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The HP1α protein is mandatory to repress the circadian clock and its output genes during the 12 h period of transcriptional repression

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The transcriptional repressions driven by the circadian core clock repressors RevErbα, E4BP4, and CRY1/PER1 involve feedback loops which are mandatory for generating the circadian rhythms. These repressors are known to bind to cognate DNA binding sites, but how their circadian bindings trigger the cascade of events leading to these repressions remain to be elucidated. Through molecular and genetic analyses, we now demonstrate that the chromatin protein HP1α plays a key role in these transcriptional repressions of both the circadian clock (CC) genes and their cognate output genes (CCGs). We show that these CC repressors recruit the HP1α protein downstream from a repressive cascade, and that this recruitment is mandatory for the maintenance of both the CC integrity and the expression of the circadian genes. We further show that the presence of HP1α is critical for both the repressor-induced chromatin compaction and the generation of "transcriptionally repressed biomolecular hydrophobic condensates" and demonstrates that HP1α is mandatory within the CC output genes for both the recruitment of DNA methylating enzymes on the intronic deoxyCpG islands and their subsequent methylation.

circadian clock | circadian DNA methylation–demethylation | circadian transcription | circadian transcriptional repression | intronic deoxyCpG-Islands

Significance

The transcriptional repressions driven by the circadian clock (CC) repressors (RevErbα, E4BP4, and CRY1/PER1) are known to involve feedback loops which generate the circadian rhythms. These repressors bind to cognate DNA binding sites, but how these bindings trigger the cascade of events leading to the repression of transcription remains to be elucidated. We now demonstrate that the chromatin protein HP1α is instrumental in the repressions of both the circadian clock genes and their output genes. Taken altogether, our data indicate that a physiopathological increase in an HP1α-induced deoxyCpG methylation leads to an impaired transcription of the circadian output genes, which is similar to that observed upon aging.

The HP1 α, β, and γ homodimer proteins are major components of the heterochromatin (1, 2). In vitro studies have revealed that HP1α could play an important role in the chromosomal organization through the physical bridging of H3K9Me3-containing nucleosomes (3, 4). The DNA binding and compaction properties of the chromatin protein HP1α then enable the recruitment of factors involved in the transcriptional repression and the generation of biomolecular heterochromatin condensates (5), thereby leading to the formation of phase-separated hydrophobic liquid droplets (6–9). On the other hand, the transcriptional repressors (RevErbα, E4BP4, and Per1/CRY1) that belong to the negative regulatory limb of the circadian clock (CC) are known to coordinate the circadian temporal expression of both the CC genes and their output genes (10–12). Of note, at the molecular level, little is known concerning i) to which extent is HP1α involved in this repressor-mediated transcriptional repression of both the core clock and its output genes and ii) how important is the circadian CpG methylation controlled by HP1α in maintaining a strict 24-h circadian expression of the CC output genes.

We have investigated, through molecular genetic studies, whether and how HP1α could possibly contribute to the transcriptional repression of both the core clock and its output genes. Our data reveal how, through epigenetic repression, HP1α plays a key role in regulating the circadian transcriptional repression of both the core clock and its output genes. We conclude that even in the presence of the RevErbα and E4BP4 transrepressors, HP1α is mandatory for the circadian repression of both the core clock and its output genes, as revealed by the constitutive expression of these genes upon HP1α ablation.

Results and Discussion

The Same Repressive Chromatin Components Are Concomitantly Associated with Their Cognate Repressed CC Enhancers and Their Cognate DeoxyCpG-Rich Islands. The transcriptional repression of the CC-controlled output genes is initiated through the binding of either the RevErbα or E4BP4 repressors to their cognate retinoic acid receptor-related orphan receptor element (RORE) and D-box enhancers (Figs. 1 *A* and *B* and 4 *A* and *B* and *SI Appendix*, Fig. S4*B*). Even though there is no RORE nor D-box binding sites within their cognate deoxyCpG islands, chip assays revealed a concomitant "in phase" circadian presence of both the RevErbα and E4BP4 repressors on deoxyCpG islands of RORE (Fig. 1*B*) and D-box (Fig. 1*A*) genes, respectively. Of note histone deacetylase 3 (HDAC3), known to be

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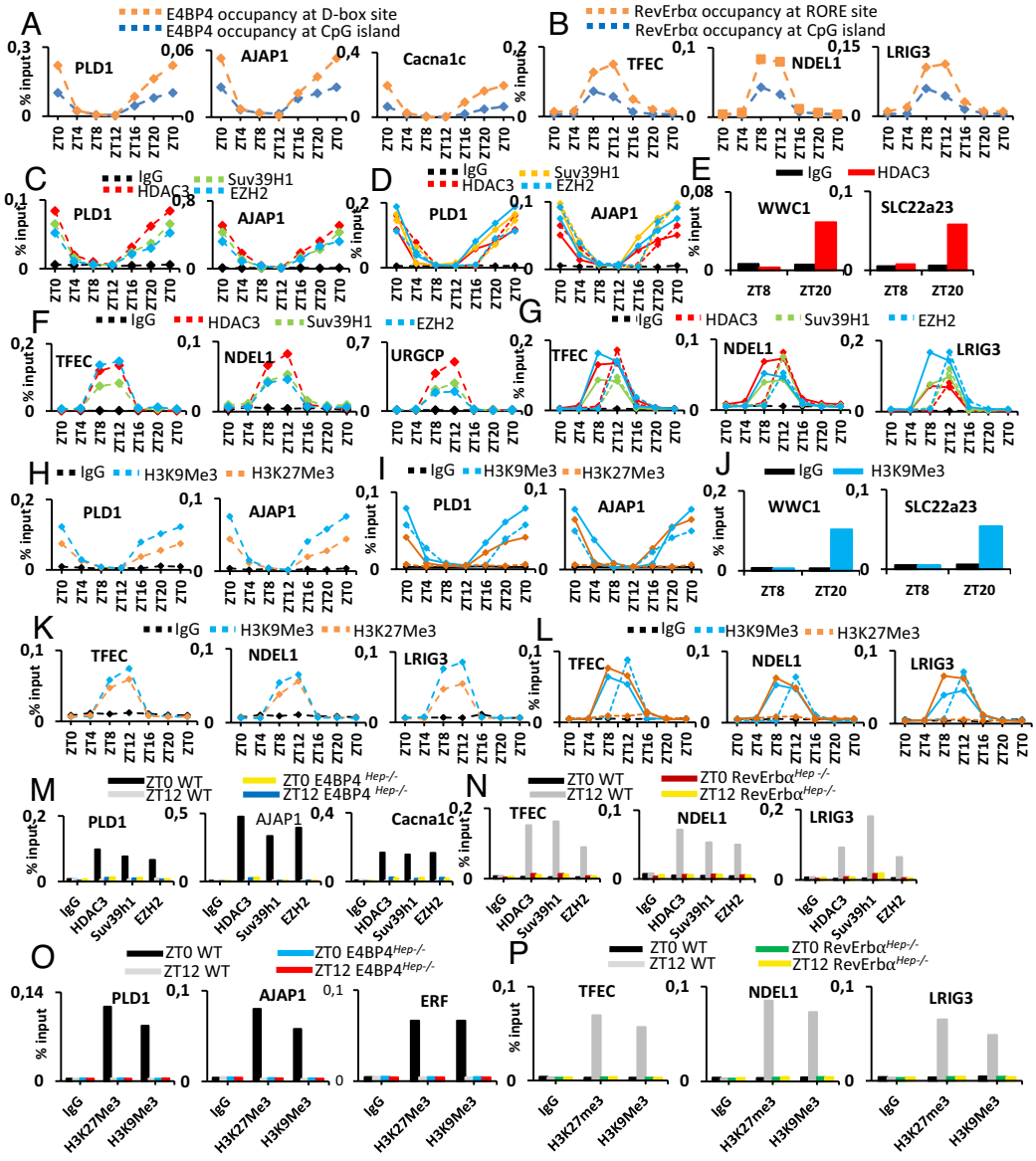

