Molecular characterization of Mybbp1a as a co-repressor on the *Period2* promoter

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ABSTRACT

The circadian clock comprises transcriptional feedback loops of clock genes. Cryptochromes are essential components of the negative feedback loop in mammals as they inhibit CLOCK-BMAL1-mediated transcription. We purified mouse CRY1 (mCRY1) protein complexes from Sarcoma 180 cells to determine their roles in circadian gene expression and discovered that Myb-binding protein 1a (Mybbp1a) interacts with mCRY1. Mybbp1a regulates various transcription factors, but its role in circadian gene expression is unknown. We found that Mybbp1a functions as a co-repressor of *Per2* expression and repressed *Per2* promoter activity in reporter assays. Chromatin immunoprecipitation (ChIP) assays revealed endogenous Mybbp1a binding to the *Per2* promoter that temporally matched that of mCRY1. Furthermore, Mybbp1a binding to the *Per2* promoter correlated with the start of the down-regulation of *Per2* expression and with the dimethylation of histone H3 Lys9, to which it could also bind. These findings suggest that Mybbp1a and mCRY1 can form complexes on the *Per2* promoter that function as negative regulators of *Per2* expression.

INTRODUCTION

Most organisms have physiological and behavioral regularities called circadian rhythms with an approximate intrinsic period of 24 h. The circadian clock is an endogenous oscillator that controls daily physiological and behavioral rhythms. Mammalian molecular oscillators located in the suprachiasmatic nucleus (SCN) in the ventral hypothalamus of the brain, constitute the master clock (1). To keep pace with the light–dark cycle, the SCN clock is entrained each day by light (2,3). The master clock subsequently synchronizes peripheral oscillators via neuronal and humoral signaling (4–6). Oscillators are located not only in the SCN but also in most peripheral tissues (7–9) and in established cell lines (4). Even in fibroblast cell lines, clock genes are induced rhythmically under specific conditions (10,11). Thus, the circadian clock is cell-autonomous (12,13). The core circadian system consists of an interacting transcriptional–translational feedback loop of clock genes in individual cells (1,14). The negative feedback loop involves the regulation of two *Period* genes (*Per1* and *Per2*) and two *Cryptochrome* genes (*Cry1* and *Cry2*) (15,16). The rhythmic transcription of the *Period* and *Cryptochrome* genes is driven by the basic helix–loop–helix–PAS protein (CLOCK-BMAL1) complex, which binds the E-box on the genes (17). This CLOCK-BMAL1-mediated transcription is in turn repressed by PER and CRY protein complexes that translocate to the nucleus (15–17).

Mammalian CRY proteins belong to the photolyase/cryptochrome protein family and were initially identified as homologs of photolyase, a DNA repair enzyme that removes UV light-induced DNA damage using visible light as an energy source (18). Animal cryptochromes are highly homologous to photolyases, but they lack the photolyase activity and the N-terminal extension that is characteristic of eukaryotic photolyases (19,20). Despite the crucial role of mCRY proteins, how they participate in core circadian system remains unclear, because little is understood about the mCRY protein complexes involved in these processes. Here we isolated mCRY1 protein complexes from cultured cells using tandem affinity purification (TAP) and identified proteins associated with mCRY1. We then investigated whether one of the novel proteins, Myb-binding protein 1a (Mybbp1a), is involved in the regulation of clock gene expression. Mybbp1a was originally identified as a cofactor that could bind to the

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negative regulatory domain of the transcription factor, c-Myb (21). It binds to several other transcription factors under various conditions (22–25). Mybbp1a has LXXLL motifs that often mediate interactions between nuclear receptors and their cofactors (26) and participate in many protein–protein interactions associated with different aspects of transcriptional regulation (27,28). Mybbp1a binds to and inhibits the coactivator function of PGC-1α, which is a key regulator of energy metabolism that also has LXXLL motifs (23). Notably, Liu *et al.* reported that PGC-1α stimulates the expression of *Bmal1* through coactivation of the ROR family of orphan nuclear receptors and that it is essential for circadian rhythms (29). We show here that Mybbp1a interacts with mCRY1 and represses *Per2* gene expression.

MATERIALS AND METHODS

Plasmid construction

The N-terminal TAP-tagged mCRY1 was constructed based on a mammalian expression vector as follows. The cDNA of mCRY1 (GenBank Accession No., NM_007771) was firstly cloned into the *EcoRI* site of pZome-1-N (Cellzome), which is a plasmid based on pBabe-puro for TAP-tagging proteins at the N-terminus, using the In-Fusion method (BD Biosciences). The resulting region corresponding to the TAP-tagged mCRY1 sequence was then cloned into pcDNA3.1 (Invitrogen) to drive its constitutive expression under the control of the CMV promoter. Full-length mCRY1 was also cloned into pcDNA3.1-His-V5 (Invitrogen). Mouse Mybbp1a cDNA was generated by RT-PCR from total RNA of NIH3T3 cells and cloned into pFlag-CMV2 (Sigma) at the *EcoRI* and *SalI* sites. Full-length mouse Mybbp1a cDNA was amplified using 5′-GATCGAATT CAGCGGAGATGAAGAGCCCCACGAAAG-3′ (forward) and 5′-GTCAGTCGACCTAAGGTGTCTGCAC TCTCCTGC-3′ (reverse) primers.

The cDNAs of mouse CLOCK (GenBank Accession No., NM_007715) and mouse BMAL1 (GenBank Accession No., NM_007489) were cloned as described (30) into pFlag-CMV2 (Sigma) and pcDNA3.1-His-Xpress (Invitrogen), respectively.

Cell culture and transfection

Sarcoma 180, NIH3T3 and COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin, transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer's protocols and harvested 24–48 h later.

Purification of TAP-tagged mCRY1 protein

Sarcoma 180 cells incubated in 100 mm dishes (total 30 dishes) were harvested 24 h after transfection with TAP-tagged mCRY1 and processed as follows at 4°C. The cells were washed with phosphate-buffered saline (PBS), suspended in 15 ml of ice-cold lysis buffer [20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40),

