

The Orphan Nuclear Receptor Rev-erb α Recruits the N-CoR/Histone Deacetylase 3 Corepressor to Regulate the Circadian *Bmal1* Gene

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Transcriptional regulation plays a fundamental role in controlling circadian oscillation of clock gene expression. The orphan nuclear receptor Rev-erb α has recently been implicated as a major regulator of the circadian clock. Expression of *Bmal1*, the master regulator of circadian rhythm in mammals, is negatively correlated with Rev-erb α mRNA level, but the molecular mechanism underlying this regulation is largely unknown. Here we show that Rev-erb α dramatically represses the basal activity of the mouse *Bmal1* gene promoter via two monomeric binding sites, both of which are required for repression and are conserved between mouse and human. Rev-erb α directly binds to the mouse *Bmal1*

promoter and recruits the endogenous nuclear receptor corepressor (N-CoR)/histone deacetylase 3 (HDAC3) complex, in association with a decrease in histone acetylation. The endogenous N-CoR/HDAC3 complex is also associated with the endogenous *Bmal1* promoter in human HepG2 liver cells, where a reduction in cellular HDAC3 level markedly increases the expression of *Bmal1* mRNA. These data demonstrate a new function for the N-CoR/HDAC3 complex in regulating the expression of genes involved in circadian rhythm by functioning as corepressor for Rev-erb α . (*Molecular Endocrinology* 19: 1452–1459, 2005)

CIRCADIAN RHYTHM IS an internal system that can sustain rhythms of about 24 h (1, 2). The mammalian central pacemaker lies in the suprachiasmatic nuclei (SCN) of the hypothalamus and controls the activity of peripheral clocks through the neuroendocrine and autonomic nervous systems (1–4). The circadian system plays a critical role in the timing of many physiological processes and in harmonizing them with daily environmental changes. The sleep/wake cycle, body temperature, blood pressure, digestive secretion, and hormone production are all regulated in a circadian manner. Dysfunction of circadian rhythms has deleterious effects on human health and may contribute to jet lag, tumorigenesis, and sleep disorders (1, 3–5).

Circadian rhythms are generated and maintained by feedback loops involving the transcription and translation of core clock genes (1, 4–8). In mammals, the transcription factors CLOCK (circadian locomotor out-

put cycles kaput) and BMAL1 [also called MOP3 (member of PAS super family 3)] form heterodimers that activate the expression of *Per* and *Cry* by binding to E-box elements within their promoters (1, 4, 7). The PER and CRY proteins then multimerize and translocate to the nucleus, where CRY proteins repress the transcriptional activity of the CLOCK-BMAL1 heterodimer (1, 4, 7). Human advanced sleep phase syndrome is associated with mutations in the *Per2* gene, which affects the turnover rate of PER2 protein (9).

Recently, the orphan nuclear receptor Rev-erb α has emerged as a critical component of the core circadian feedback loop that control cyclic expression of the *Bmal1* gene both in the SCN and in the peripheral clock liver (10–12). Rev-erb α is a unique nuclear receptor that lacks the activation function 2 domain that is required for ligand-dependent activation of transcription by other members of the nuclear receptor superfamily (13, 14). Therefore, Rev-erb α constitutively behaves as an unliganded receptor, repressing transcription by binding to corepressor molecules (13–15). Rev-erb α preferentially recruits the corepressor N-CoR (13, 15–18), which brings with it a multiprotein complex that includes histone deacetylase 3 (HDAC3) (15, 19, 20). HDAC3 alters local chromatin structure by removing acetyl groups from histone and creating a chromatin structure that is not favorable for accessibility of the basal transcription machinery (19, 20).

Rev-erb α monomers bind to a highly specific DNA sequence consisting of the classic nuclear receptor half-site hexamer AGGTCA with AANT (where N is any

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Abbreviations: ChIP, Chromatin immunoprecipitation; CLOCK, circadian locomotor output cycles kaput; C_t , threshold cycle; dRORE, distal RORE; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; HDAC3, histone deacetylase; N-CoR, nuclear receptor corepressor; pRORE, proximal RORE; ROR, retinoid-related orphan receptors; RORE, ROR binding element; SCN, suprachiasmatic nuclei; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA.