Fig. 1. Components of repressive chromatin complexes are concomitantly present on enhancers and intronic CpG islands. (*A*) qPCR chip assays with wild type (WT) liver extracts, showing the circadian binding of E4BP4 to its D-box DBS: DNA binding sequence. and its concomitant presence on the CpG islands of genes, as indicated (PLD1, Adherens Junctions Associated Protein 1 (AJAP1), and Channel Subunit Alpha1 C (Cacna1c)). The X-axis represents the % input for the chip assay, and the Y-axis represents the time points (in ZT) for the experiments. For the selection of E4BP4 controlled D-box-containing target genes, see in the accompanying paper "The circadian demethylation of a unique intronic deoxymethylCpG-rich island boosts the transcription of its cognate circadian clock output gene" under the section "A single intronic deoxyCpG-rich island undergoing a circadian demethylation–methylation is present within each CC-controlled output gene containing a D-box, a RORE or an E-box, but not within the core clock genes, nor their immediate output genes" (*B*) qPCR chip assays with WT liver extracts, showing the circadian binding of RevErbα to its RORE DBS and its concomitant presence on the CpG islands of genes, as indicated (Transcription factor EC (TFEC), Nude Neurodevelopment Protein 1 Like 1 (NDEL1), and Leucine Rich Repeats And Immunoglobulin Like Domains 3 (LRIG3)). For the axis, see *A*. For the selection of RevErbα controlled RORE-containing target genes, see in the accompanying paper "The circadian demethylation of a unique intronic deoxymethylCpG-rich island boosts the transcription of its cognate circadian clock output gene" under the section "A single intronic deoxyCpG-rich island undergoing a circadian demethylation–methylation is present within each CC-controlled output gene containing a D-box, a RORE or an E-box, but not within the core clock genes, nor their immediate output genes" (*C* and *F*) qPCR chromatin immunoprecipitation (CHIP) assays with WT liver extracts, showing the circadian recruitment of HDAC3, Suv39h1, and EZH2 on the CpG island of genes containing either a D-box (*C*) or a RORE (*F*), as indicated. For the axis, see *A*. (*D* and *G*) qPCR chip assays with WT liver extracts, showing the circadian association of HDAC3, Suv39h1, and EZH2 on the promoter (dashed lines) and the enhancer (solid lines) regions of D-box (*D*) and RORE (*G*)-containing genes, as indicated. For the axis, see *A*. (*E*) qPCR chip assays with WT liver extracts, showing the circadian binding of HDAC3 to the CpG islands of two E-box-containing BMAL1 target genes, as indicated (WWC1 and SLC22a23). For the axis, see *A*. (*H* and *K*) qPCR chip assays with WT liver extracts, showing the circadian association of the repressive histones (H3K9Me3 and H3K27Me3) on CpG islands of genes containing either a D-box (*H*) or a RORE (*K*), as indicated. For the axis, see *A*. (*I* and *L*) qPCR chip assays with WT liver extracts, showing the circadian presence of the repressive histones (H3K9Me3 and H3K27Me3) on the upstream enhancer (solid line) and proximal-promoter regions (dashed line) of genes, as indicated containing either a D-box (*I*) or a RORE (*L*). (*J*) qPCR chip assays (as for *I* and *L*), showing the circadian association of H3K9Me3 to the CpG islands of two E-box-containing genes, as indicated. For the axis, see *A*. (*M* and *N*) qPCR chip assays showing the lack of recruitment of HDAC3, Suv39h1, and EZH2 on CpG islands of D-box-containing genes in E4BP4$^{hep-/-}$ mice (*M*), and of RORE-containing genes in RevErbα$^{hep-/-}$ mice (*N*), as indicated. (*O*) qPCR chip assays showing, in E4BP4$^{hep-/-}$ mice, the lack of recruitment of repressive histones (H3K9Me3 and H3K27Me3) on CpG islands of D-box-containing genes, as indicated. (*P*) As for *O*, but for RORE-containing genes in RevErbα$^{hep-/-}$ mice, as indicated. For the figures, the X-axis represents the % input for the chip assay, and the Y-axis represents the time points (in ZT) for the experiments.

selectively recruited by nuclear Receptor Corepressor (NCOR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) on enhancer sites repressed by RevErbα (at ZT8) and E4BP4 (at ZT20) (13) (Figs. 1 *D* and *G* and 4 *A* and *B*), was also found on deoxyCpG islands with zeniths between ZT20-ZT0 for D-box genes (Figs. 1*C* and 4*B*) and ZT8-ZT12 for RORE genes