5 mM EDTA] supplemented with protease inhibitor mixture (Roche Diagnostics) and lysed for 30 min. Cell lysates were prepared by centrifugation at $12\,000 \times g$ for 20 min. TAP-tagged mCRY1 was batch-purified using 1.5 ml (50% slurry) of IgG beads (GE Healthcare). Lysates were incubated with the beads for 1.5 h, and then washed three times with lysis buffer. Bound, tagged proteins were released from the beads by incubation with 600 units of TEV protease (Invitrogen) in 5 ml of reaction buffer (50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT) overnight. The supernatant from the TEV reaction was collected and diluted 1:7 with $CaCl_2$-binding buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% β-mercaptoethanol, 2 mM $CaCl_2$). The diluted eluate was batch-purified by binding to calmodulin-coated beads (Stratagene) in 1.2 ml (50% slurry) for 2 h. After extensive washing with $CaCl_2$-binding buffer, tagged proteins were released from the beads with 5 ml of elution buffer (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.05% β-mercaptoethanol, 0.5% NP-40, 7.5 mM EGTA). To validate the purity of the mCRY1 protein complexes, eluates from each step were resolved by SDS–PAGE and then visualized by silver staining (Wako Pure Chemical) as well as by Western blotting using anti-mCRY1 antibody.
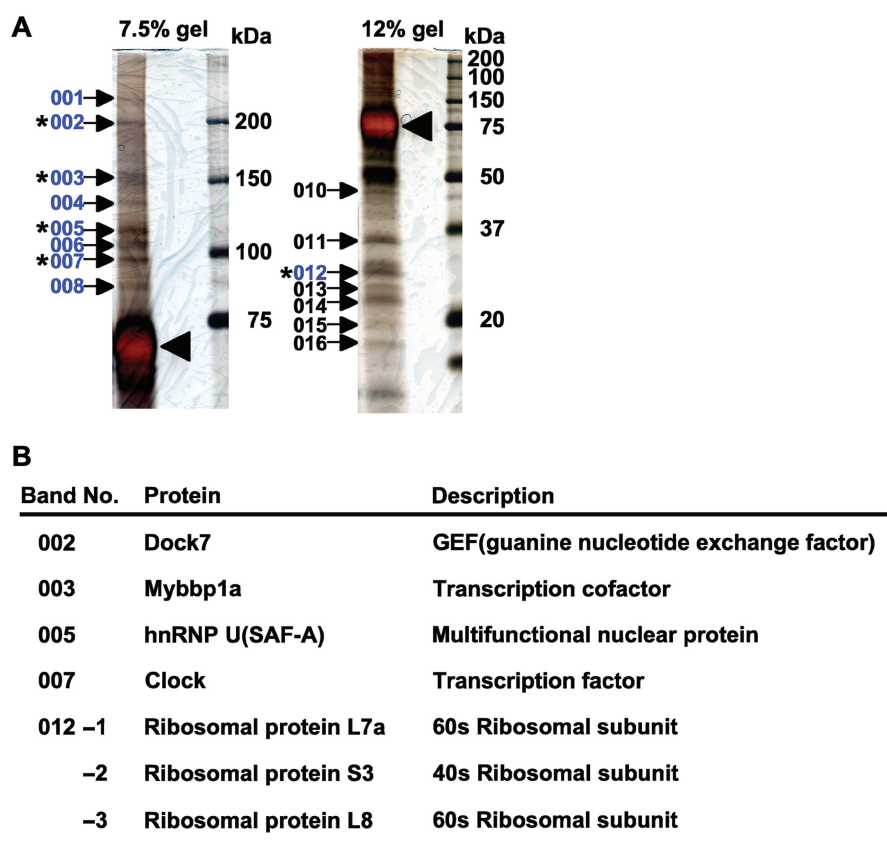
Protein identification

Purified mCRY1 protein complexes were concentrated by trichloroacetic acid (TCA) precipitation, resolved by SDS–PAGE and visualized by silver staining (Silver Stain MS Kit, Wako Pure Chemical). Excised bands on gels (Figure 1A) were digested *in situ* with trypsin. The tryptic digest was analyzed by either MALDI-TOF-MS (Voyager-DE STR; Applied Biosystems) or nano LC-MS/MS (MAGIC 2002 nano LC; Michrom Bioresources Inc.) and Q-Tof 2 (Waters Micromass). The MALDI-TOF-MS and nano LC-MS/MS data were searched against the public NCBI databases using MS-Fit (http://prospector.ucsf.edu/) and Mascot software (MatrixScience), respectively. Any matches found were manually evaluated and confirmed.

Immunoprecipitation

Cells were harvested 24–48 h after transfection and lysed by suspension in ice-cold lysis buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA) supplemented with protease inhibitor mixture (Roche Diagnostics). Lysates were collected after centrifugation at $12\,000 \times g$ for 20 min and those containing Flag-tagged proteins were immunoprecipitated with anti-Flag M2 agarose beads (Sigma), washed three times with lysis buffer, resolved by SDS–PAGE and Western blotted.

Antibodies

Flag-tagged proteins were detected using anti-Flag M2 antibody (Sigma) and horseradish peroxidase-conjugated anti-mouse IgG antibody (Chemicon). Anti-myc antibody was obtained from Roche Diagnostics. Anti-mCRY1 antisera were raised against purified, bacterially produced protein. Briefly, a GST-fusion protein containing CRY1 C-terminal residues 438 to 606 was expressed in

Figure 1. Identification of protein components of TAP-purified mCRY1 complexes. (**A**) Protein complexes eluted after final TAP-purification. Numbers on left side indicate bands excised from gels. Blue characters indicate bands analyzed by MS. Identified bands are marked with asterisks. Numbers on right side represent molecular weight markers. Bands corresponding to purified mCRY1 are indicated by arrowhead. Gel concentrations are indicated on top. (**B**) Characterization of proteins that interacted with mCRY1 identified by MS analysis.

E. coli and purified using glutathione Sepharose beads (GE Healthcare). The GST moiety was then cleaved using PreScission protease (GE Healthcare). Rabbit polyclonal antisera were then raised against the purified protein. Anti-mouse Mybbp1a antisera were raised against the GST-fusion protein containing Mybbp1a C-terminal residues 1187 to 1344 expressed in *E. coli* as described earlier and horseradish peroxidase-conjugated anti-rabbit IgG antibody was obtained from GE Healthcare.

Transient luciferase assays

Luciferase assay proceeded as described (31). Briefly, the mouse *Per2* promoter region (−789 to +331) was isolated and cloned into the pGL3-Basic vector (Promega). The construct was cotransfected with phRG-TK (Promega) into NIH3T3 cells as an internal control. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Transcriptional activities were normalized relative to *Renilla* luciferase activities.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assays proceeded as described (31). Briefly, NIH3T3 cells were cross-linked with 1% formaldehyde and then washed with PBS. The cells were lysed on ice for 20 min with lysis buffer (25 mM Tris–HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 3 mM EDTA). The DNA was sheared by sonication into 2.0 kbp fragments. The chromatin fractions were cleared using *E. coli* DNA and protein A/G beads (Santa Cruz), and then incubated with anti-Mybbp1a, anti-mCRY1 or anti-dimethyl-histone H3 (Lys9) (Upstate) antibodies overnight at 4°C, followed by protein A/G beads. Chromatin immunocomplexes were washed once for 10 min each at 4°C with wash buffers 1 (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA), 2 (20 mM Tris–HCl, pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA) and 3 (10 mM Tris–HCl, pH 8.0, 250 mM LiCl, 1% NP-40, 1% deoxycholate and 1 mM EDTA), followed by three washes with TE buffer. Immunocomplexes were removed from the beads with 1% SDS and 0.1 M NaHCO$_3$ and then heated along with DNA input samples overnight at 65°C to reverse the cross-links. Samples were then purified with SV Minicolumns (Promega). Target regions were amplified by PCR using the following primer set: mouse *Per2* promoter, 5′-GGCCGTCCTAT TTGCCCTCAAG-3′ (forward) and 5′-GCGAGTAGGC TCGTCCACTTC-3′ (reverse); *G3PDH*, 5′-ACCACAGT CCATGCCATCAC-3′ (forward) and 5′-TCCACCACCC TGTTGCTGTA-3′ (reverse).