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nucleotide) as the 5' flanking sequence (13). The retinoid-related orphan receptor (ROR) binds to the same site, referred to as an RORE, and constitutively activates transcription as a monomer (21–23). Rev-erb α can inhibit transcription as a monomer by competing with ROR for their shared binding site (22, 24, 25). However, monomeric binding of Rev-erb α is insufficient for corepressor binding, which requires two Rev-erb α half-sites (13, 18). When two ROREs are present as a direct repeat separated by two bases the two Rev-erb α molecules bind cooperatively, but functional corepressor recruitment is also achieved when two monomeric binding sites are present in proximity to one another (18).

Rev-erb α is widely expressed, and its mRNA is induced during adipocyte differentiation (26). Several genes have been found to be repressed by Rev-erb α , including human ApoCIII gene (24), N-myc (27), and Rev-erb α itself (28), suggesting diverse functions of this nuclear receptor *in vivo*. The role of Rev-erb α in circadian biology involves the regulation of the *Bmal1* gene expression (11, 12, 25). Loss of the *Bmal1* gene in mice results in immediate and complete loss of circadian rhythmicity in constant darkness and impaired locomotor activity in light-dark cycles, suggesting the role of *Bmal1* as a nonredundant and essential component of the circadian pacemaker in mammals (29, 30). *Bmal1* mRNA displays a cyclic expression pattern (29), and this is disrupted in mice lacking Rev-erb α (11, 31). Compared with wild type, the Rev-erb α mutant mice displayed a sustained high level of *Bmal1* expression (11). Rev-erb α mRNA is normally expressed in a phase opposite to the *Bmal1* mRNA, suggesting a negative regulatory role, but the detailed mechanisms underlying this repression are unclear.

Here we show that the Rev-erb α directly binds to and represses the *Bmal1* gene promoter via two ROREs, each of which is necessary for this effect. Using chromatin immunoprecipitation, we demonstrate that this process involves active recruitment of the N-CoR/HDAC3 complex, leading to histone deacetylation. In liver cells, the *Bmal1* mRNA is induced by knockdown of this pathway, indicating that the endogenous gene is controlled by this mechanism. Thus, the ability of Rev-erb α to repress the *Bmal1* gene depends upon recruitment of the N-CoR/HDAC3 complex, leading to histone modification of this critical circadian gene.

RESULTS

The Orphan Nuclear Receptor Rev-erb α Represses the Basal Activity of the Mouse *Bmal1* Promoter

Two potential Rev-erb α /ROR monomer binding sites spaced by 26 bp have been identified in the proximal promoter region of the *Bmal1* gene (11, 32). Because ROR is active as a monomer on such sites (25), we will refer to them as ROREs. Both the distal and proximal

ROREs, here referred to as ROREd and ROREp, respectively, are highly conserved in mouse and human (Fig. 1A), but the function of both RORE sites in mediating gene repression by Rev-erb α has not been tested. We first determined whether Rev-erb α can repress the activity of the proximal *Bmal1* promoter by using a reporter vector containing approximately 1 kb of the mouse *Bmal1* promoter driving the luciferase gene. Cotransfection of Rev-erb α dramatically repressed the expression of this gene, but not the parent luciferase vector (Fig. 1B). The magnitude of repression was dependent upon the amount of transfected Rev-erb α (Fig. 1C). Moreover, repression of the *Bmal1* promoter by Rev-erb α was observed in multiple cell types including 293T, Hela and NIH3T3 cells (Fig. 1D), suggesting this phenomenon is not a cell type-specific response but a more general effect.

The C Terminus of Rev-erb α Is Required for Repression of the *Bmal1* Gene

One mechanism by which Rev-erb α might inhibit *Bmal1* gene expression is via DNA binding competition with constitutively active ROR orphan receptors (25). This possibility was tested using a C-terminal truncated mutant [1–236] that retains full DNA binding activity but lacks the corepressor interaction domain (13, 17). In contrast to full-length Rev-erb α (wild type), Rev-erb α [1–236] failed to repress the promoter activity of *Bmal1* (Fig. 2A), implying that the C terminus of Rev-erb α is necessary for repression of the *Bmal1* promoter. Although it had no activity on its own, Rev-erb α [1–236] dose-responsively abrogated repression due to full-length Rev-erb α (Fig. 2B), indicating that DNA binding is required for repression of the *Bmal1* gene by Rev-erb α . This could be overcome by increasing concentrations of Rev-erb α (Fig. 2C), suggesting that the two proteins competed for DNA binding.