(Figs. 1*F* and 4*A*), while lacking in RevErbα$^{hep-/-}$ and E4BP4$^{hep-/-}$ mutant mice (Fig. 1 *M* and *N*). The recruitment of HDAC3, known to interact with SUV39H1 (14, 15) (an H3K9 methyltransferase) and EZH2 (16, 17) (an H3K27 methyltransferase), then leads to the circadian recruitment of the H3K9Me3 and H3K27Me3 repressive histones on both D-box and RORE enhancers (18) (Fig. 1 *D*, *G*, *I*, and *L*). SUV39H1, EZH2, H3K9Me3, and H3K27Me3 histones were similarly found on the deoxyCpG islands, with zeniths between ZT20-ZT0 for D-box genes (Figs. 1 *C* and *H* and 4*B*) and ZT8-ZT12 for RORE genes (Figs. 1 *F* and *K* and 4*A*). Importantly, the recruitment of these methylated histones on both deoxyCpG islands, D-box, and "repressed" RORE enhancers precedes their final recruitments on the proximal-promoter region (Fig. 1 *C*, *D*, *F*–*I*, *K*, and *L*), and, as expected, all of these circadian recruitments were lacking in E4BP4$^{hep-/-}$ and RevErbα$^{hep-/-}$mutant mice (Figs. 1 *M*–*P* and 4 *A* and *B*).

Thus, the bindings of the RevErbα and E4BP4 CC repressors on their cognate enhancer sites result, not only in the recruitment of these transrepressors on intronic deoxyCpG islands devoid of cognate binding sites, but also in the binding of histone deacetylases (HDAC3) and histone methyltransferases (SUV39H1 and EZH2), thereby leading within the chromatin to a transition from "active" (H3K9Ac and H3K27Ac) to "repressive" (H3K9Me3 and H3K27Me3) histones (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S4*B*). Note that this transfer of repressive components from a repressed enhancer to an intronic CpG island is carried out through a mechanism which is similar to that observed upon gene transactivation, as shown in our accompanying PNAS paper entitled "The circadian demethylation of a unique intronic deoxymethylCpG-rich island boosts the transcription of its cognate circadian clock output gene" (19).

The RevErbα and E4BP4 Transrepressors Control the Selective Circadian Binding of HP1α on the CC Output Genes. It is well established that the tri-methylated H3K9 histone "H3K9Me3" is required in vitro for the recruitment of the HP1 proteins (α, β, or γ) to the nucleosome core (2). As the recruitment of H3K9Me3 to the CC output genes which contain either a D-box or a RORE is circadian (Fig. 1 *H*, *I*, *K*, and *L*), we examined which of the three HP1 proteins (α, β, or γ) could be recruited in a circadian manner in vivo. Chip assays with WT mouse liver chromatin extracts and either HP1α, β, or γ monoclonal antibodies revealed a selective circadian recruitment of HP1α, not only on D-box (*SI Appendix*, Fig. S1*A*) and RORE (*SI Appendix*, Fig. S1*B*) enhancer regions, but also on their cognate deoxyCpG islands (Fig. 2 *A* and *B*). All of these HP1α recruitments were restricted to the circadian active phase for genes expressed during the rest phase (i.e., D-box genes; see Figs. 2*A* and 4*B* and *SI Appendix*, Fig. S1*A*) and to the rest phase for genes expressed during the active phase (i.e., RORE genes; see Figs. 2*B* and 4*A* and *SI Appendix*, Fig. S1*B*), whereas, as expected, no such recruitments could be detected in HP1α$^{hep-/-}$ mutant mice (Fig. 2 *D* and *E* and *SI Appendix*, Fig. S1 *C* and *D*). Of note, these recruitments preceded the binding of HP1α to the proximal-promoter and gene body regions (i.e., ZT16 for D-box genes and ZT8 for RORE genes; see Fig. 2 *A* and *B* and *SI Appendix*, Fig. S1 *A* and *B*), while no Hp1α recruitment was detected upon disruption of the CC (E4BP4$^{hep-/-}$ or RevErbα$^{hep-/-}$ mutant mice; see Fig. 2 *F* and *G* and *SI Appendix*, Fig. S1 *E* and *F*).

We conclude that the Hp1α recruitments on enhancers, deoxyCpG islands, and proximal-promoter regions are dependent on both i) the binding of a transrepressor (either E4BP4 or RevErbα) to its cognate enhancer site and ii) the RevErbα-induced recruitment of the H3K9Me3 repressive histone (Fig. 4 *A* and *B*).

Hp1α Is Mandatory for the Circadian Binding of the DNMT3a DNA Methylating Enzyme on DeoxyCpG Islands. The chromodomain of Hp1α is known to directly interact with the bromodomain of DNA methyltransferases (20). It is also known that, upon binding of the DNMT3a enzyme in somatic and embryonic stem (ES) cells, the methyldeoxyCpG-binding transcriptional repressor proteins MeCP2 and MBD4 bind together with DNMT3a, on the methyl deoxyCpG-rich regions of the heterochromatin (21). We now show i) that the alternate methylation–demethylation of intronic deoxyCpG islands is controlled by the circadian core clock (Fig. 4 *A* and *B*) and ii) that, upon repression of the D-box and RORE genes, the enzymes involved in DNA methylation (DNMT3a, MeCP2, MBD4) are bound to the deoxyCpG islands.

Chip analyses were carried out with liver extracts of HP1α$^{hep-/-}$ mutant mice to determine whether HP1α i) is mandatory for deoxyCpG methylation within the deoxyCpG islands and ii) could possibly act as a "bridge" between the methylated histone H3K9Me3 and methylated DNA. No binding of the DNA methylating enzymes (DNMT3a, MeCP2, MBD4) was detected in HP1α$^{hep-/-}$ mutants, while the DNA demethylating enzymes (TDGα, GADD45α, AID, and Apobec2) were constitutively bound to the deoxyCpG islands of both D-box and RORE genes (Fig. 2 *H* and *I*), thus leading to the constitutive demethylation of these deoxyCpG islands.

We conclude that, upon the circadian recruitment of HP1α, the CC repressors (either E4BP4 or RevErbα) are involved in the methylation of the deoxyCpG islands which are permanently demethylated in the absence of HP1α as indicated by the constitutive recruitment of DNA demethylating enzymes.