RT-PCR

Total RNA was prepared using ISOGEN (Nippon Gene) and transcribed to cDNA using SuperScript III First-Strand Synthesis System (Invitrogen). We used the following PCR primers for mouse *Mybbp1a*, mouse *Per2* and control *G3PDH*: *Mybbp1a*, 5′-GACACAGAGGACTCA GAGGAC-3′ (forward) and 5′-TGACGGGGCTCTGGG TGTCA-3′ (reverse); *Per2*, 5′-CAGCGACCGGCCCTTG ATGCTCG-3′ (forward) and 5′-ATGATGTCTGGCTC ATGAGGTG-3′ (reverse); *G3PDH*, 5′-ACCACAGTCC ATGCCATCAC-3′ (forward) and 5′-TCCACCACCCT GTTGCTGTA-3′ (reverse). The expression levels of each gene were calculated using Image Gauge (Fuji Photo Film) and normalized to *G3PDH* expression.

Real-time RT-PCR

Total RNA was prepared from the mouse liver using guanidine thiocyanate followed by RNAiso (Takara) and digested with DNase I. Complementary DNA was synthesized using PrimeScript RT reagent kits (Takara). Real-time RT-PCR proceeded using SYBR Premix Ex Taq II (Takara) and LightCycler (Roche Diagnostics). Reaction conditions were 95°C for 10 s, followed by 45 cycles at 95°C for 5 s, at 57°C for 10 s and 72°C for 10 s. We used the following PCR primers for mouse *Mybbp1a*, mouse *Per2*, mouse *Cry1* and control *β-actin*: *Mybbp1a*, 5′-TGCCCTGAAGCGCCTAATCAC-3′ (forward) and 5′-GGATGTCACACAATGGGATGTC-3′ (reverse); *Per2*, 5′-TTCCACTATGTGACAGCGGA GG-3′ (forward) and 5′-CGTATCCATTCATGTCGG GCTC-3′ (reverse); *Cry1*, 5′-AGGAGGACAGATCCCA ATGGA-3′ (forward) and 5′-GCAACCTTCTGGATGC CTTCT-3′ (reverse); *β-actin*, 5′-CACACCTTCTACAAT GAGCTGC-3′ (forward) and 5′-CATGATCTGGGTCA TCTTTTCA-3′ (reverse). The relative mRNA levels of each gene were normalized to *β-actin* expression.

Peptide pull-down assay

Biotinylated peptides were bound to streptavidin beads (Sigma). COS-1 cells were then transfected with Flag-tagged Mybbp1a, lysed as described earlier and incubated with peptide-bound beads overnight at 4°C. The beads were washed three times with lysis buffer and then bound proteins eluted by boiling in SDS sample buffer were resolved by SDS–PAGE and Western blotted using anti-Flag antibody. The biotinylated peptides (Upstate) for pull-down assays were as follows: dimethyl-histone H3 (Lys9), ARTKQTAR[dimethyl K]STGGKAPRKQ LA-GGK-biotin; acetyl-histone H3 (Lys9), ARTKQTAR [acetyl K]STGGKAPRKQLA-GGK-biotin; unmodified-histone H3, ARTKQTARKSTGGKAPRKQLA-GGK-biotin.

Real-time luciferase assay

Real-time luciferase assays proceeded as described (31). Briefly, the mouse *Per2* promoter region (−789 to +331) was cloned into reporter plasmid pGL3-dLuc (*Per2*-dLuc) containing a rapid degradation domain at the C-terminus of firefly luciferase. After transfection with a reporter plasmid, NIH3T3 cells were synchronized by stimulation with 100 nM dexamethasone for 2 h, and then the medium was replaced with DMEM (2 ml per 3.5 cm dish) containing 100 mM luciferin (Wako Pure Chemical). Luciferase activity was measured 2 h later using Kronos AB-2500 (ATTO).

RESULTS

## Expression of TAP-tagged mCRY1 in Sarcoma 180 cells

We identified proteins that interact with mCRY1 using TAP, which is a rapid method of purifying protein complexes for proteome analysis (32). We decided to purify mCRY1 protein complexes from Sarcoma 180 cells because they are derived from a mouse sarcoma, they rapidly proliferate and generate large volumes of cell lysates, and circadian oscillation of the clock genes can be induced in these cells (Supplementary Figure S1A) (33). Exogenously overexpressed mCRY1 predominantly localizes in the nucleus of cultured cells (15). Supplementary Figure S1C shows that transiently overexpressed TAP-tagged mCRY1 was also located in the nucleus of Sarcoma 180 cells.

Purification of mCRY1-protein complexes from Sarcoma 180 cells and identification of proteins associated with mCRY1

We purified TAP-tagged mCRY1 and associated proteins from total lysates of Sarcoma 180 cells. The eluate from calmodulin beads was concentrated, resolved by SDS–PAGE and silver-stained (Figure 1A and Supplementary Figure S2). Visualized protein bands were excised and those that migrated to a position corresponding to higher molecular weight than that of exogenously expressed mCRY1 (bands from 001 to 008 in Figure 1A) were preferentially analyzed by MS along with one randomly selected band with a lower molecular weight (band 012).

We identified a small GTPase-related protein involved in signal transduction (band 002), a transcription cofactor (band 003, Mybbp1a, discussed in detail later), a multifunctional nuclear protein that participates in the processing of pre-mRNA and the regulation of chromatin structure (band 005), a circadian oscillator protein, CLOCK (band 007) and three ribosomal proteins (band 012) (Figure 1B). We assessed the specificity of mCRY1 interactions of all of these proteins except for band 002, which was too large for exogenous expression in mammalian cells and band 007 (CLOCK), which is known to interact with mCRY1 (16,34).

