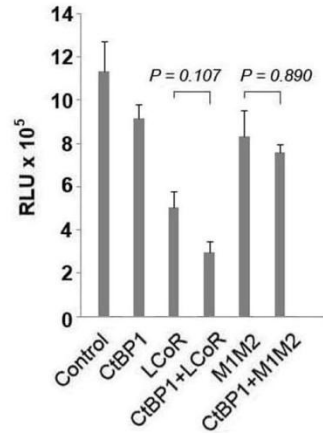
CDKN1A - 2.5kbp



Co-Expression Comparison	P-value
LCoR vs M1M2	0.984
CtBP1+LCoR vs CtBP1+M1M2	0.281

B

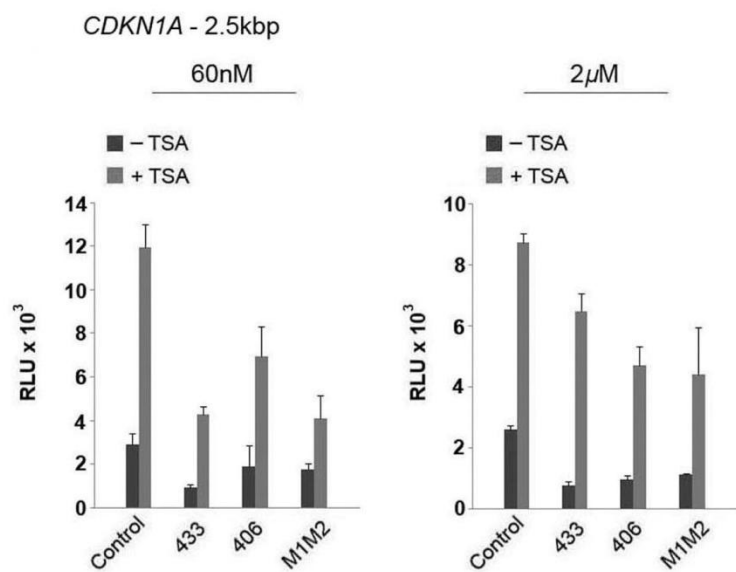
CDH1



Co-Expression Comparison	P-value
LCoR vs M1M2	0.007
CtBP1+LCoR vs CtBP1+M1M2	< 0.001