HP1α Is Mandatory to Generate a Repressing Chromatin Compaction of the CC-Controlled Output Genes. The in vitro studies of Machida et al. (3) have demonstrated that an HP1α-induced chromatin compaction generated upon HP1α binding results in the formation of "a symmetric dimer" which bridges two adjacent H3K9Me3 nucleosomes. Further yeast studies (22) have shown that the binding of Swi6 (the yeast homolog of HP1) to H3K9Me3 results, not only in bridging two adjacent nucleosomes, but also in restructuring them to transiently expose buried histone core residues involved in weak multivalent interactions between the nucleosomes. Both Machida et al. (3) and Sanulli et al. (22) studies led us to examine how Hp1α could possibly be instrumental in maintaining a repressed chromatin state in vivo. Chip analyses using liver extracts from HP1α$^{hep-/-}$ mutant mice revealed the presence of a mixture of acetylated (H3K9Ac and H3K27Ac) and methylated (H3K9Me3, H3K27Me3, and H3K4Me1) histones, on both the D-box and RORE enhancers, as well as on the proximal-promoter regions of the D-box (*SI Appendix*, Fig. S2 *A* and *B*) and RORE (*SI Appendix*, Fig. S2 *C* and *D*) genes. Of note, in HP1α$^{hep-/-}$ mice, qPCR RNA analyses (*SI Appendix*, Fig. S2 *G* and *H*) revealed, within the D-box and RORE enhancer regions of these CC output genes, a constitutive lower level of expression of both messenger ribonucleic acid (mRNA)(Fig. 3 *A* and *B*) and enhancer RNA (eRNA; *SI Appendix*, Fig. S2 *E* and *F*). Furthermore, chip assays on deoxyCpG islands indicated the presence of a mixture of acetylated and methylated histones, similar to those found on enhancers of D-box and RORE genes (Fig. 3 *C* and *D*), while, in HP1α$^{hep-/-}$ mice, qPCR RNA analyses showed a constitutive lower level of expression of an island-encoded RNA (*SI Appendix*, Fig. S2 *G* and *H*). Of note upon ablation of the HP1α protein which results in an "open" chromatin structure, chip assays performed on liver whole-cell extracts from HP1α$^{hep-/-}$ mice revealed the YY1 constitutive bindings to cognate sites located upstream to the enhancer, downstream from the CpG island, and within the

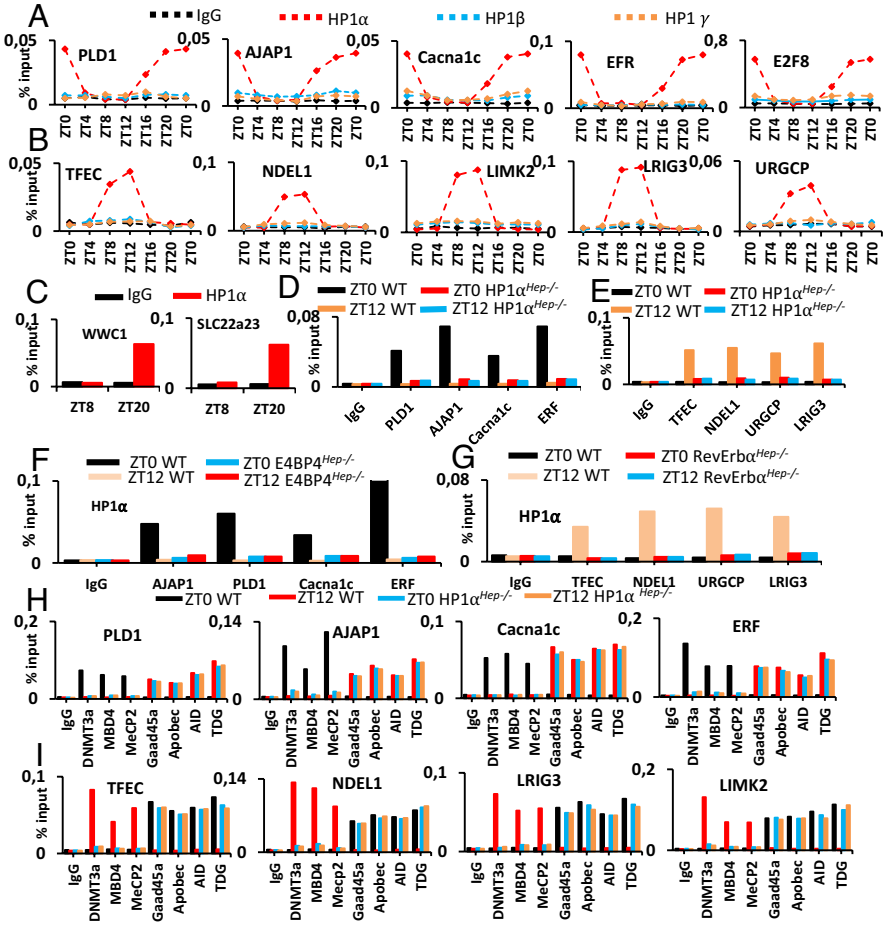

Fig. 2. Recruitment of HP1α is involved in the CC and is mandatory for the circadian DNA methylation of the CpG islands (*A*) qPCR chip assays with WT liver extracts showing the binding of HP1α, but not of HP1β nor HP1γ, on CpG islands of D-box-containing genes (as indicated) at a time when these genes are not transcribed. The X-axis represents the % input for the chip assay, and the Y-axis represents the time points (in ZT) for the experiment (*B*) as under (*A*), but for RORE-containing genes, at a time when these genes are not transcribed. (*C*) qPCR chip assays with WT liver extracts, showing the circadian binding of HP1α to the CpG islands of two E-box-containing genes, as indicated. X-axis represents the % input for the chip assay, and the Y-axis represents the time points (in ZT) for the experiment. (*D* and *E*) qPCR chip assays with liver extracts of HP1α$^{hep-/-}$ mice, showing the loss of recruitment of HP1α on CpG islands of D-Box (*D*) and RORE (*E*)-containing genes, as indicated. (*F*) qPCR chip assays with liver extracts of E4BP4$^{hep-/-}$ mice, showing the lack of HP1α binding on CpG islands of D-box-containing genes, as indicated. (*G*) qPCR chip assays with liver extracts of RevErbα$^{hep-/-}$ mice, showing the lack of HP1α binding on CpG islands of RORE-containing genes, as indicated. (*H* and *I*) qPCR chip assays with liver extracts of HP1α$^{hep-/-}$ mice, showing the loss of binding of DNA methylating enzymes and the constitutive binding of DNA demethylating enzymes on CpG islands of output genes (as indicated) containing either a D-box (*H*) or a RORE (*I*).

proximal-promoter region of D-box (*SI Appendix*, Fig. S3*A*) and RORE genes (*SI Appendix*, Fig. S3*B*), thus indicating a loss in chromatin compaction within the CC output genes.