Full-length mouse cDNAs were cloned into expression vectors with the Flag epitope. COS-1 cells were cotransfected with Flag-tagged proteins together with TAP-tagged mCRY1, and pull-down assays were performed using IgG beads. Figure 2A (Supplementary Figure S3A and C) shows Flag-tagged protein-mCRY1 complexes in the precipitates. To further confirm specific interaction, we prepared a V5-His-tagged mCRY1 expression construct without the TAP tag to show that the identified proteins interacted with mCRY1 and not with the TAP tag. Flag-tagged proteins were cotransfected with V5-His-tagged

A

B
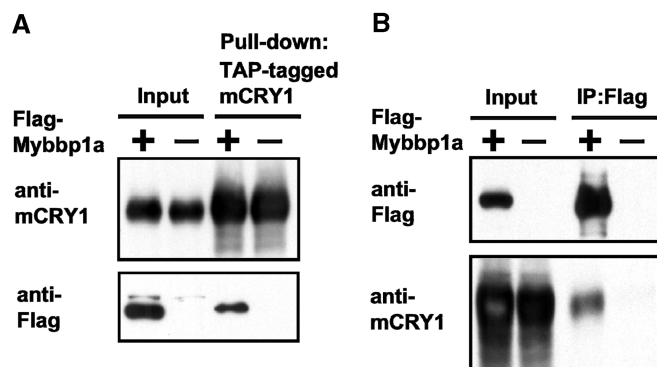
Figure 2. Interaction between Mybbp1a and mCRY1 confirmed by reciprocal co-precipitation. (**A**) TAP-tagged mCRY1 co-transfected with Flag-Mybbp1a into COS-1 cells. Pull-down assays were performed using IgG beads with high-affinity to TAP-tagged protein. (**B**) Flag-Mybbp1a co-transfected with V5-His-tagged mCRY1 into COS-1 cells. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody. Symbols (+) or (–), presence or absence of Flag-Mybbp1a-expression plasmids, respectively.

mCRY1 into COS-1 cells, and immunoprecipitated with anti-Flag antibody. Figure 2B (Supplementary Figure S3B and D) shows mCRY1 in protein complexes precipitated with anti-Flag antibody. Therefore, these results confirmed that these five proteins (the three ribosomal proteins comprising band 012, as well as bands 003 and 005) interacted with mCRY1.

Band 003 was identified as Mybbp1a, which has been characterized as a transcription cofactor (21–24,35). Some evidence indicates that Mybbp1a binds to several transcription factors, although its effect on transcription varies among genes. We postulated that Mybbp1a is involved in the transcriptional regulation of clock genes. We initially generated an antibody specific to mouse Mybbp1a (Supplementary Figure S4A) and then immunohistochemically examined the expression of Mybbp1a in the mouse SCN. Supplementary Figure S4B shows intense Mybbp1a expression in the SCN cells and predominant location in the nucleolus, which is in agreement with previous findings (21,36). However, a small amount of Mybbp1a in the nucleoplasm can regulate transcription factors (24).

Mybbp1a represses transcriptional activity of the *Per2* promoter

Although Mybbp1a binds to several transcription factors, which indicates a role in transcriptional regulation, the function of Mybbp1a in circadian gene expression remains unknown. To determine whether Mybbp1a regulates *Per2* gene expression, we performed reporter assays in NIH3T3 cells in which *Per2* is a core oscillator for maintenance of the circadian clock. We cotransfected the *Per2*-luciferase reporter (Figure 3A) with a Mybbp1a expression plasmid. Figure 3B shows that Mybbp1a dose-dependently repressed *Per2* promoter activity (left panel). We then tested the effect of Mybbp1a on CLOCK-BMAL1-dependent transactivation (Figure 3B, right panel). Increasing amounts of Mybbp1a dose-dependently

repressed *Per2* promoter activity regardless of the presence of CLOCK-BMAL1. We also examined whether or not Mybbp1a represses *Per2* promoter activity via an E2-box. We used a reporter containing a mutant E2-box that lacks transcriptional activation by CLOCK-BMAL1 (31) (Supplementary Figure S5A). A mutation of the E2-box (mut E2-box reporter) resulted in the same transcriptional repression (up to 44%) as the wild type (wt E2-box reporter) (Supplementary Figure S5B, left panel).

To determine whether such repression occurs through direct action of Mybbp1a on the *Per2* gene promoter, we performed ChIP assays in NIH3T3 cells transfected with Flag-Mybbp1a. The polymerase chain reaction (PCR) showed that Mybbp1a bound to the *Per2* promoter (Figure 3C). A region of the *G3PDH* gene that was also included in PCR from precipitated DNA confirmed that Flag-Mybbp1a specifically precipitated the *Per2* promoter. To determine whether Mybbp1a binds to the *Per2* promoter together with mCRY1, we also immuno-precipitated endogenous Mybbp1a and mCRY1. The results of ChIP assays showed that endogenous Mybbp1a and mCRY1 specifically bound to the *Per2* promoter (Figure 3D).

Moreover, mCRY1 also repressed the activity of the mut E2-box reporter by 69% (Supplementary Figure S5B, right panel), which was less than that of the wt E2-box reporter (93%). This finding suggests that mCRY1 also represses the *Per2* promoter activity, at least in part, in an E2-box-independent manner.

Mybbp1a binding to the *Per2* promoter is involved in negative regulation of *Per2* expression

Circadian oscillators are operative even in cell lines cultured *in vitro* (4,37,38). We examined the role of Mybbp1a on the expression of *Per2* in the cell-autonomous clock after inducing circadian gene expression in NIH3T3 cells with dexamethasone (13). Reverse transcription (RT)-PCR showed that *Mybbp1a* mRNA expression temporally fluctuated (Figure 4A), but without a circadian rhythm. In contrast, the transcription of *Per2* oscillated with a robust circadian rhythm and rhythmic *Per2* expression peaked at 30 and 54 h. The overall fluctuation in the mRNA level of *Mybbp1a* seemed to be generated from a primary response to dexamethasone stimulation. Thus, we examined the temporal expression profile of *Mybbp1a* mRNA in livers of mice maintained under light/dark cycles (Figure 5). Circadian fluctuation was absent in the *Mybbp1a* mRNA (Figure 5, top panel). Conversely, the rhythms of *Per2* and *Cry1* expression were robustly circadian and rhythmic *Per2* and *Cry1* mRNA expression peaked at Zeitgeber times (ZT) 14 and ZT 17, respectively (Figure 5, middle and bottom panels). Therefore, we concluded that *Mybbp1a* mRNA did not oscillate in a circadian manner.