Fig.6

A



B

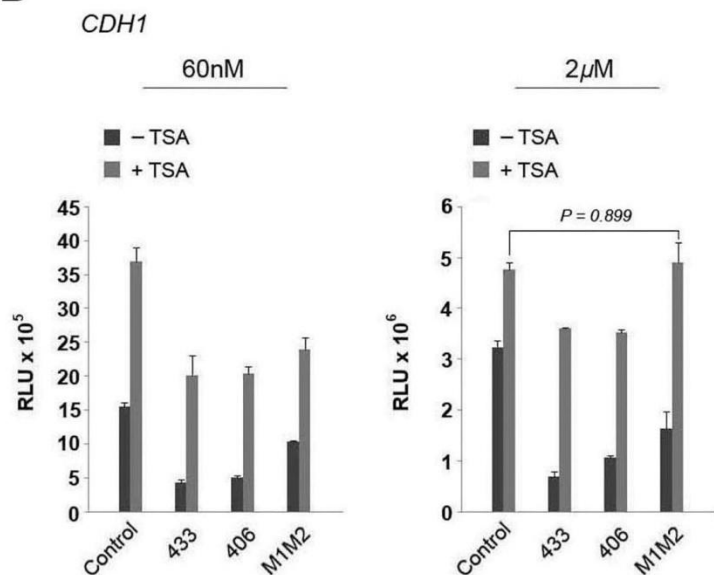
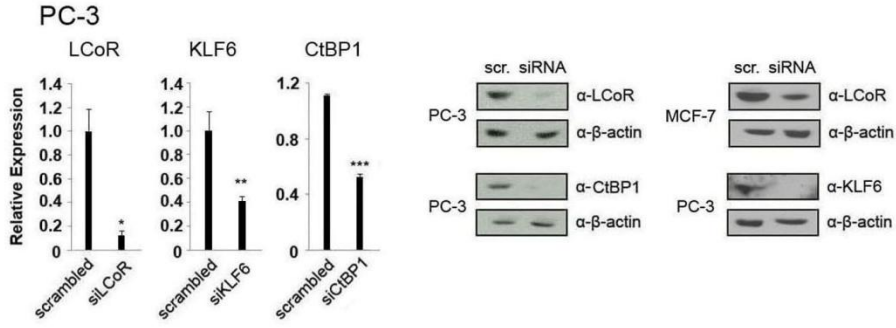
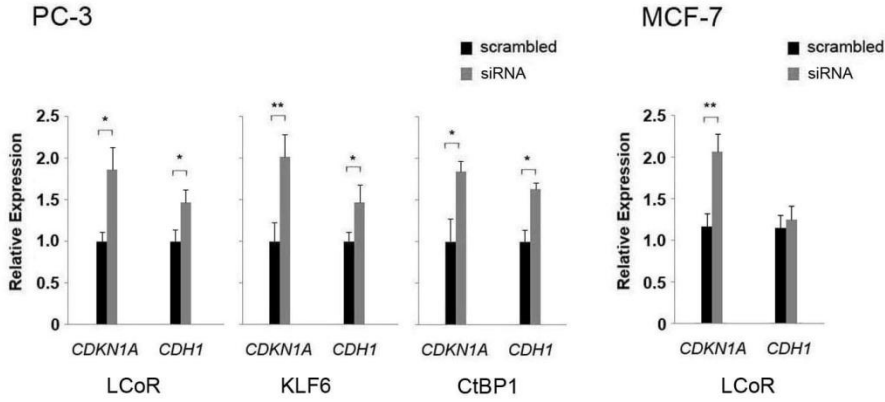


Fig.7

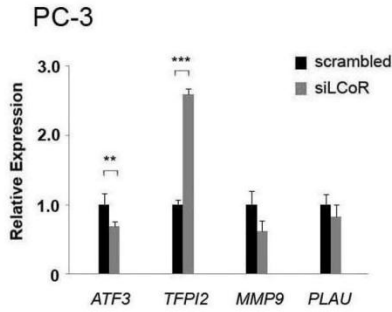
A



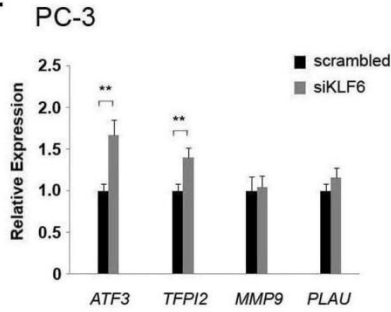
B



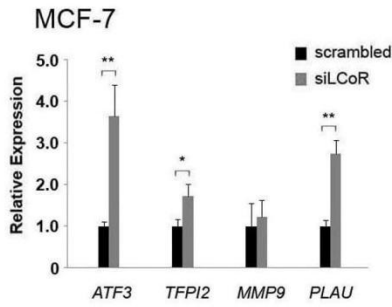
C



E



D



F

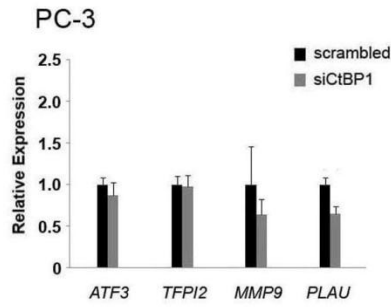
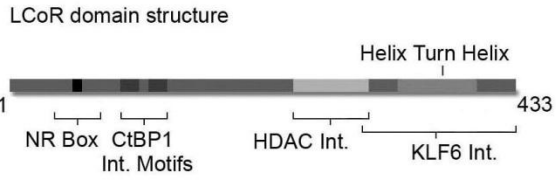


Fig.8

A



B