Taken the above data altogether, we conclude that the lack of the HP1α protein results in a partial loss of both the chromatin compaction and the epigenetic silencing of the CC output genes, thus leading to their partially constitutive expression.

The Circadian Recruitment of HP1α Is Also Crucial for the Epigenetic Silencing of the Core Clock Genes and Their E4BP4-Immediate Output Gene. HP1α was recruited on enhancers and proximal-promoter regions of both the core clock genes and the E4BP4-immediate output gene (Fig. 3*E*). In both cases, these Hp1α recruitments on D-box (PER1/2) and RORE (BMAL1, RevErbα, and E4BP4) enhancers preceded their recruitment on the proximal-promoter region (Fig. 3*E*). Both the core clock and immediate CC output genes were constitutively expressed in HP1α$^{hep-/-}$ mice (*SI Appendix*, Fig. S3*C*). Importantly, in marked contrast to that of Hp1α, the in vivo hepatocyte-specific deletion of either HP1β or HP1γ did not affect the circadian expression of both the core clock and its immediate output genes (*SI Appendix*, Fig. S3*D*), thus demonstrating that the circadian recruitment of Hp1α is mandatory for the epigenetic silencing of the core clock and its immediate output genes. Chip assays performed with liver extracts of HP1α$^{hep-/-}$ mice similarly revealed, for both the core clock and its immediate CC output genes, a constitutive YY1 binding to cognate sites located upstream to the enhancers and within the proximal-promoter regions (Fig. 3*H*), in keeping with the constitutive lower level of expression of the core clock and its immediate output genes upon mutation of HP1α, but not of HP1β nor HP1γ (*SI Appendix*, Fig. S3*D*).

Taken altogether, our data demonstrate that the binding of HP1α is mandatory for the circadian chromatin compaction of the core clock and its immediate output genes. As we have shown that the circadian binding of HP1α is controlled by the clock repressors RevErbα and E4BP4, it appears that within the circadian repression cascade, the recruitment of Hp1α is downstream from the bindings of these "repressors" and "repressive histones." Accordingly, we found that the in vivo selective ablation of HP1α in mouse hepatocytes results in a partially constitutive expression of both the core clock and the CC output genes. Thus, even though the clock repressors RevErbα and E4BP4 are enhancer-bound during the phase of gene repression, the circadian binding of HP1α is mandatory for both the "full" chromatin

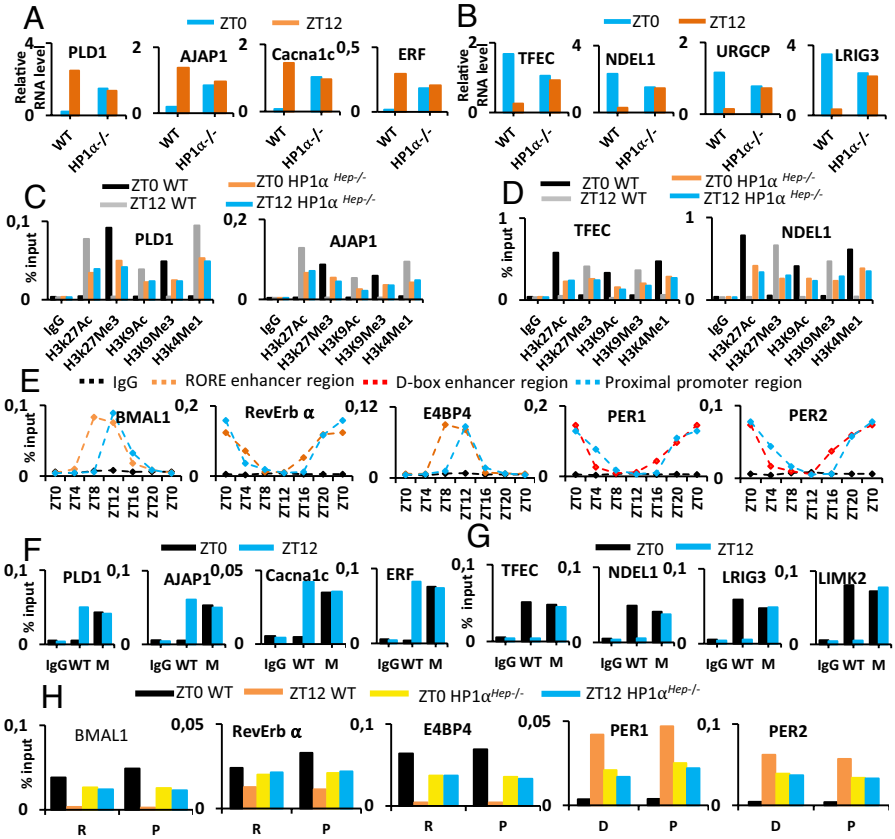

Fig. 3. Recruitment of HP1α is mandatory for both the epigenetic silencing of the core clock and its CC output genes. (*A* and *B*) qPCR mRNA transcript analysis showing the constitutive expression of D-box (*A*) and RORE (*B*)-containing genes, as indicated, in liver extracts of HP1α$^{hep-/-}$ mice. The X-axis represents the relative RNA level, and the Y-axis represents the time points (in ZT) for the experiment (*C* and *D*) qPCR chip assays with liver extracts of HP1α$^{hep-/-}$ mice, showing the presence of active H3K27Ac, H3K9Ac, and H3K4Me1, and repressive H3K27Me3 and H3K9Me3 histones modifications on CpG islands of D-box (*C*) and RORE-containing genes (*D*), as indicated. The X-axis represents the % input for the chip assay. (*E*) qPCR chip assays with WT liver extracts, showing the circadian binding of HP1α on the proximal-promoter (blue), D-box DBS (red), and RORE DBS (orange) of the core clock genes and E4BP4-immediate output gene, as indicated. The X-axis represents the % input for the chip assay, and the Y-axis represents the time points (in ZT) for the experiment. (*F* and *G*) qPCR chip assays on liver extracts of HP1α$^{hep-/-}$ mice, showing at ZT0 and ZT12 the constitutive recruitment of the YY1 protein on its cognate sites located near the CpG islands of D-box (*F*) and RORE (*G*)-containing genes. Wild type and mutants are represented by WT and M, respectively. The X-axis represents the % input for the chip assay. (*H*) qPCR chip assays on liver extracts of HP1α$^{hep-/-}$ mice, showing the constitutive recruitment of the YY1 protein on its cognate sites located near the D-box (D) DBS, the RORE (R) DBS and to the promoter regions (P) of core clock and immediate output genes. The X-axis represents the % input for the chip assay.