We postulated that Mybbp1a cooperatively represses *Per2* transcription together with mCRY1 via *Per2* promoter interaction. To test this hypothesis, we analyzed the temporal binding of endogenous Mybbp1a and mCRY1 on the *Per2* promoter by ChIP assays of NIH3T3 cells after dexamethasone stimulation. The ChIP findings
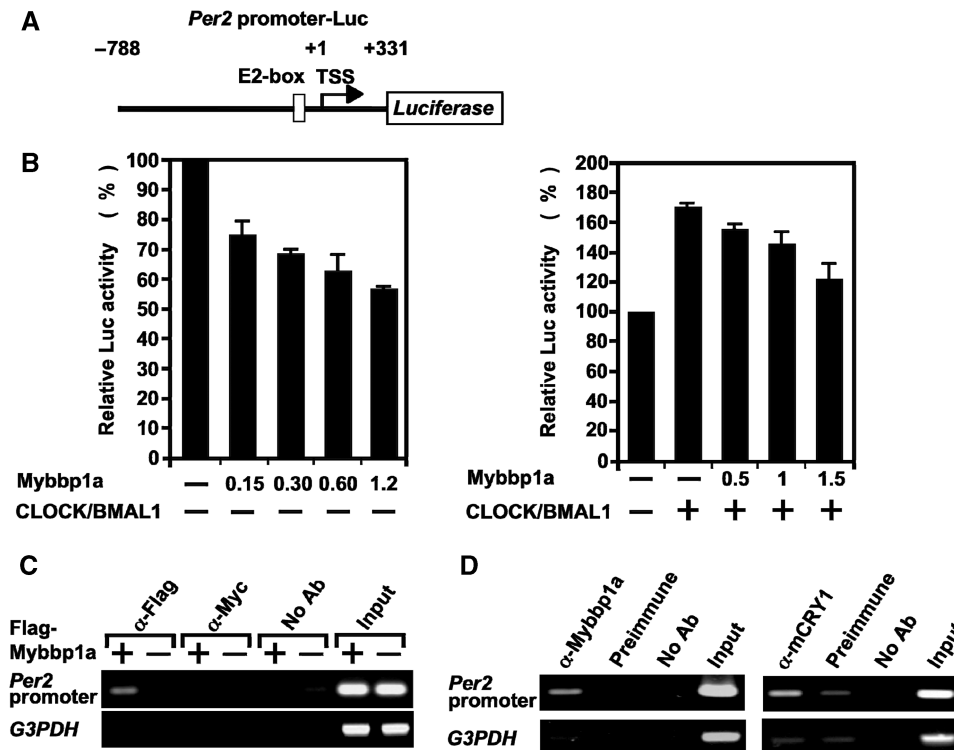
Figure 3. Mybbp1a represses transcriptional activity of *Per2* promoter. (**A**) Schematic representation of reporter gene containing mouse *Per2* promoter (*Per2* promoter-Luc). Arrow and open box indicate transcription start site (TSS) and E2-box, respectively. (**B**) Repression of *Per2* promoter activity by Mybbp1a in NIH3T3 cells. Co-transfection with increasing amounts of Mybbp1a expression plasmid (0.15, 0.30, 0.60 and 1.2 μg) and *Per2* promoter-Luc without CLOCK and BMAL1 (left panel). Co-transfection with increasing amounts of Mybbp1a expression plasmid (0.5, 1.0 and 1.5 μg) and *Per2* promoter-Luc with CLOCK and BMAL1 (right panel). Symbols (+) or (–), presence or absence of expression plasmids, respectively. Expression levels were calculated relative to luciferase activities without Mybbp1a and CLOCK-BMAL1. Note difference in *y*-axis scale between left and right panels. (**C**) Binding of Mybbp1a to *Per2* promoter. NIH3T3 cells were transfected with Flag-tagged Mybbp1a and then analyzed by ChIP assays using indicated antibodies, followed by PCR amplification with primers for *Per2* promoter. Symbols (+) or (–), presence or absence of Flag-Mybbp1a-expression plasmids, respectively. (**D**) Identification of endogenous proteins on *Per2* promoter region. NIH3T3 cells were analyzed by ChIP assays using either anti-Mybbp1a or anti-mCRY1 antibodies. Preimmune: preimmune serum of same animals in which antisera were raised. No Ab: without antibody. *G3PDH* primers served as negative control.

showed that the peaks of Mybbp1a binding to the *Per2* promoter were almost identical to those of mCRY1 binding (Figure 4B), suggesting that the two proteins function as coordinate regulators of *Per2* expression.

Histone H3 Lys9 dimethylation is linked to transcriptional repression (39–41). Therefore, we considered that Mybbp1a would cooperatively repress *Per2* transcription with mCRY1 via interaction with histone H3 dimethylated Lys9 on the *Per2* promoter. We analyzed the temporal binding of histone H3 dimethylated Lys9 to the *Per2* promoter. Figure 4B shows that the peaks of histone H3 dimethylated Lys9 binding to the *Per2* promoter were almost identical to those of both Mybbp1a and mCRY1. The correlation among Mybbp1a binding, mCRY1 binding and histone H3 dimethylated Lys9 on the *Per2* promoter suggests that these three proteins cooperatively regulate *Per2* expression.
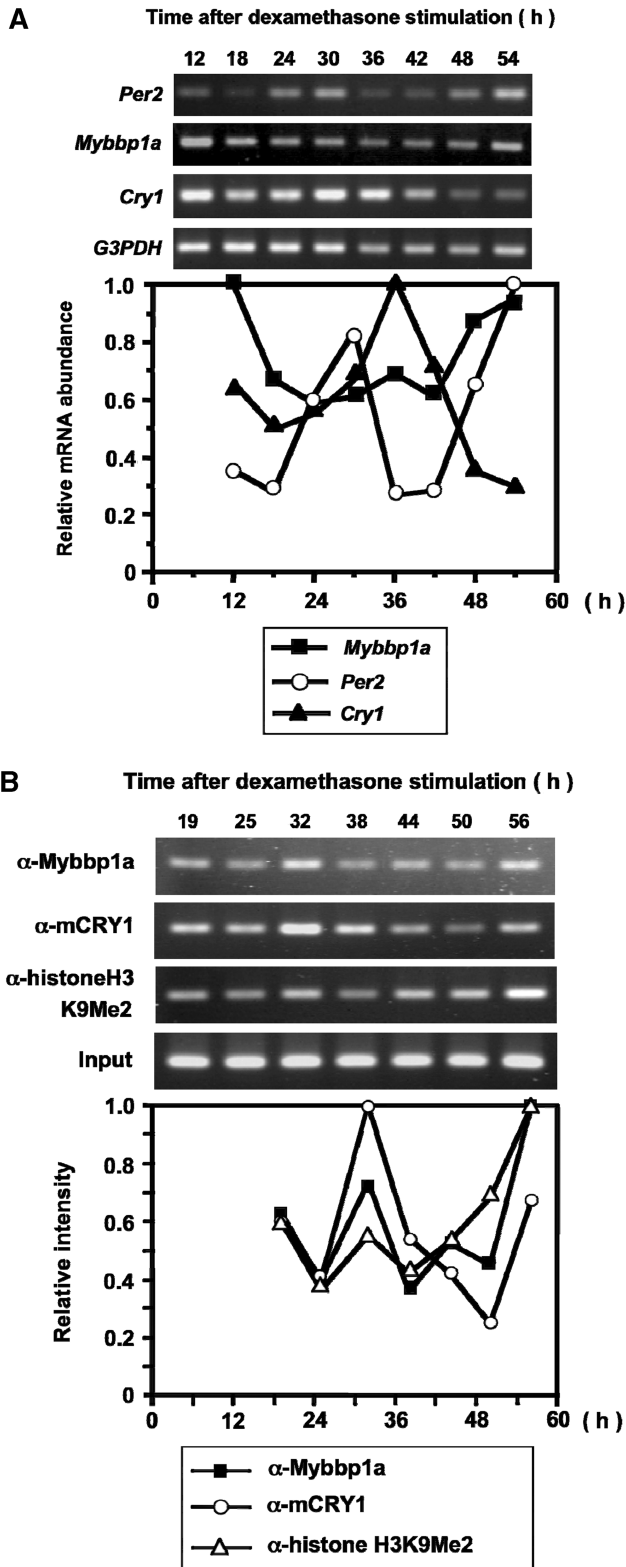
Mybbp1a binds to dimethylated histone H3 on Lys9