Rev-erb α Binds to the *Bmal1* Gene Promoter in Association with Cellular N-CoR/HDAC3

The ability of Rev-erb α to bind and recruit active corepressor complexes to the *Bmal1* promoter was determined using chromatin immunoprecipitation (ChIP) focusing on the region of the *Bmal1* promoter that contained the putative Rev-erb α binding sites (Fig. 3A). In the absence of transfected Rev-erb α , relatively little N-CoR/HDAC3 could be identified in association with this region of the *Bmal1* promoter (Fig. 3B). After transfection, epitope-tagged Rev-erb α was readily identified at the promoter, along with endogenous corepressor N-CoR silencing mediator for retinoids and thyroid hormone receptor (SMRT) (using an antibody that recognizes a common epitope) and associated HDAC3 (Fig. 3B). Of note, acetylation of histones H3 and H4 in this region of the *Bmal1* promoter decreased markedly, consistent with active deacetylation due to Rev-erb α mediated recruitment of the

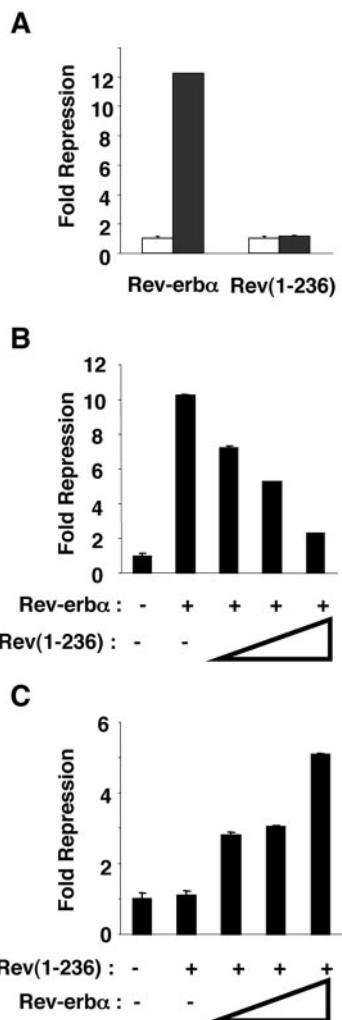


Fig. 2. The C Terminus of Rev-erb α Is Necessary for the Suppression of the Promoter Activity of the *Bmal1* Gene

A, HEK 293T cells were transfected with 50 ng of *Bmal1*-luc reporter alone or together with full-length hRev-erb α expression vector (300 ng) or C-terminal deletion mutant Rev-erb α 1–236 (300 ng). B, Rev-erb α 1–236 inhibits repression by wild-type Rev-erb α in a dose-dependent manner. The amount of full-length Rev-erb α expression vector (300 ng) was kept constant in each transfection mixture. An increasing dose of Rev-erb α 1–236 (200, 300, and 400 ng) was added into transfection mixture along with 50 ng of *Bmal1*-luciferase reporter plasmid. C, Increasing concentrations of full-length Rev-erb α (200, 300, and 400 ng) repress the *Bmal1* promoter in the presence of 300 ng of Rev-erb α 1–236 expression vector. Luciferase activities of all experiments are expressed as the mean \pm SEM of at least three independent experiments performed in triplicate.

endogenous *Bmal1* promoter was critical for basal repression of this gene. To test this we used small interfering RNA (siRNA) to reduce the expression of HDAC3 in HepG2 cells (Fig. 5B). Under these conditions, endogenous *Bmal1* mRNA was markedly induced (Fig. 5C). These data clearly implicate HDAC3 in the basal repression of the *Bmal1* gene in liver cells.

