

The Polycomb Group Protein EZH2 Is Required for Mammalian Circadian Clock Function^{*S}†

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We examined the importance of histone methylation by the polycomb group proteins in the mouse circadian clock mechanism. Endogenous EZH2, a polycomb group enzyme that methylates lysine 27 on histone H3, co-immunoprecipitates with CLOCK and BMAL1 throughout the circadian cycle in liver nuclear extracts. Chromatin immunoprecipitation revealed EZH2 binding and di- and trimethylation of H3K27 on both the *Period 1* and *Period 2* promoters. A role of EZH2 in cryptochrome-mediated transcriptional repression of the clockwork was supported by overexpression and RNA interference studies. Serum-induced circadian rhythms in NIH 3T3 cells in culture were disrupted by transfection of an RNA interfering sequence targeting EZH2. These results indicate that EZH2 is important for the maintenance of circadian rhythms and extend the activity of the polycomb group proteins to the core clockwork mechanism of mammals.

The circadian clock mechanism in the mouse is driven by interacting positive and negative transcriptional feedback loops (1, 2). The negative feedback loop is essential for clockwork function and involves CLOCK·BMAL1 enhanced expression of three *Period* genes (*mPer 1–3*) and two *Cryptochrome* genes (*mCry1* and *mCry2*) (3–5). Negative feedback is provided by a CRY·PER complex that rhythmically enters the nucleus to inhibit CLOCK·BMAL1-mediated transcription via a mechanism that does not substantially alter CLOCK·BMAL1 binding to *mPer1* and *mPer2* promoters (3, 6). Other clock-controlled genes may be regulated by other mechanisms,

as CLOCK·BMAL1 binds rhythmically to the promoter of the *Dbp* gene (7).

The positive transcriptional feedback loop involves the regulation of *Bmal1* transcription by CLOCK·BMAL1-mediated transcription of the nuclear orphan receptor genes *Rev-erba* and *Rora* (8–11). The orphan receptor gene products act on the *Bmal1* promoter to generate a circadian rhythm in *Bmal1* RNA levels that is antiphase to the *mPer* and *mCry* RNA rhythms. The positive feedback loop appears to provide stability to the core clock mechanism (12).

Changes in chromatin structure due to post-translational modifications of histones are required for transcriptional regulation of gene expression (13, 14), and circadian genes are no exception (7, 15–18). Previously, we showed in the liver clock that the promoter regions of *mPer1* and *mPer2* undergo rhythmic acetylation of histone H3 that correlates with their transcriptional activation (16). We proposed that at the time of transcriptional inhibition the mCRY proteins disrupt a CLOCK·BMAL1-coactivator complex thereby reducing histone acetyltransferase activity. As histone deacetylase activity is constantly associated with the CLOCK·BMAL1 nuclear complex, the balance between acetylation and deacetylation of H3 on circadian promoters appears to be regulated by the rhythmic regulation of histone acetyltransferase activity, with deacetylation predominating during transcriptional repression. Other groups have also reported H3 acetylation rhythms at circadian promoters (17, 18).

Our search for other chromatin remodeling activities involved in clockwork function has focused on mechanisms that would link deacetylase activity with other chromatin remodeling events involved in transcriptional inhibition by the mCRY proteins. One such link could be provided by the polycomb group (PcG)⁵ proteins, which elicit chromatin-mediated transcriptional repression by both the deacetylation and methylation of H3 (19–21).

PcG proteins were first described in *Drosophila* where they maintain transcriptional repression of Hox/homeotic genes in a stable, heritable manner throughout development. As multi-protein complexes, the PcG proteins of flies and mammals can

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⁵ The abbreviations used are: PcG, polycomb group proteins; PRC, polycomb repressive complex; EZH2, enhancer of zeste; mEZH2, mouse EZH2; hEZH2, human EZH2; H3K27, histone H3 lysine 27; qPCR, quantitative polymerase chain reaction; RNAi, RNA interference; siRNA, small interfering RNA; shRNA, short hairpin RNA; ChIP, chromatin immunoprecipitation; CT, circadian time; pol II, RNA polymerase II; luc, luciferase.

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be divided into at least two distinct groups, the Pc-repressive complex 1 (PRC1) and PRC2 (22). The current model of transcriptional repression by PcG proteins proposes that the binding of the PRC2 to responsive promoters initiates histone deacetylation and the methylation of lysine 27 in H3 (H3K27). H3K27 di- and trimethylation then recruit the PRC1 that represses gene expression by inhibiting transcriptional initiation (23).

We have focused our clockwork studies on the PRC2, which in mammals is comprised of the histone methyltransferase EZH2 (enhancer of zeste), SUZ12 (suppressor of zeste), EED (extra-sex comb), RbAp48, a histone-binding protein shown to be associated with histone deacetylase 1 (24), and other proteins (22). The *mEzh2*, *mSuz12*, and *mEed* genes are essential for mouse development, as the deletion of each causes embryonic lethality (25–28). The EZH2-containing PRC2 of mammals is involved in X-chromosome inactivation, genomic imprinting during germ line development, the regulation of stem cell pluripotency, and the promotion of cancer metastasis (29, 30).