We postulated that Mybbp1a binds to the histone H3 N-terminal tail, because Mybbp1a binding to the *Per2* promoter correlated with histone H3 dimethylated

Lys9 (Figure 4B). We performed pull-down assays using histone H3 N-terminal peptide-immobilized beads to confirm this notion. Figure 6 shows that Mybbp1a preferentially bound to the dimethylated peptide at Lys9. On the other hand, Mybbp1a also relatively weakly bound to the acetylated and unmodified peptide at Lys9. To further confirm these results, we quantified the band intensity of bound Mybbp1a to each peptide. We found that the intensity of bands of Mybbp1a bound to the peptide dimethylated at Lys9 was 2-fold higher than that of Mybbp1a bound to the acetylated and unmodified peptide at Lys9 (Supplementary Figure S6), confirming that Mybbp1a preferentially binds to histone H3 dimethylated Lys9. This result was consistent with the finding that the temporal binding of Mybbp1a to the *Per2* promoter correlated with the dimethylation of histone H3 Lys9.

Mybbp1a can interact with CLOCK

Although Mybbp1a represses transcriptional activity of the *Per2* promoter, its function in the regulation of *Per2* gene expression remains unclear. Because endogenous CLOCK was present in mCRY1-interacted proteins

A

B

identified by MS (Figure 1B), we postulated that Mybbp1a also interacts with CLOCK and forms protein complexes. Supplementary Figure S5C (top panel) confirms that Mybbp1a interacts with CLOCK.

The functional relation between Mybbp1a and CLOCK-BMAL1 is also suggested by the results of Luc assays using mut E2-box reporter (Supplementary Figure S5D). Although the level of mut E2-box reporter activity repression by Mybbp1a alone was similar to that of the wt E2-box reporter, mut E2-box reporter activity was further reduced (12%, $P < 0.05$) when Mybbp1a coexisted with CLOCK-BMAL1. On the other hand, only CLOCK-BMAL1 did not repress the level of mut E2-box reporter activity. In addition, the level of wt E2-box reporter activity repressed by Mybbp1a in the presence of CLOCK-BMAL1 (39%) was more than that (22%) in the absence of CLOCK-BMAL1. These results suggest that the related action of Mybbp1a and CLOCK-BMAL1 is involved, at least in part, in repression of the *Per2* promoter and that this process is independent of the E2-box.

Effect of Mybbp1a on circadian oscillation in the cell-autonomous clock

We conducted real-time reporter assays to determine whether or not Mybbp1a regulates the circadian expression of *Per2* in the cell-autonomous clock. Supplementary Figure S7A shows that Mybbp1a overexpression reproducibly and significantly repressed about 40% of the *Per2* promoter activity although the inter-experimental luciferase values varied. These results were consistent with those of conventional Luc assays (Figure 3B). Although Mybbp1a overexpression down-regulated the *Per2* promoter activity in real-time reporter assays, the period length of *Per2* oscillation did not significantly differ. To further understand the potential role of Mybbp1a in the cell-autonomous clock, we performed knockdown experiments using small interfering RNA (siRNA) for Mybbp1a. The levels of luciferase activity in real-time reporter assays after introducing Mybbp1a siRNA (Supplementary Figure S7B), and the period length of *Per2* did not significantly differ. Therefore, further studies are required to understand the involvement of Mybbp1a in circadian oscillation in the cell-autonomous clock.

DISCUSSION

Although mammalian CRY proteins play crucial roles in the regulation of circadian gene expression, precisely how this is accomplished remains unclear. A recent study has shown that CRYs and PERs form large complexes of

Figure 4. Temporal binding of Mybbp1a to *Per2* promoter correlates with mCRY1 binding. (**A**) Temporal expression profile of *Mybbp1a* mRNA in NIH3T3 cells. Cells were stimulated with dexamethasone and then total RNA isolated at various time points was analyzed by RT-PCR. Products of PCR were resolved by electrophoresis in 2% agarose gels and stained with ethidium bromide (top panels). Levels of mRNA were normalized to *G3PDH* expression and peak values of individual curves were set to 1 (bottom panel). (**B**) Oscillatory binding

of Mybbp1a to the *Per2* promoter. NIH3T3 cells were stimulated with dexamethasone, and then analyzed at each time point by ChIP assays using indicated antibodies and primers for *Per2* promoter. Products of PCR were resolved by electrophoresis in 2% agarose gels and stained with ethidium bromide (top panels). Relative band intensities were normalized to input intensities. Peak values of individual curves were set to 1 (bottom panel).
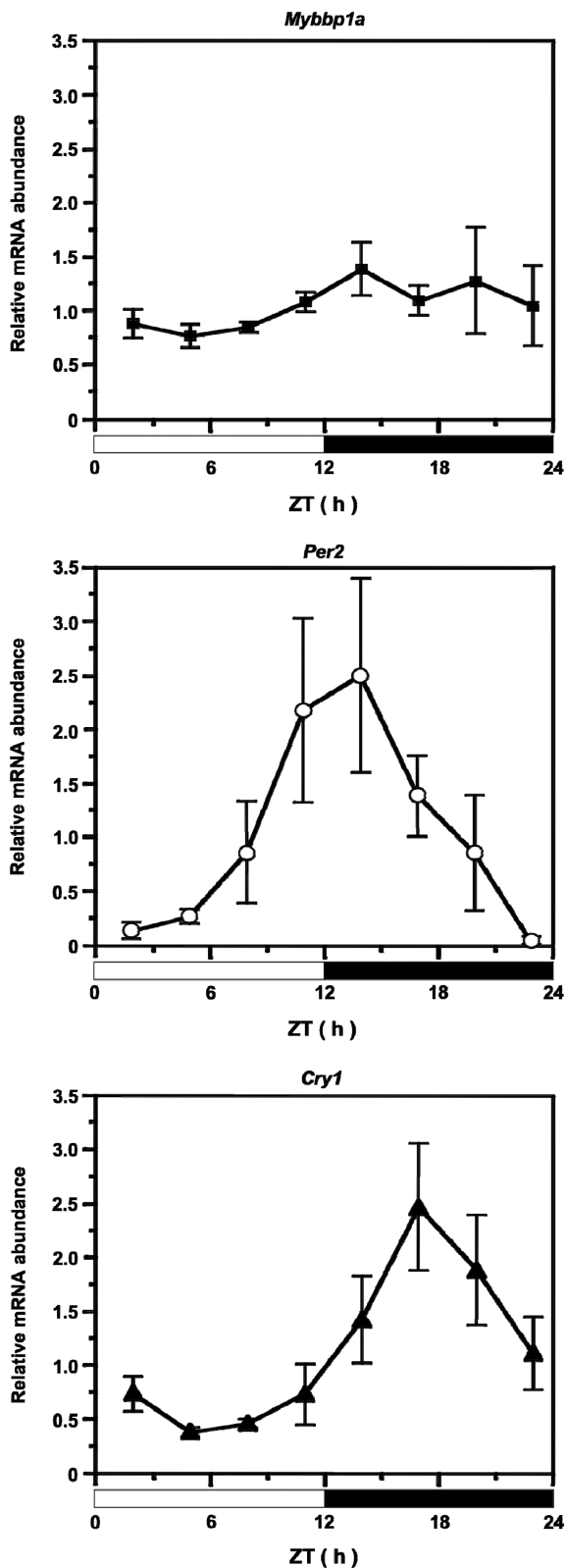
Figure 5. Temporal expression profile of *Mybbp1a* mRNA in mouse liver. Mice were maintained under 12 h light: 12 h dark cycles (light on at ZT 0) and liver samples were obtained at each time point. Levels of *Mybbp1a* mRNA (top panel) were determined using real-time RT-PCR. Expression levels were normalized to *β-actin* mRNA. Values are means ± SEM of three mice per time point. Expression profiles of *Per2* and *Cry1* mRNA are also shown in middle and bottom panels, respectively.