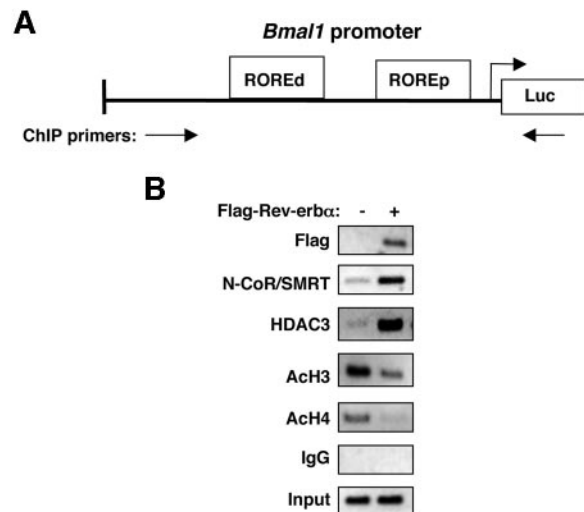


Fig. 3. Nuclear Receptor Corepressors-HDAC3 Complexes Are Recruited to the Rev-erb α -Binding Region of the *Bmal1* Promoter

A, Schematic illustrating the *Bmal1* promoter and the region amplified by PCR after ChIP. B, ChIP assay for exogenous Rev-erb α (Flag-epitope), and endogenous HDAC3, N-CoR/SMRT, and acetylated histone H3 and H4. The ChIP assay data were representative of at least three independent experiments.

DISCUSSION

Oscillation of clock gene mRNA is a hallmark of circadian rhythms (4, 7). The orphan nuclear receptor Rev-erb α has been suggested to regulate the expression of the clock gene *Bmal1*, but the molecular mechanism has not previously been explored. Here we have shown that Rev-erb α represses basal transcription of the *Bmal1* gene by binding to two closely spaced monomeric ROREs, both of which are required for this repressive function. Binding of two Rev-erb α molecules leads to recruitment of the nuclear receptor corepressor-HDAC3 complex, which triggers histone deacetylation at the *Bmal1* promoter. Consistent with this mechanism, we have demonstrated that endogenous HDAC3 actively represses the basal expression of the endogenous *Bmal1* gene in liver cells.

BMAL1, along with its heterodimer partner CLOCK, are major positive components of the molecular circadian clock (7, 8, 29). BMAL1-CLOCK heterodimers activate transcription of negative clock components, including Rev-erb α itself as well as PER and CRY, leading to and propagating oscillatory gene expression (10, 30, 35, 36). Intriguingly, *Bmal1* is the only positive clock component whose mRNA displays robust circadian oscillation in both the SCN and peripheral clocks (11, 12, 25). ROR α strongly activates the *Bmal1* promoter in transient transfection assays (25), but its expression is normally constant (11). By contrast, the expression of Rev-erb α , the major negative regulator of *Bmal1*, is cyclical (11, 12). In absence of the *Rev-erb\alpha* gene, hepatic *Bmal1* gene expression

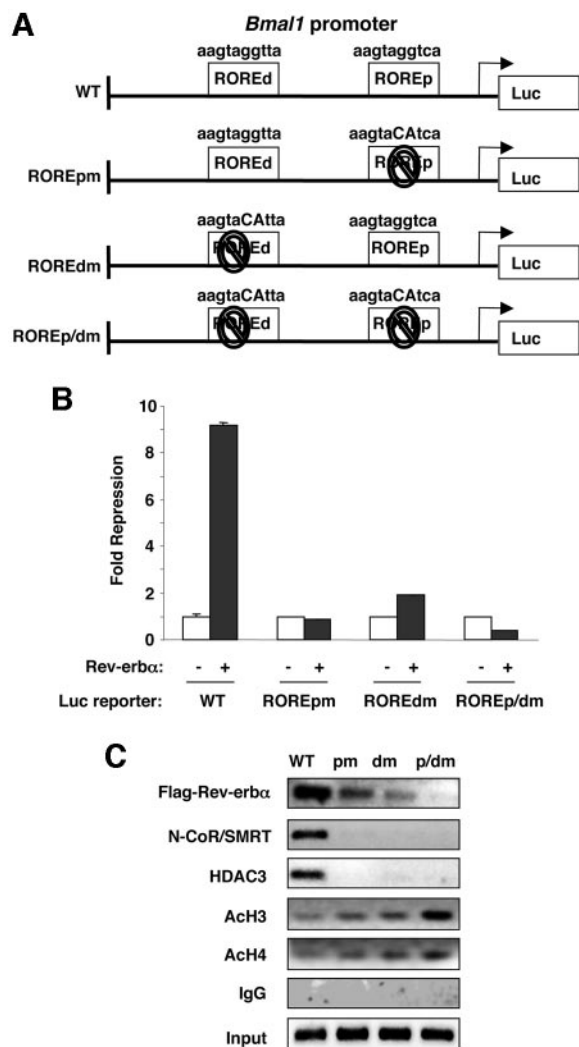


Fig. 4. Both Monomeric Sites in the *Bmal1* Promoter Are Required for Rev-erb α -Mediated Gene Repression of the *Bmal1* Promoter