Here we investigate the role of mEZH2 in the molecular circadian clock mechanism. We found that mEZH2 is a component of the CLOCK·BMAL1 complex *in vivo*, and luciferase reporter assays show that mEZH2 augments transcriptional repression mediated by the mCRY proteins. Furthermore, circadian rhythms in mouse fibroblasts in culture are disrupted by RNA interference (RNAi) knockdown of mEZH2.

EXPERIMENTAL PROCEDURES

Animals and Collections of Liver Tissue—BALB/c mice were obtained from the Charles River Laboratories and housed in a 12 h light/12 h dark cycle for at least 10 days prior to tissue collections. At selected times on the first cycle in constant darkness, animals were killed and livers were collected. Nuclei were purified by sucrose gradient centrifugation immediately after collection and stored at -80°C , as described previously (3, 16).

Immunoprecipitation Assays—Immunoprecipitation was performed on nuclear extracts using the anti-CLOCK (anti-CLK-1-GP), anti-BMAL1 (anti-BM1-2-GP), or anti-EZH2 (Upstate Biotechnologies) antibodies, as described (3). The immunoprecipitates were Western blotted and probed with the following antibodies: anti-CLOCK, anti-BMAL1, anti-EZH2, anti-PER1 (PER1-1-GP) (3), anti-PER2 (PER2-1-GP) (3), anti-CRY1 (anti-C1-GP) (31), and anti-CRY2 (anti-C2-GP) (31).

Chromatin Immunoprecipitations—Formaldehyde-cross-linked nuclei were incubated with antibodies against RNA polymerase II (pol II) (Covance), EZH2, or anti-dimethyl-H3K27 or trimethyl-H3K27 (32), as described previously (16). DNA was isolated from the immunoprecipitates and subjected to quantitative polymerase chain reaction (qPCR) using primer pairs directed to the promoter regions of *mPer1* or *mPer2*, as described (16). Each qPCR was performed using the ABI-PRISM 7000 Sequence Detection System (Applied Biosystems) with a SyberGreen Master Mix (Qiagen). To ensure specific amplification, every run was followed by a dissociation phase analysis (7000 SDS, Applied Biosystems). The data were normalized to input control, which consisted of PCR reactions from cross-linked chromatin before immunoprecipitation.

RNA Interference Assays—Small interfering RNA (siRNA) oligonucleotides targeting *mEzh2* were transfected into NIH 3T3 cells using Lipofectamine 2000 (Invitrogen). mEZH2 protein expression in the presence or absence of siRNA oligonucleotides was visualized by Western blot analysis using anti-EZH2 antibody. The percentage of siRNA knockdown was determined by normalizing the levels of mEZH2 to Tubulin (Sigma).

Short hairpin RNA (shRNA) constructs targeting *mEzh2* were cloned into the lentivirus vector, and pLL3.7. Lentiviruses carrying *mEzh2*-shRNA constructs were made in HEK 293T cells and purified by Millipore filtration (0.45 μm) followed by ultracentrifugation as described by Rubinson and colleagues (33). NIH 3T3 cells were infected with these *mEzh2*-shRNA lentiviruses and after 48 h, cells were subjected to formaldehyde cross-linking as described by Cao and Zhang (34). Chromatin immunoprecipitations (ChIP) using anti-EZH2 or anti-trimethyl-H3K27 were performed as described above. ChIP DNA, on the *mPer1* promoter, was detected by qPCR as described above.

Transcriptional Assays—Luciferase reporter gene assays were performed in HeLa or NIH 3T3 cells, as described previously (35), using either the *mPer1* reporter (4) or the *mPer2* reporter (36).

Real-time Bioluminescence Assays—NIH 3T3 cells were cotransfected with *mPer2-luc* or *Bmal1-luc* reporter plasmid (0.3–0.4 μg) and with shRNA plasmids (0.6–0.7 μg) using Lipofectamine (Invitrogen). The *mPer2-luc* reporter construct was generated by cloning the 5' region from -1.8 to $+0.1$ kb of the *mPer2* promoter into the pGL3 R2.1 plasmid (Promega), which expresses a destabilized form of luciferase. The region from -318 to $+5$ of *mBmal1* was subcloned into the pGL3 R2.1 plasmid to generate the *Bmal1-luc* reporter. Oligonucleotides encoding shRNA sequences were cloned into the pLL3.7 expression plasmid (kindly provided by Luk Van Parijs, MIT) (33).

Twenty-four hours after transfection, cells were synchronized using serum shock (37). The medium was then replaced with serum-free medium containing 0.1 mM luciferin. Luciferase activity was recorded in real time by counting each culture for 1 min, at 15-min intervals, using a photomultiplier tube assembly (LM2400, Hamamatsu) that was housed within an incubator maintained at 37°C with 95% air/5% CO_2 .

RESULTS AND DISCUSSION

mEZH2 Is a Component of the CLOCK·BMAL1 Complex *in Vivo*—Immunoprecipitation studies revealed that mEZH2 is present within clock-relevant protein complexes throughout the circadian cycle (Fig. 1). mEZH2 interaction with clock proteins was examined by incubating liver nuclear extracts with CLOCK, BMAL1, or EZH2 antisera and probing the immune complexes for interactions by Western blot analysis. These studies focused on circadian times (CT) before (CT 06 and 09) and during (CT 15 and 18) the expected peaks in negative feedback of the circadian clockwork in the liver (3).