Western blot: anti-Flag antibody
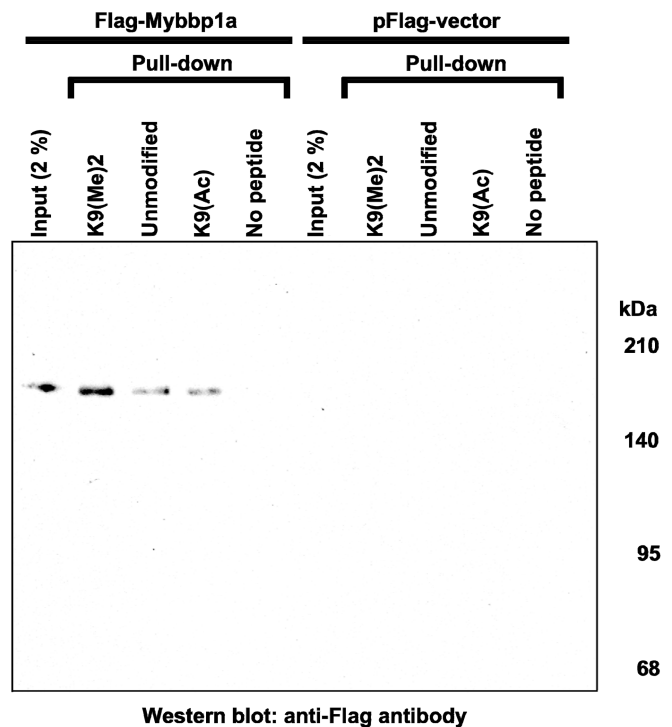
Figure 6. Specific binding of Mybbp1a to histone H3 dimethylated Lys9. Pull-down assays of lysates from COS-1 cells transfected with Flag-Mybbp1a using histone H3 N-terminal peptides that were modified or not at Lys9. Bound proteins eluted from beads previously bound to peptides were analyzed by Western blotting against anti-Flag antibody. Control experiments were performed with lysates from COS-1 cells transfected with empty vector (pFlag-vector). K9(Me)2, dimethylated Lys9; Unmodified, unmodified Lys9; K9(Ac), acetylated Lys9 (indicated on top).

which the abundance and size distribution fluctuate during the day (42). Therefore, we postulated that CRY proteins interact with other proteins that might play important roles in circadian oscillator function.

The present study focused on the roles of large proteins that interact with mCRY1, because their functions are frequently linked to processes in higher-order organisms such as diseases and the nervous system (43,44), and such proteins can bind to many partners (45). However, to accumulate functional information about such large proteins in the molecular clock mechanism is hampered by technical limitations. From this viewpoint, we preferentially selected protein bands with an apparently high molecular weight for MS analysis (Figure 1A).

We identified seven proteins that interact with mCRY1 (Figure 1B). The presence of endogenous CLOCK indicated that TAP-MS can identify endogenous proteins that interact with mCRY1, because studies using transiently expressed proteins found that mCRY1 interacts directly with CLOCK (16,46). To our knowledge, we present herein the first direct evidence that endogenous CLOCK actually associates with mCRY1 in cell lines cultured *in vitro*. This finding suggests that mCRY1 can indeed form protein complexes with endogenous CLOCK even though cell-specific proteins are involved in mediating this process.

Although the focus of the present study was band 003, we are also interested in the role of band 005 (hnRNP U) in the circadian clock. The functions of both the glucocorticoid receptor, for which the ligand is a major cue for circadian oscillation (47) and β-TrCP, the F-box protein of the SCF complex ubiquitin ligase, the substrates for which are circadian oscillator proteins (48,49) are modified by hnRNP U. Thus, hnRNP U appears to play a role(s) in circadian gene expression. Tamaru *et al.* reported that hnRNP U protein and transcript levels robustly change in a circadian manner in both the SCN and hippocampus of the mouse brain (50). We found that hnRNP U plays an important role in circadian *Bmal1* expression through chromatin alteration (51). However, the involvement of mCRY1 in the regulation of hnRNP U function in cell-autonomous clock should be elucidated in future studies.

Band 003 was identified as Mybbp1a, and we discovered that it negatively regulates *Per2* transcription, which is consistent with its ability to repress the transcription activity of several factors (23–25). Our results indicate that Mybbp1a represses *Per2* gene expression through binding to the *Per2* promoter (Figure 3B, C and D). As Mybbp1a lacks DNA-binding domains (35), it might bind to the *Per2* promoter through interaction with other factors. The ChIP assays showed that endogenous mCRY1 bound to the *Per2* promoter (Figure 3D), indicating that Mybbp1a binds to this promoter through mCRY1 or forms complexes containing mCRY1 on the promoter.

A non-canonical E-box (E2-box) located 20 bp upstream of the *Per2* transcription start site is a functional element for CLOCK-BMAL1 binding (52). Luc assays using a mutant of E2-box revealed that Mybbp1a represses the *Per2* promoter activity independently of E2-box (Supplementary Figure S5B, left). In addition, mCRY1 not only inhibits CLOCK-BMAL1-mediated transactivation via the E2-box, but also represses *Per2* promoter activity in an E2-box-independent manner (Supplementary Figure S5B, right). Therefore, Mybbp1a appears to be involved in E2-box-independent repression via mCRY1 rather than the inhibition of CLOCK-BMAL1-mediated transactivation.

We postulated that Mybbp1a plays an important role in *Per2* oscillation in the cell-autonomous clock. After stimulation with dexamethasone, the expression profile of *Mybbp1a* mRNA did not indicate clear circadian oscillation in NIH3T3 cells (Figure 4A). We also found that *Mybbp1a* mRNA did not exhibit any significant circadian oscillation in the mouse liver (Figure 5, top panel). In addition, Mybbp1a immunoreactivity in the mouse SCN was not associated with significant circadian rhythms (data not shown). These results indicated that even if Mybbp1a participates in clock gene regulation, its expression is not directly governed by the circadian clock system.