compaction and the epigenetic gene silencing. We also demonstrate that the circadian binding of HP1α is mandatory for the recruitment of the DNA methylating enzymes on the deoxyCpG islands of CC output genes (Fig. 4 *A* and *B*). As upon HP1α ablation, the circadian DNA methylation is impaired, which leads to the constitutive binding of DNA demethylating enzymes on deoxyCpG islands, we conclude that, among the CC output genes, HP1α plays a crucial role in the circadian DNA methylation of the deoxyCpG islands (Fig. 4 *A* and *B*).

A Circadian Biomolecular Repressive Condensate, Instrumental in the Transcriptional Repression of the CC Output Genes, Is Generated upon HP1α Binding and Methylation of the DeoxyCpG Islands. During the "transcriptional repression phase" of the circadian genes, a transrepressor (either RevErbα or E4BP4) is recruited on the deoxyCpG-rich islands (Fig. 1 *A* and *B*), in association with HDAC3 and the histone methyltransferases Suv39H1 and EZH2, both of which known to be present on cognate repressed enhancers (Figs. 1 *C*–*G* and 4 *A* and *B* and *SI Appendix*, Fig. S4*B*). These recruitments lead to the formation of a "repressed chromatin" upon conversion of the active acetylated H3K9Ac and H3K27Ac histones to repressive tri-methylated H3K9Me3 and H3K27Me3 histones (Fig. 1 *H*–*L* and *SI Appendix*, Fig. S4 *A* and *B*). HP1α is then recruited by the repressive H3K9Me3 histone (Fig. 2 *A* and *B*), which generates both an heterochromatin structure (Fig. 3 *C* and *D*) and a "hydrophobic condensate" within the intronic deoxyCpG island. In addition, HP1α controls the methylation of these intronic deoxyCpG-rich islands through the recruitment of the DNA methylating enzyme DNMT3a which, together with the proteins MBD4 and MeCP2, stabilizes the deoxyCpG methylation within the intronic island (Fig. 2 *H* and *I* and *SI Appendix*, Fig. S4*A*). It appears therefore that the repressive methylated histones and HP1α, together with DNA methylation of the intronic deoxyCpG-rich islands, generate in vivo a "repressive biomolecular hydrophobic condensate" similar to that described in vitro by Li et al. (5). We conclude that, upon binding of a transrepressor (either RevErbα or E4BP4) to the enhancer, a single transcriptionally repressing hydrophobic condensate is assembled within the intronic methylated deoxyCpG island. This "condensate," which includes the methylated histones, HP1α, DNA methylating enzymes, and methylated deoxyCpGs (*SI Appendix*, Fig. S4*A*), is enriched in RevErbα or the E4BP4 transrepressors, HDAC3, and in two histone methyltransferases (Suv39H1 and EZH2), all of which being assembled on RevErbα or E4BP4 repressed enhancers, and subsequently associated with the deoxyCpG islands (*SI Appendix*, Fig. S4*B*).

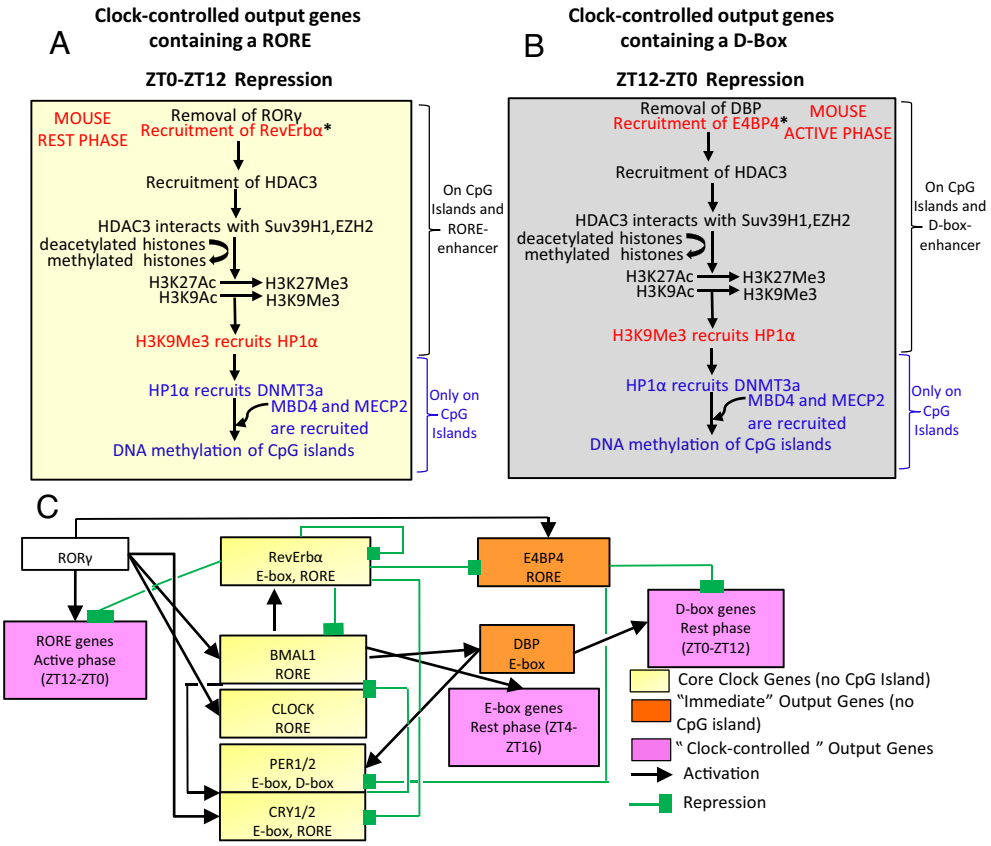

Fig. 4. (*A*) Schematic representation showing, in the liver, the mechanism through which the DNA methylation of CpG islands controls the circadian repression of RORE-containing output genes. The asterisk * indicates a non-specific binding on CpG islands. (*B*) Schematic representation showing, in the liver, the mechanism through which the DNA methylation of CpG islands controls the circadian repression of D-box-containing output genes. The *, as under *A*, indicates a non-specific binding on CpG islands. (*C*) Schematic representation of the CC. The core clock genes are shown on a yellow background, the "immediate" output genes (DBP and E4BP4) are on an orange background, and the clock-controlled output genes (D-box, RORE, and BMAL1-dependent E-box genes) are on a pink background. Black arrows indicate "activation" and green bars "repression" of transcription.