Immunoprecipitated CLOCK was most abundant at CT 18 (Fig. 1A), as described previously (3). BMAL1 and mEZH2 copurified with CLOCK at all times examined. The negative cir-

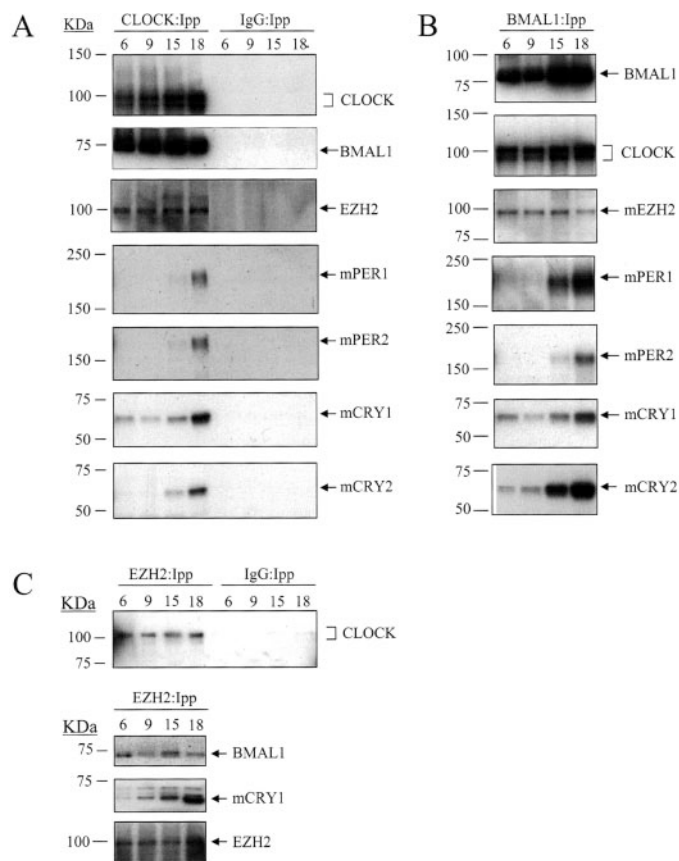


FIGURE 1. mEZH2 co-precipitates with CLOCK and BMAL1 in vivo. Liver extracts from CT 06, 09, 15, and 18 were immunoprecipitated (1pp) with antibodies against CLOCK or guinea pig IgG (A), BMAL1 (B), and EZH2 or rabbit IgG (C). Immune complexes generated by each antibody were Western blotted and probed for the indicated proteins. Each time point represents liver nuclei from six mice.

circadian clock regulators mPER1, mPER2, mCRY1, and mCRY2 co-purified with CLOCK chiefly at CT 18, consistent with the roles of the mPER and mCRY proteins in negative transcriptional regulation (3).

Immunoprecipitated BMAL1 was also most abundant in the nucleus at CT 15 and 18 and co-purified the other proteins examined, including mEZH2, in a pattern similar to that found in CLOCK immunoprecipitates (Fig. 1B). mEZH2 co-precipitated CLOCK and BMAL1 at the four times examined (Fig. 1C), while mEZH2 co-precipitated mCRY1 at CT 15 and 18. mEZH2 association with CLOCK or BMAL1 did not exhibit a prominent circadian variation, as its association was relatively constant over the circadian cycle, suggesting a non-oscillatory pattern of H3K27 methylation on CLOCK·BMAL1-regulated promoters.

Recruitment of mEZH2 and the Methylation of H3K27 at the *mPer1* and *mPer2* Promoters—We next determined whether mEZH2 is bound to the *mPer1* and *mPer2* promoters and whether the binding correlates with the methylation of H3K27, as mEZH2 primarily methylates H3 at lysine 27 (22, 23). This was examined in liver nuclei using formaldehyde-cross-linked ChIP, followed by qPCR analysis of the chromatin complexes. The *mPer1* and *mPer2* promoters were evaluated because their protein products are rate-limiting for negative feedback and for perpetuating the clockwork oscillation (3).

As a positive control, we found rhythmic binding of pol II to each promoter (Fig. 2A), which is synchronous with the circadian rhythm in steady state RNA levels of *mPer1* and *mPer2*, as described previously (16). mEZH2 binding to each promoter did not vary over time ($p > 0.05$, one-way analysis of variance). This is consistent with the co-precipitation of mEZH2 with the CLOCK·BMAL1 complex throughout the circadian cycle (see Fig. 1). We then examined the di- and trimethylation patterns of H3K27 on the *mPer1* and *mPer2* promoters, as both states are associated with repressive histone methylation (32). The di- and trimethylation patterns of H3K27 exhibited a significant, but shallow, circadian variation on the *mPer1* promoter ($p < 0.01$) (Fig. 2A); the methylation state rhythms were synchronous, but antiphase to the rhythm in pol II binding, suggesting involvement of H3K27 methylation in transcriptional repression of the clockwork. There were no significant circadian variations in the di- or trimethylation patterns of H3K27 on the *mPer2* promoter, however. These patterns of di- and trimethylation of H3K27 are consistent with the apparent non-rhythmic promoter recruitment of mEZH2 (Fig. 2A) and the co-precipitation of mEZH2 with CLOCK·BMAL1 (Fig. 1).