ChIP assays after dexamethasone stimulation revealed that Mybbp1a binding to the *Per2* promoter was almost identical to the peaks of both mCRY1 and histone H3 dimethylated Lys9 binding and that the binding peaked at circadian intervals (Figure 4B; see 32 and 56 h). This correlation suggests that the three proteins coordinately regulate *Per2* expression. Furthermore, the peaks of Mybbp1a, mCRY1 binding and histone H3 dimethylated Lys9 on the *Per2* promoter appeared ahead of the trough of *Per2* expression (Figure 4A and B). Thus, Mybbp1a and mCRY1 binding apparently repress *Per2* expression in a circadian manner. As *Mybbp1*a mRNA does not oscillate in a proper circadian manner, rhythmic Mybbp1a binding to the *Per2* promoter is probably controlled by post-translational mechanisms. The circadian rhythmicity of Mybbp1a binding to the *Per2* promoter might be mediated by the temporal activation of mCRY1.

Our pull-down assays showed that Mybbp1a preferentially bound to histone H3 dimethylated peptide at Lys9 as compared with both histone H3 acetylated peptide at Lys9 and histone H3 unmodified peptide (Figure 6 and Supplementary Figure S6). These results suggest that Mybbp1a, mCRY1 and histone H3 dimethylated Lys9 form complexes on the *Per2* promoter and that these complexes negatively regulate *Per2* expression.

We also confirmed that Mybbp1a is expressed in the nucleolus of SCN cells (Supplementary Figure S4B), although its function in the nucleolus remains unclear. However, increasing evidence indicates that Mybbp1a functions in nuclear mRNA transcription by pol II, even though it is mainly located in the nucleolus (22–25,53). Thus, its expression in SCN cells supports the notion that Mybbp1a regulates clock gene expression.

We showed that Mybbp1a can interact with CLOCK (Supplementary Figure S5C). In addition, CLOCK-BMAL1 is involved in the suppressive effect of Mybbp1a on the *Per2* promoter independently of the E2-box (Supplementary Figure S5D). Therefore, one possibility is that Mybbbp1a, mCRY1 and CLOCK-BMAL1 form complexes in an E2-box-independent manner that negatively regulate the *Per2* expression. Another possibility is that this negative regulation may be, at least in part, due to indirect effects via mediators that are controlled by CLOCK-BMAL1. Mybbbp1a and mCRY1 complexes may negatively regulate such mediators expression via E-box through a mechanism requiring chromatin remodeling. However, more studies are needed to reach a firm conclusion.

We postulated that gain- and/or loss-of-function experiments using real-time reporter assays would reveal the role of Mybbp1a in the circadian expression of *Per2*. We found that Mybbp1a overexpression significantly decreased levels of luciferase activity, but did not affect the period length of *Per2* oscillation (Supplementary Figure S7A). On the other hand, siRNA-mediated knockdown of Mybbp1a did not significantly change the oscillation profile (Supplementary Figure S7B). These results indicate that the function of Mybbp1a is not essential for circadian clock oscillation. However, Fan *et al.* demonstrated using mCRY1 proteins that can permeate cells, that CRY1 protein cycling is not necessary for circadian clock function in mouse fibroblasts (54). Yamanaka *et al.* also revealed that circadian oscillation persisted in rat-1 cells even under conditions of constitutive mCRY1 protein overexpression (55). Therefore, a post-translational cyclic regulatory mechanism probably contributes to maintain the circadian oscillation. Our findings indicated that Mybbp1a

participates in the regulation of *Per2* gene transcription, but its precise role in the cell-autonomous clock awaits further investigation.

In addition, Mybbp1a is a negative regulator of PGC-1α, which is a transcription coactivator that regulates energy metabolism (23). PGC-1α activates *Bmal1* gene expression and might integrate the circadian-clock and energy metabolism (29). Thus, we speculate that the Mybbp1a-mCRY1 complex also plays a role in circadian gene expression through the modulation of PGC-1α activity.

Transcription-permissive chromatin states are dynamically established in a circadian-time-specific manner (56–60). Mybbp1a might be involved in coupling circadian regulators to chromatin remodeling through histone modifications. One hypothesis is as follows, considering our finding that Mybbp1a preferentially bound to histone H3 dimethylated Lys9. After histone H3 Lys9 dimethylation is initiated, Mybbp1a binds to histone H3 dimethylated Lys9 and forms complexes with mCRY1 on the *Per2* promoter. This complex represses transcription activity of the *Per2* promoter in cooperation with unknown factors (Figure 7). In fact, Mybbp1a plays an important role in transcriptional regulation via histone modification. Owen *et al.* reported that Mybbp1a functions as a co-repressor of NF-κB-dependent transcription by competing with p300 histone acetyltransferase activity (24). Fan *et al.* indicated that Gal4-fused Mybbp1a possesses intrinsic repressive activity according to the results of reporter gene assays and that this activity is diminished by the histone deacetylase inhibitor, trichostatin A (23). Similarly, mCRY1 represses transcription by recruiting the histone deacetylase complex to promoter sites (59). The repressive activity of Mybbp1a and the mCRY1 complex are apparently involved in histone modification, for example, the deacetylation and di- and trimethylation of H3 at Lys9 (Figure 7). However, the modulation of histone methylation by the Mybbp1a-mCRY1 complex should be elucidated in the future. Further investigation into the effect of Mybbp1a on the clock gene promoter will provide more information about circadian clock gene regulation at the chromatin level.
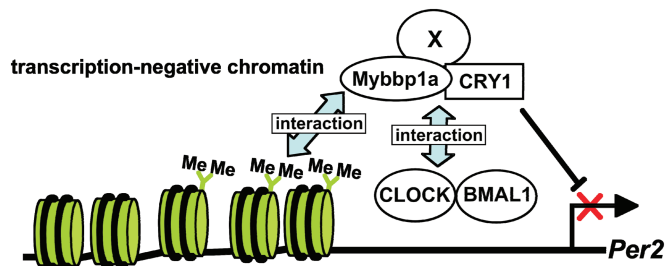
Figure 7. Regulation of circadian transcription through histone tail modification. Mybbp1a and mCRY1 (and CLOCK) form complexes on *Per2* promoter and repress *Per2* transcription although Mybbp1a did not directly bind to mCRY1. After Mybbp1a-independent histone H3 Lys9 dimethylation, Mybbp1a binds to histone H3 dimethylated Lys9. Mybbp1a–mCRY1 complexes then recruit unknown chromatin remodeling factor(s) (indicated as X, e.g. histone deacetylase) to *Per2* promoter and support establishment of transcription-permissive chromatin independently of E2-box. Consequently, *Per2* gene expression is repressed.

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