In conclusion, it appears that, during the "repressed phase" of the circadian cycle, a "circadian repressive biomolecular condensate" is involved in the transcriptional repression of the CC-controlled genes, through modulation of the local concentrations of the repressing components RevErbα and E4BP4, which are involved as transrepressors within the enhancer region (*SI Appendix*, Fig. S4 *A* and *B*). Upon the appearance of both transrepressors, methylated histones, and HP1α, the intronic deoxyCpG islands are methylated for the next 12-h period during which the transcription of the CC-controlled output genes is repressed by the RevErbα and E4BP4 transrepressors (Fig. 4 *A* and *B* and *SI Appendix*, Fig. S4 *A* and *B*). At this stage, the methylated deoxyCpG islands harbor repressive components (e.g., transrepressors, HDAC3, Suv39H1, EZH2, H3K9Me3, H3K27Me3, HP1α, DNA methylating enzymes, and methylated deoxyCpGs) assembled under the control of the RevErbα and E4BP4 transrepressors, thus leading to the formation of a repressive biomolecular hydrophobic condensate which disappears during the last 4 h of the repression period.

Taken altogether, our present data reveal that upon the circadian binding of the RevErbα and E4BP4 transrepressors to their cognate binding sites during the circadian phase of gene repression, the methylation of the intronic deoxyCpG islands boosts the transcriptional repression of the CC output genes. As it is known that upon aging, there is an increase in methylation of the deoxyCpG island (23, 24) which is directly controlled by HP1α (Fig. 2 *H* and *I*), it appears that any physiopathological increase in HP1α may result in an increased deoxyCpG methylation of the CC output genes, which could lead to an impaired circadian transcription of these genes.

We suggest that, during the circadian phase of gene transcription, such an above pathophysiological increase in deoxyCpG methylation could be selectively prevented by inhibiting the binding of the HP1α protein to the chromatin histones through the administration of an inhibitor of HP1α methylation conjugated with an antibody directed against the circadian transactivator, either retinoic acid receptor-related orphan receptor gamma (RORγ) or DBP.

Materials and Methods

Reagents and Resources. E4BP4 (Santacruz sc-9549X), RevErbα (Abcam ab115552), HDAC3 (Abcam ab7030), Suv39h1 (Active motif 39785), EZH2 [Diagenode C15410039(pAb-039-050)], HP1α [Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) (In house) HP-1H5], HP1β [IGBMC (In house) HP-1A9], HP1γ [IGBMC (In house) HP-1G6], growth arrest and DNA-damage-inducible 45 alpha protein (GADD45α) (Santacruz sc-792X], activation-induced cytidine deaminase (AID) (Santacruz sc-25620X), thymine DNA glycosylase alpha (TDGα) (Active Motif 61437), DNMT3a (Abcam ab13888), MDB4 (Santacruz sc-398249X), MECP2 (Abcam ab2828), H3K9Ac (Abcam ab4441), H3K9Me3 (Abcam ab8898), YY1 (Abcam ab38422), H3K4Me1 (Active motif 39297), H3K27Ac (Active motif 39133), and H3K27Me3 (Active motif 39155).

Assay Kits. GeneJET Genomic DNA Purification Kit (Fermentas K0721), ExiLERATE LNA qPCR cDNA Synthesis Kit (Exiqon 303301), SYBR Green Mix (Qiagen 204143), Superscript II cDNA Synthesis Kit (Invitrogen 18064-014), GoTaq Hot Start Polymerase (Promega M5005), and Taq DNA Polymerase (Roche 13929320).

Mice Models.

Mice. Eight- to 12-wk-old C57BL6/J male wild-type mice (Charles River Laboratories) were used for experimental purpose. Mice were provided food and water ad libitum, under 12-h light (6 AM to 6 PM; ZT0-ZT12) and 12-h dark (6 PM to 6 AM; ZT12-ZT0) conditions. Hepatocyte-specific ablation of HP1α (HP1$\alpha^{hep-/-}$), HP1β (HP1$\beta^{hep-/-}$), HP1γ (HP1$\gamma^{hep-/-}$), RevErbα (RevErb$\alpha^{hep-/-}$), RORα/RORγ (RORα/RORγ$^{hep-/-}$), and E4BP4 (E4BP4$^{hep-/-}$) was generated by crossing "floxed" female mice with albumin-CreERT^2 floxed male mice (25), and subsequent tamoxifen injections were given for 5 d. All floxed mice were generated and maintained in IGBMC/Institut Clinique de la Souris (ICS). Genotyping was performed by PCR on genomic DNA isolated from mouse tails. All experiments were performed under light–dark (L/D) conditions, with ZT0 being the start of the light period (6 AM) and ZT12 the start of the dark period (6 PM). Mice were sacrificed at 4 h interval starting at ZT0 and fed a "normal" laboratory chow diet. Breeding, maintenance, and experimental manipulations were approved by the Animal Care and Use Committee of IGBMC/ICS.

Mutant mice strains. Mouse mutant strains were generated at our Mouse Clinical Institute (ICS) from C57BL/6J mice (Charles River Laboratories). Mice: Alb-CreERT^2/E4BP4$^{hep-/-}$ (ICS); Alb-CreERT^2/RevErbα$^{hep-/-}$ (ICS); Alb-CreERT^2/RORα/RORγ$^{hep-/-}$ (ICS); Alb-CreERT^2/HP1α$^{hep-/-}$ (ICS); Alb-CreERT^2/HP1β$^{hep-/-}$ (ICS), and Alb-CreERT^2/HP1γ$^{hep-/-}$ (ICS).