To provide a more direct link between mEZH2 and the methylation of H3K27 on clock gene promoters, we analyzed mEZH2 binding to the *mPer1* and *mPer2* promoters in NIH 3T3 cells using RNAi. We first evaluated the ability of lentivirus-mediated expression of three shRNA constructs to reduce mEZH2 expression (supplemental Fig. S1). Two of the shRNA sequences, *mEzh2*-shRNA-A and *mEzh2*-shRNA-B, decreased endogenous mEZH2 levels by 70%, while the third sequence (*mEzh2*-shRNA-C) was without effect (Fig. 2B); similar dose-dependent reduction in mEZH2 was found using siRNA constructs (supplemental Fig. S2). We then showed by ChIP and qPCR that the lentivirus-mediated RNAi knockdown of mEZH2 levels caused a corresponding decrease ($\sim 70\%$; $p < 0.01$) in mEZH2 binding and trimethyl-H3K27 levels at both the *mPer1* and *mPer2* promoters (Fig. 2, C and D). These data are consistent with the notion that mEZH2 bound to the clock gene promoters methylates H3K27.

EZH2 Enhances mCRY-mediated Transcription Repression—As the methylation of H3K27 is associated with transcriptional repression in other systems (22, 24), and the mCRY proteins are potent inhibitors of transcription in the circadian clockwork (39, 40), we investigated whether EZH2 influences mCRY-mediated transcriptional repression. This was evaluated using a luciferase reporter-based system to assay CLOCK·BMAL1-mediated transcription from the *mPer1* or *mPer2* promoters in cultured HeLa cells.

Study of the *mPer1* promoter showed that human EZH2 (hEZH2) overexpression enhanced the dose-dependent transcriptional repression by mCRY1 (Fig. 3A, red bars) using very low concentrations of mCRY1 that at their highest level have a small ($< 20\%$) repressive effect in the absence of hEZH2 (Fig. 3A, lanes 3–6). Furthermore, a mutation in the histone methyltransferase domain of hEZH2 (Δ hEZH2) (41) impaired its ability to enhance mCRY1-mediated transcription repression in cell culture (Fig. 3A, green bars). When mEZH1, a structural homolog of EZH2 that is not a component of the PRC2 (22) was overexpressed in cell culture, it was unable to enhance mCRY1-

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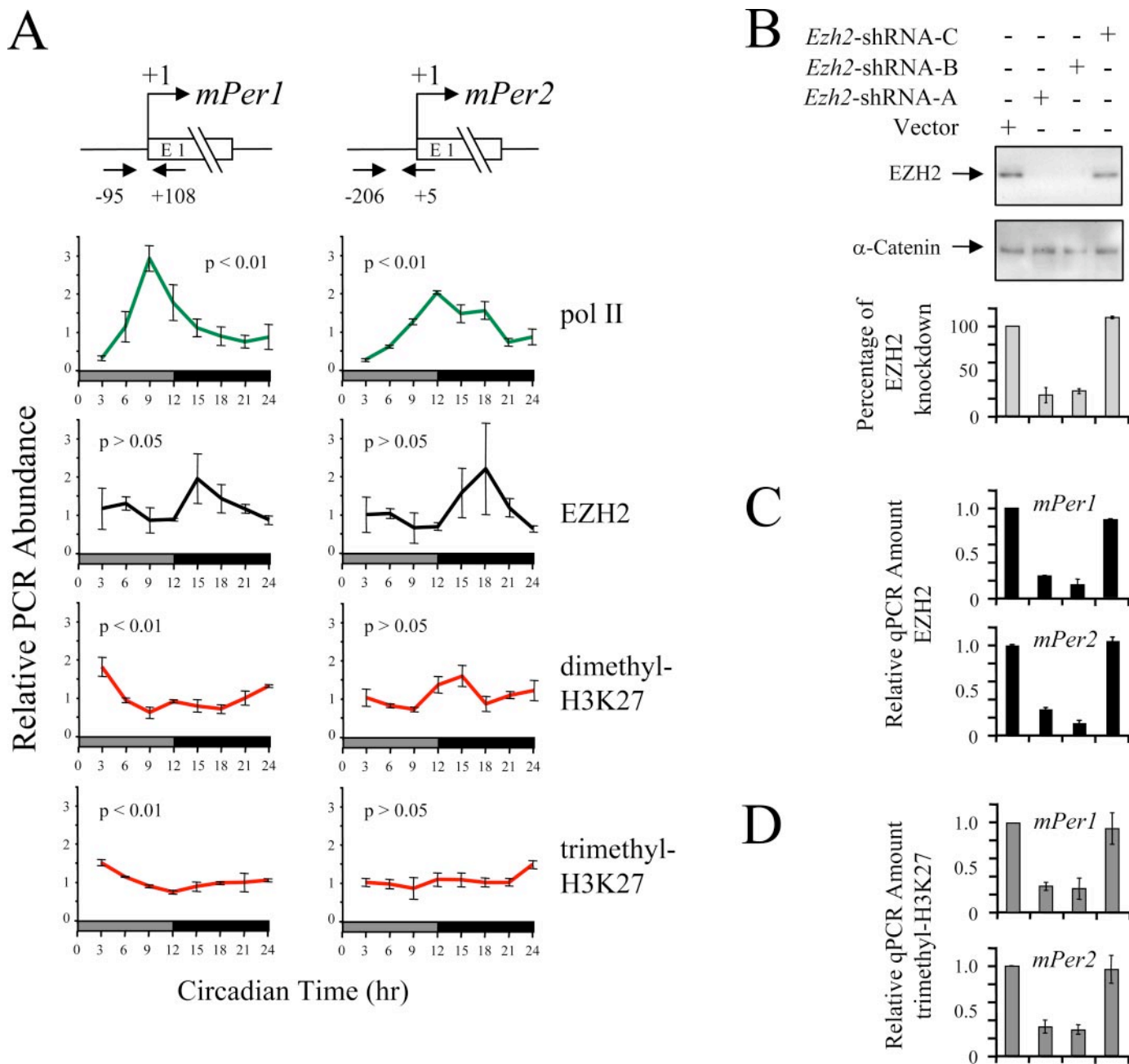