A, The schematic presentation of various *Bmal1* promoter luciferase constructs. The mutated nucleotides are shown in *capital letters*. Pm, Proximal mutation; dm, distal mutation. B, Mutation of either Rev-erb α binding site abrogates repression. Cotransfection of Rev-erb α (400 ng) with 50 ng *Bmal1* promoter constructs. C, Rev-erb α binds to proximal and distal binding sites, but both are required for recruitment of corepressor complex. ChIP assay was performed to detect exogenous Rev-erb α (Flag-epitope), and endogenous HDAC3, N-CoR/SMRT, Acetyl-H3 and Acetyl-H4 on various *Bmal1* promoter luciferase constructs. The ChIP assay data were representative of at least three independent experiments. WT, Wild type.

remains at a nearly constant, high-level of expression (11).

Our results demonstrate that Rev-erb α is indeed recruited to the *Bmal1* promoter, and this is mediated by binding to two closely spaced ROREs that allows Rev-erb α to actively repress basal transcription of the *Bmal1* gene by recruiting the N-CoR/HDAC3 core-

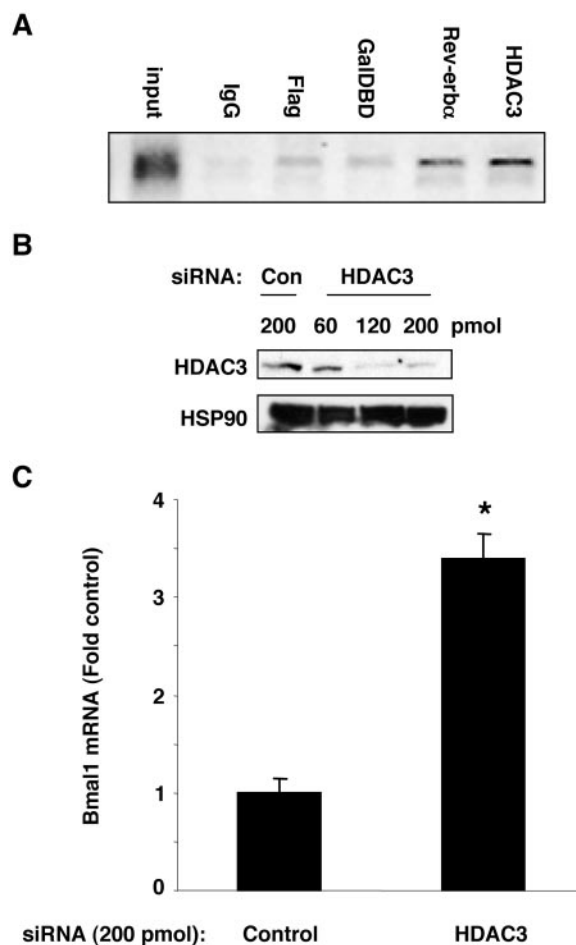


Fig. 5. Rev-erb α Recruits HDAC3 to Repress the Expression of the *Bmal1* Gene in Human Liver Cells

A, CHIP assay of the *Bmal1* promoter was performed in human HepG2 cells with the indicated antibodies. B, siRNA knockdown of HDAC3 in HepG2 cells assessed by immunoblot. C, HDAC3 knockdown induces endogenous *Bmal1* gene expression. After siRNA transfection (as shown in B), total RNA was prepared and *Bmal1* gene expression was analyzed relative to GAPDH control by quantitative real-time PCR. The fold change was calculated as the relative abundance of *Bmal1* mRNA in the cells receiving HDAC3 siRNA divided by the relative abundance of *Bmal1* mRNA in the cells receiving control siRNA, which was set to 1. Results are expressed as the mean \pm SEM of two independent experiments performed in duplicate. The *P* value of paired Student's *t* test is less than 0.01. GalDBD, Galactosidase DNA binding domain; HSP, heat shock protein.