Chemicals, peptides, and recombinant proteins. TRI reagent (MRC TR118), Tamoxifen (Sigma T5648), bovine serum albumin (BSA) (MP Bio 160069), Sepharose-G beads (Sigma P3296), Complete Protease inhibitor (Roche 11873580001), and Dyna beads (Invitrogen 112.01D).

List of "oligonucleotide primers" used in the present study. See *SI Appendix*, Table S1 for CHIP, mRNA qPCR, and eRNA qPCR primers.

Methods Details.

mRNA transcript determination. Freshly liver samples isolated at the indicated circadian time points (ZT) were used for total RNA isolation using TRI reagent (Molecular Research Center). The RNA quality was checked by gel electrophoresis and spectrophotometric analysis (280/260 nm). One microgram of total RNA was reverse-transcribed using random hexamers and Superscript II reagents (Invitrogen) as per manufacturer instructions. The synthesized DNA was used for qRT-PCR and amplified using gene-specific forward and reverse primers (from two adjacent exons) with SYBR green (QIAGEN) and expressed relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA levels, as previously described (26). At each time point, three mice were sacrificed for RNA transcript determination, and all experiments were replicated at least three times.

Chromatin immunoprecipitation (ChIP) assays and qPCR analyses. ChIP was performed as reported in ref. 26. Briefly, 30 μg of liver or 30 μg of distal ileum was homogenized with a Dounce homogenizer in 10 mL phosphate-buffered saline (PBS). Samples were crosslinked with 1% formaldehyde for 15 min at room temperature. Crosslinking was stopped by addition of 2 M Glycine (to reach a 0.125 M final concentration) at room temperature for 5 min. Cells were pelleted followed by resuspension on ice in 500 μL of lysis buffer (50 mM Tris-HCl, pH 8.1, 1% (w/v) Sodium Dodecyl Sulfate (SDS), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100) in the presence of protease inhibitors cocktail (Sigma). Following cell lysis, samples were sonicated (Bioruptor, Diagenode) to generate fragments containing an average length of 200 to 500 base pairs of DNA. Cellular debris was removed by centrifugation at 4 °C for 10 min (10,000 g), and the supernatant was precleared with Protein A/G-Sepharose beads (Roche) which have been preblocked with salmon sperm DNA and BSA for 1 h at 4 °C. The beads were then pelleted (100 g for 1 min) and discarded. Ten percentage of the supernatant lysate was stored from each sample as the "Input" source. The remaining supernatant lysate was diluted 8 times in chip dilution buffer [16.7 mM Tris-HCl, pH 8.1, 0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, 50 mM protease inhibitor cocktail (Sigma)], and incubated overnight at 4 °C on a flip-flop rocker in the presence of cognate primary antibodies (see methods). Seventy microliters of Protein A/G-Sepharose beads (preblocked with salmon sperm DNA and BSA) were then added for 90 min at 4 °C. Immunoprecipitated complexes attached to the beads were then recovered by centrifugation at 500 g for 1 min, washed at 4 °C in a low salt buffer [20 mM Tris-HCl, pH 8.1, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 150 mM NaCl], followed by washes in a high salt buffer [20 mM Tris-HCl, pH 8.1, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 500 mM NaCl], in a LiCl buffer [10 mM Tris-HCl, pH 8.1, 250 mM LiCl, 1% (v/v) Nonidet P-40 (NP-40), 1% (w/v) sodium deoxycholate, 1 mM EDTA], and finally twice in 1 mL of Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The "beads bound chromatin" was then released by intermittent vortexing (2 × 30 min) at room temperature in 2 × 150 μL of elution buffer (1% w/v SDS and 100 mM $NaHCO_3$). One microliter of 10 mg/mL RNase A and 5 M NaCl (to reach a 200 mM final concentration) were added to the eluate and incubated overnight at 65 °C. One microliter of Proteinase K (200 μg/mL) was added for 1 h at 55 °C. The released DNA was finally purified using the QIAGEN PCR purification kit to a final volume of 50 μL and used for Q-PCR analysis with the SYBR green reagent (QIAGEN). PCR cycles were within the linear range of amplification. For each chip assay and qPCR analyses, three mice were sacrificed at each time point and each experiment was performed in triplicate.

eRNA transcript determination. Freshly liver samples, isolated at indicated circadian time points (ZT), were used for total RNA isolation using TRI reagent (Molecular Research Center). The quality of the purified RNA was checked by spectrophotometric analysis (280/260 nm) and gel electrophoresis. Two hundred nanograms of total RNA was reverse-transcribed using strand-specific primers for RORE, D-box enhancer regions or CpG islands of output genes, and a reverse transcriptase (cDNA enzyme from ExiLERATE LNA™ qPCR, cDNA Synthesis Kit) as per manufacturer instructions. The synthesized cDNA was used for qRT-PCR with SYBR green (Qiagen) and expressed relative to GAPDH levels, as described in ref. 26. For eRNA transcript determination, three mice were sacrificed at each time point and each experiment was performed in triplicates.

BMAL1 ChIP-seq and RNA-seq data analysis. For identification of CpG islands that undergo circadian methylation/demethylation in BMAL1-dependent "E-box" genes, the BMAL1 ChIP-seq peaks were downloaded from *SI Appendix*, Table S1 of Beytebiere et al. (27) (Accession No.: GSE110604) and converted to mm9 using University of California, Santa Cruz (UCSC) liftover tool. RNA expression values were downloaded from Gene Expression Omnibus (GEO) session GSE54651, file: GSE54651_Liv_quant.txt. ChIP-seq and RNA-seq data were fused with the Ensemble Gene ID and analyzed against the MeDIP-seq: Methylated DNA immunoprecipitation sequencing data from the accompanying paper entitled "The circadian demethylation of a unique intronic deoxymethylCpG-rich island boosts the transcription of its cognate circadian clock output gene" to identify the list of BMAL1-dependent E-box genes that show concomitantly BMAL1 binding on the E-box, circadian expression of mRNA transcript, and demethylation of CpG islands.

Identification of YY1 motif in core clock and CC-output genes. Find Individual Motif Occurrences (FIMO) (28) was used for searching the YY1 motif (open-access database (JASPAR), MA0095.2) in the mouse genome (mm9) with default parameters set at a *P* value cutoff of 1e5.

Data, Materials, and Software Availability. MeDIP-seq deposited data from liver and Ileum samples of WT mouse are available under GEO Accession no: GSE182147. All study data are included in the article and/or *SI Appendix*.

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