FIGURE 2. mEZH2 is bound to the *mPer1* and *mPer2* promoters and is associated with the di- and trimethylation of H3K27. *A*, *mPer1* and *mPer2* promoter profiles. Nuclei fractions were purified from liver tissues collected at the circadian times indicated and used for ChIP. The *top graph* shows location of primers. The *bottom four graphs* show quantitative PCR values for pol II binding, EZH2 binding, dimethyl-H3K27, and trimethyl-H3K27. The data are plotted relative to the mean value (1.0) for each antibody. Each value is the mean \pm S.E. of three independent experiments. *p* values were calculated using one-way analysis of variance. *B*, RNAi-mediated knockdown of endogenous mEZH2. NIH 3T3 cells were infected with a lentivirus-based construct expressing shRNA sequences targeting *mEzh2*, and protein expression was assessed by Western blot analysis. The graph shows the quantification of *mEzh2* knockdown by each shRNA lentivirus construct. Band intensities were analyzed by densitometry and normalized to those of α -Catenin. Values are mean \pm S.E. of three experiments. The targeted sequences are: *mEzh2*-shRNAi-A, 5'-AAGCACAATGCAACACCAA-3'; *mEzh2*-shRNAi-B, 5'-GCTTTGGACAACAAGCCTT-3'; *mEzh2*-shRNAi-C, 5'-AATGCTCTTGGTCAATATA-3'. *C*, effect of shRNA lentivirus targeting constructs on the recruitment of mEZH2 to the *mPer1* and *mPer2* promoters analyzed by ChIP according to the procedure described by Cao and Zhang (34). *D*, effect of shRNA lentivirus targeting constructs on the trimethylation of H3K27 on the *mPer1* and *mPer2* promoters. ChIP experiments were performed as described for *C*.

mediated transcriptional repression (Fig. 3A, lanes 7–10), showing the specificity of EZH2 enhancement. Similar responses were found in experiments using mCRY2 and the *mPer1* promoter (supplemental Fig. S3A). The mCRY1-mediated transcription repression was also enhanced by hEZH2, but not mEZH1 or Δ hEZH2, in experiments using the *mPer2* promoter (supplemental Fig. S3B). Taken together, these data sup-

port a role for EZH2 in the negative limb of the circadian clock mechanism by augmenting mCRY-mediated transcription repression.

mEZH2 Knockdown Inhibits mCRY-mediated Transcription Repression—We utilized RNAi to determine the effects of reducing endogenous mEZH2 levels on the ability of the mCRY protein to inhibit CLOCK·BMAL1-mediated transcription