pressor complex. Thus, although DNA binding by Rev-erb α competitively prevents ROR α from activating the *Bmal1* promoter (25), the architecture of the gene favors active, enzymatically mediated repression of the *Bmal1* gene. In support of this, our knockdown experiments have demonstrated a direct link between HDAC3 and the regulation of the endogenous *Bmal1* gene. Modulation of this activity would be predicted to influence the circadian clock. Indeed, HDAC inhibitors have been shown to alter circadian rhythms (10, 37).

Our data strongly suggest that Rev-erb α and its associated N-CoR/HDAC3 complex is one likely target of this intervention. To our knowledge, this is the first report addressing the potential function of corepressor complexes in regulating circadian rhythms. Manipulations that selectively affect corepressor function may be useful remedies for circadian phase shift.

MATERIALS AND METHODS

Plasmids

The mouse *Bmal1*-luciferase vector (*Bmal1-luc*) was generously provided by Dr. Masaaki Ikeda from University of Saitama Medical School (Japan) (32). The mutations in RORE sites were created by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). The expression vector of pcDNA-Flag-Rev-erb α (wild type) and Rev-erb α (1–236) were created using standard subcloning techniques. All plasmids were confirmed by automatic sequencing analysis.

Mammalian Cell Culture and Transfection

293T cells and HepG2 cells were maintained in DMEM (high glucose) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate and 1 mM nonessential amino acid (all from Invitrogen Life Technologies, Carlsbad, CA). Cells were grown at 37 C in 5% CO₂. All transient transfection assays were performed using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. For repression assay, cells were grown in 24-well plates and 0.05 μ g of *Bmal1*-luciferase reporter, 0.4 μ g of pcDNA or pcDNA-Flag-Rev-erb α expression vector, and 0.1 μ g of β -galactosidase expression vector were added to each well. At 24 h after transfection, a luciferase assay kit (Promega, Madison, WI) was used to determine relative levels of the luciferase gene product. Light units were normalized to the cotransfected β -galactosidase expression plasmid. Fold repression was calculated as the activity of a given reporter after transfection with control expression vector divided by the activity of the same reporter in the presence of Rev-erb α expression vector.

ChIP Assay

A ChIP assay was performed according to the protocols of Upstate Biotechnology (Lake Placid, NY) with minor modifications (15). 293T cells growing in a 100-mm plate were transfected with 12 μ g of either pcDNA or pcDNA-Flag-Rev-erb α construct expression vector and 1.5 μ g of *Bmal1*-luc vector. After overnight incubation, cells were cross-linked with 1% formaldehyde for 10 min at room temperature in PBS. Cells were washed three times with PBS and then collected in ice-cold PBS with 1 \times protease inhibitor (Roche Molecular Biochemicals). Cell pellets were obtained by centrifugation at 1000 \times *g* in PBS for 4 min and resuspended in 500 μ l of hypotonic buffer [50 mM Tris-HCl (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40] with protease inhibitor. After incubation on ice for 10 min, the cell lysates were centrifuged for 8 min at 2000 \times *g*. The pellets were resuspended in 300 μ l of sodium dodecyl sulfate (SDS)-containing sonication buffer [0.01% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)] with protease inhibitor and sonicated three times for 20 sec each time followed by centrifugation at 14,000 \times *g* for 10 min. Supernatants were collected and diluted 5-fold with dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl] with protease inhibitor