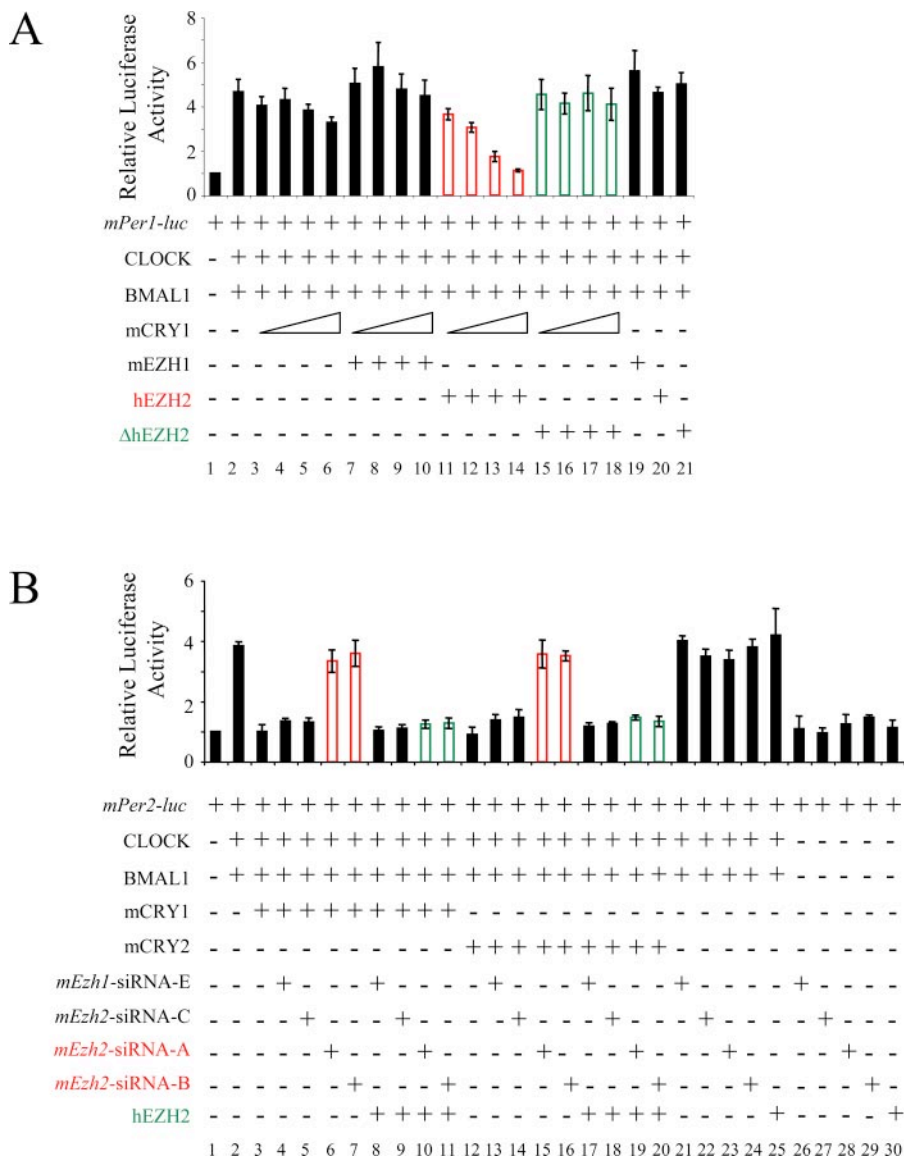


FIGURE 3. mEZH2 is essential for mCRY-mediated transcriptional repression in cell culture. *A*, mEZH2 overexpression. HeLa cells were co-transfected with *mPer1* reporter (25 ng) in the presence (+) or absence (-) of expression plasmids (100 ng of CLOCK and BMAL1; 400 ng of mEZH1, hEZH2, and ΔhEZH2). Increasing concentrations of the mCRY1 plasmid were transfected: 0.25, 0.5, 1.0, and 2.5 ng. Each value is the mean ± S.E. of three replicates. The results shown are representative of three independent experiments. *B*, effect of EZH2-RNAi-mediated knockdown on the expression of *mPer2* reporter. NIH 3T3 cells were co-transfected with *mPer2-luc* reporter (25 ng) in the presence (+) or absence (-) of expression plasmids (100 ng of CLOCK, BMAL1, mCRY1, and mCRY2; 400 ng of hEZH2) and double strand siRNA (500 pmol) targeting *mEzh1* or *mEzh2*. Values are the mean ± S.E. of three independent experiments. A nonspecific siRNA sequence (GFP-siRNA, 5'-GCCA-CAACGUCUAUAUCAUTT-3') was co-transfected as a siRNA-specific control.

from the *mPer2* promoter in NIH 3T3 cells. We used mCRY protein concentrations that inhibited CLOCK·BMAL1-mediated transcription by >80% and targeted endogenous *mEzh2* expression with the three characterized siRNA sequences (supplemental Figs. S1 and S2). The study of the *mPer2* promoter revealed that transient transfection with either of two siRNA constructs targeting *mEzh2* (siRNA-A and -B, at 500 pmol each) completely inhibited the ability of the mCRY proteins to repress transcription (Fig. 3*B*, red bars). The siRNA construct that did not knock down mEZH2 (*mEzh2*-siRNA-C) was ineffective in suppressing mCRY-mediated repression (Fig. 3*B*, lanes 5 and 14).

through their repressive actions, are essential for the maintenance of circadian rhythms both *in vivo* and *in vitro* (4, 5, 39, 40, 42). We thus directly examined the effects of mEZH2 knock down on the oscillatory machinery of the circadian clock by using a real-time circadian gene expression assay in cell culture (43). Molecular oscillations in serum-shocked NIH 3T3 cells were monitored continuously by real-time recording of luciferase activity from either a *mPer2-luciferase* (*luc*) reporter or a *Bmal1-luc* reporter in the presence or absence of shRNAs to disrupt mEZH2. Read-out from these reporters monitors real-time molecular oscillation from the positive (via *Bmal1-luc*) and negative (via *mPer2-luc*) transcriptional feedback loops (1, 2).

The siRNA-induced suppression of mCRY-mediated transcription repression was reversed by co-transfection with a *hEzh2* construct that was insensitive to the transfected siRNAs, as the siRNA sequences used specifically target the *mEzh2* sequence (Fig. 3*B*, green bars, and supplemental Fig. S1). An siRNA sequence targeting *mEzh1* (siRNA-E), which knocked down co-transfected mEZH1 expression by 80% (supplemental Fig. S2*B*), did not alter the ability of the mCRY proteins to inhibit CLOCK·BMAL1-mediated transcription (Fig. 3*B*, lanes 4 and 13).

Importantly, siRNA knockdown of endogenous mEZH2 alone did not inhibit CLOCK·BMAL1-mediated transcription (Fig. 3*B*, lanes 21–24), indicating that these RNAi sequences, on their own, do not have transcriptional repressive activity. In addition, the expression of the mCRY proteins was not different between co-transfections with the *mEzh*-targeting siRNAs (supplemental Fig. S4).

These data indicate that mEZH2 is necessary for mCRY-mediated transcription repression. It also favors the hypothesis that the methylation of H3K27 at the *mPer1* and *mPer2* promoters, mediated by mEZH2, is a chromatin marker for the mCRY proteins to repress transcription.

mEZH2-targeting shRNAs Disrupt Circadian Oscillations in Cultured Mouse Fibroblasts—Up to this point, we have provided data suggesting that the methylation of H3K27 by mEZH2 on the *mPer1* and *mPer2* promoters is necessary for mCRY-mediated transcriptional repression. Moreover, previous studies have shown that the two-mCRY proteins,

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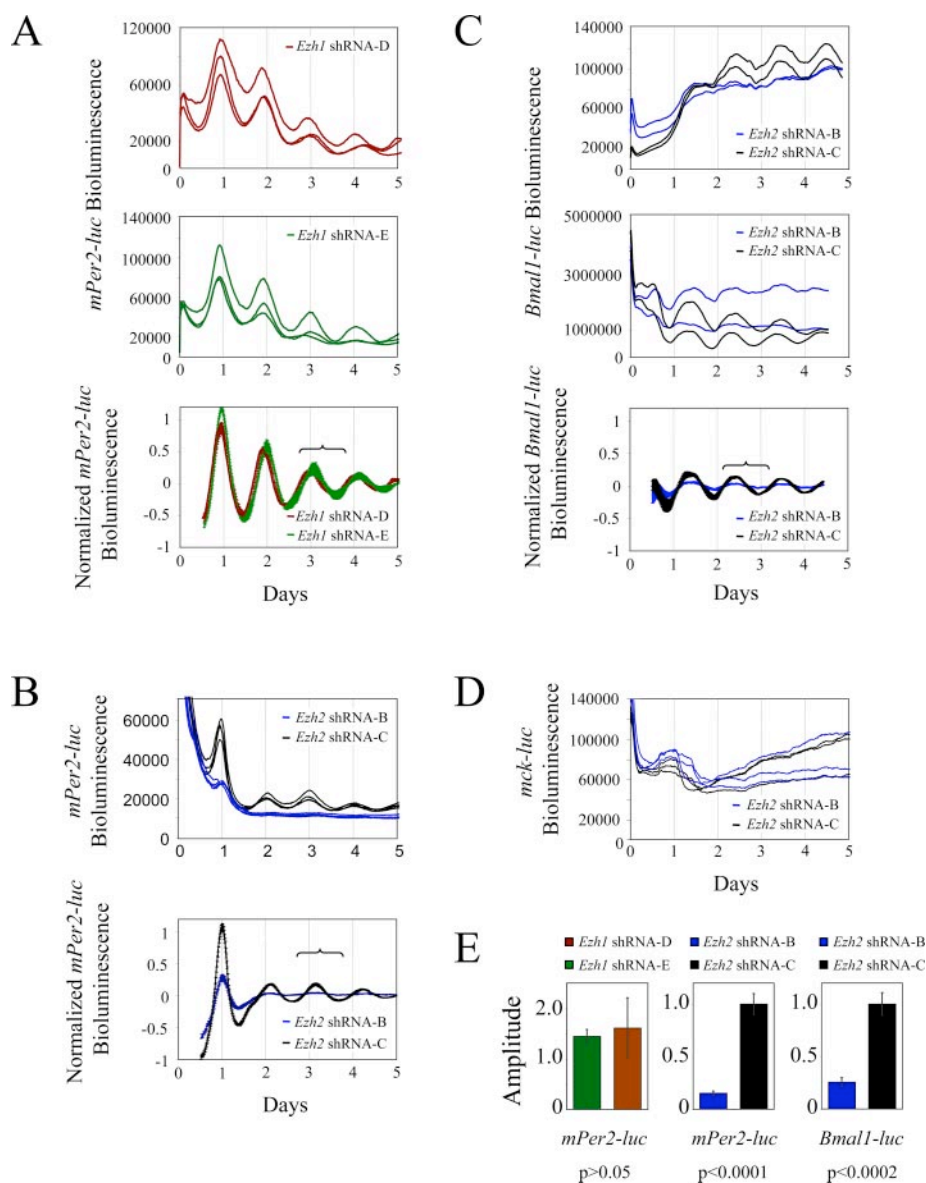


FIGURE 4. mEZH2 knockdown disrupts molecular oscillations in fibroblasts. Luciferase activities of either the *mPer2-luc* or *Bmal1-luc* reporter were recorded in real time following co-transfection with the reporter construct and various shRNA sequences. *A*, the top two graphs show *mPer2-luc* activity after co-transfection of *mEzh1*-shRNAi-D or *mEzh1*-shRNAi-E, respectively. The bottom graph shows the normalized bioluminescence. *B*, the top graph shows the *mPer2-luc* activity after co-transfection of *mEzh2*-shRNAi-B or *mEzh2*-shRNAi-C. The bottom graph shows the normalized bioluminescence. *C*, the top two graphs show the *Bmal1-luc* activity after co-transfection of *mEzh2*-shRNAi-B or *mEzh2*-shRNAi-C. The bottom graph shows the normalized bioluminescence. *D*, *mck-luc* activity after co-transfection of *mEzh2*-shRNAi-B or *mEzh2*-shRNAi-C. *E*, graphical representation of the effect of shRNA targeting *mEzh1* or *mEzh2* on the *mPer2-luc* and *Bmal1-luc* rhythm amplitude. For *A–D*, the data for each culture were normalized to the average luciferase activity over the duration of the recordings and then de-trended by subtracting a centered 24-h moving average. In *E*, each value is the mean \pm S.E. of three to four cultures. At the bottom of *A–C* are the normalized bioluminescence data that were used to calculate the amplitude rhythms shown in *E*. The amplitudes were calculated for a full cycle (defined by bracket width in the corresponding bottom panels shown in *A–C*) that were determined by measuring the absolute area above and below “0” for the third cycle for *mPer2-luc* (days \sim 2.5–3.5; *A* and *B*) or the second cycle for *Bmal1-luc* (days \sim 2–3; *C*) and expressed relative to control (normalized bioluminescence from co-transfection with *mEzh2*-shRNAi-C) from the same experiments. The data are plotted as mean \pm S.E., and the “*p*” values are shown in each graph.