followed by preclearing with 2 μ g of sheared salmon sperm DNA and protein A Sepharose [50 μ l of a 50% slurry in 10 mM Tris-HCl (pH 8.1)-1 mM EDTA] for 2 h at 4 C. Immunoprecipitation with the following antibodies was performed at 4 C overnight: anti-Flag, anti-HDAC3, anti-N-CoR/SMRT, anti-acetyl histone H3 and H4 (Upstate Biotechnology) and normal rabbit IgG and normal mouse Ig (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoprecipitated complexes were collected with protein A Sepharose beads followed by sequential washes in low-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 150 mM NaCl], high-salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), 500 mM NaCl], LiCl wash buffer [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)], and Tris-EDTA buffer. Precipitates were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃), and 5 M NaCl was added to reverse cross-links at 65 C for 6 h. DNA fragments were purified with a PCR purification kit (QIAGEN, Valencia, CA). A total of 1–3 μ l of purified sample was used in 23–28 cycles of PCR using one pair of primers encompassing both ROREs region of mouse *Bmal1* promoter. The primers used for PCR used in Fig. 3 are as follows: forward: 5'-ggattggtcggaaagtacgtt-3'; reverse: 5'-aggaaccaggcgctatctct-3'. The primers used for PCR used in Fig. 4 are as follows: forward: 5'-ttgggcacagcgattggtg-3'; reverse: 5'-taaacaggcactccgctccc-3'. The ChIP assay in HepG2 cells was performed using the same protocol. However, the optimal sonication condition of HepG2 cells was 25 sec for 4 times at output 8. The rabbit anti-Rev-erb α antibody was raised against the peptide: GSLQVAMEDSSRVSPSK. The primers used to amplify RORE region of human *Bmal1* promoter in HepG2 cells are as follows: forward: 5'-cgacattagggaggcaga-3'; reverse: 5'-tttcggccctaaagtctca-3'.

siRNA Expression

The control siRNA (D-00122001) and HDAC3 *SMARTpool* siRNA (M-00349600) were both purchased from Dharmacon (Lafayette, CO). HepG2 cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) with some modifications. In brief, HepG2 cells were transfected when they were about 30–50% confluence. The siRNA oligonucleotides were diluted in Opti-MEM reduced serum (Invitrogen Life Technologies) and then mixed with Lipofectamine 2000 prediluted in OptiMEM. The transfection reactions were incubated for about 25 min. Meanwhile, the HepG2 cells were washed once with OptiMEM and covered with fresh OptiMEM. After incubation time, the transfection reaction was added into the HepG2 cells and incubated at 37 C for 12 h. About 2 ml of fresh growth medium were added back to transfected cells. The HepG2 cells were exposed to siRNA treatment for total 96 h and then subjected to either immunoblotting or quantitative RT-PCR.

Immunoblotting

The protein lysate was prepared in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.4% deoxycholate, 0.1% SDS]. About 20 μ g of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were probed with primary antibodies in Tris-buffered saline containing 5% nonfat dry milk followed by horseradish peroxidase-conjugated antirabbit or anti-mouse antibody (Pierce, Rockford, IL) at 1:5,000 dilution. Blots were visualized by ECL (Amersham Biosciences, Piscataway, NJ) and the following antibodies and dilutions: anti-HDAC3 antibodies (ABCAM Inc., Cambridge, MA) at 1:2,000 and anti-Hsp90 (heat shock protein 90) antibody at 1:10,000 (Santa Cruz Biotechnology).

Quantitative RT-PCR

Total RNA was prepared using an RNeasy kit (QIAGEN). cDNA was synthesized from RNA treated with deoxyribonuclease followed by reverse transcription reaction (Invitrogen Life Technologies) according to manufacturer's instructions. mRNA transcripts were quantified by SYBR GREEN PCR kit (Applied Biosystems), using a Prism 7900 thermal cycler and sequence detector (Applied Biosystems). The primers used in the real-time PCR were the following: *hBmal1*—forward, 5'-tgtggcgctcactgtgt-3'; reverse, 5'-ttctgctgatcctgtcatctct-3'; glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)—forward, 5'-gaaggtgaaggtggagtc-3'; reverse, 5'-gaagatgg-tgatgggattc-3'. Both pairs of primers were designed using Primer express software and the primer efficiencies were greater than 95%. All the reactions were performed in quadruplicate, and each threshold cycle (C_t) value was an average of the values obtained from each reaction. The ΔC_t values were determined by subtracting the *Gapdh* C_t values from the *Bmal1* C_t values. The fold change in expression of the *Bmal1* in the HDAC3 siRNA-treated group relative to that in control siRNA-treated group was expressed as $2^{-\Delta\Delta C_t}$, in which $\Delta\Delta C_t$ equals difference between the ΔC_t value of HDAC3 siRNA-treated cells and the ΔC_t value of the control group, which was normalized to 1.

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