Following a serum pulse that synchronizes the intrinsic circadian oscillators in cultured fibroblasts (37, 42, 44), *mPer2-luc* manifested a robust circadian oscillation in bioluminescence for four cycles that were unaltered by co-transfection of shRNAs targeting *mEzh1* (Fig. 4*A*). However, when *mEzh2*-shRNA-B was co-transfected, which knocks down endogenous mEZH2 (supplemental Fig. S2*A*), there was a marked reduction in the *mPer2-luc* oscillation,

which was damped to the point of arrhythmicity during the last 4 days of study (Fig. 4*B*). By contrast, co-transfection with *mEzh2*-shRNA-C, which does not knock down endogenous mEZH2 (supplemental Fig. S2*A*), did not alter the five-day circadian oscillation in reporter activity (Fig. 4*B*). *Bmal1-luc* rhythmic activity was also unaltered by *mEzh2*-shRNA-C, but co-transfection with *mEzh2*-shRNA-B caused the rhythm to dampen to arrhythmicity (Fig. 4*C*).

To determine whether these *mEzh2*-targeting shRNAs have an overall inhibitory effect on transcription, we used an E-box-containing luciferase reporter construct derived from the muscle creatine kinase (*mck*) gene. This *mck-luc* reporter is insensitive to CLOCK-BMAL1-mediated transcriptional regulation (45). In contrast to the effect on both *mPer2-luc* and *Bmal1-luc* reporters, co-transfection with the *mEzh2*-shRNA-B did not affect the bioluminescence from the *mck-luc* reporter (Fig. 4*D*). In fact, the bioluminescence from the *mck-luc* reporter shows no difference when co-transfected with either *mEzh2*-shRNA-B or *mEzh2*-shRNA-C (Fig. 4*D*). Therefore, the *mEzh2*-shRNA-B effect on bioluminescence is specific to the *mPer2-luc* and *Bmal1-luc* reporters, and it is not due to an overall transcriptional defect. We calculated the amplitude difference in rhythmic bioluminescence from the co-transfected circadian-luciferase reporters with *mEzh2*-shRNA-B or *mEzh2*-shRNA-C. Co-transfection with *mEzh2*-shRNA-B showed a 75–80% decrease in rhythm amplitude of the *Bmal1-luc* and *mPer2-luc* reporters, respectively (Fig. 4*E*). The amplitude in rhythmic bioluminescence from *mPer2-luc* was

not significantly affected by the *mEzh1*-targeting shRNAs (Fig. 4*E*). These data show that functional targeting of *mEzh2* by shRNA disrupts circadian oscillations in cell culture by decreasing rhythm amplitude in circadian gene expression.

Collectively, these data reveal an unexpected role for EZH2 in circadian clock function. The primary involvement of EZH2 is through the methylation of H3K27 on circadian promoters, which

is necessary for the mCRY proteins to inhibit transcription. One feature of this mechanism not yet resolved is how the relatively constitutive methylation at circadian promoters interfaces with the mCRY-containing negative regulatory complex. The simplest possibility relies on the rhythmic binding of the mCRY-repressor complex to the CLOCK·BMAL1·EZH2 complex on the chromatin of the methylated *mPer1* and *mPer2* promoters. Another possibility is the association of the mCRY complex with members of the PRC1, which would provide a mechanism for the rhythmic inhibition of CLOCK·BMAL1-mediated transcription by rhythmically bringing associated PRC1 members to the responsive promoters, repressing transcription.

A recent study has shown circadian rhythms in the methylation patterns of lysine 4 and lysine 9 on H3 of the *Rev-erba* promoter (46). Moreover, RNAi knockdown of WDR5, a member of a methyltransferase complex involved in cell differentiation (47, 48), disrupts both the lysine 4 and lysine 9 methylation rhythms in cell culture but does not substantially alter molecular oscillations of the clockwork itself (46). In contrast, our studies provide strong evidence for a critical role of EZH2 and methylation of lysine 27 on histone H3 in the core clock mechanism.

The PcG proteins participate in a number of events in which long term transcriptional repression is required, such as regulating repression of homeotic genes during development (22, 49). The methylation of H3K27 was also shown to play an important role in vernalization, a temperature-dependent flowering rhythm observed in *Arabidopsis thaliana* during seasonal transitions (38, 50). Our results now provide strong evidence of a vital role of EZH2, a component of the PRC2, in a more dynamic process, namely the daily transcriptional activity of the clockwork.

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