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Transcriptional program of Kpna2/Importin-α2 regulates cellular differentiation-coupled circadian clock development in mammalian cells

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The circadian clock in mammalian cells is cell-autonomously generated during the cellular differentiation process, but the underlying mechanisms are not understood. Here we show that perturbation of the transcriptional program by constitutive expression of transcription factor *c-Myc* and DNA methyltransferase 1 (*Dnmt1*) ablation disrupts the differentiation-coupled emergence of the clock from mouse ESCs. Using these model ESCs, 484 genes are identified by global gene expression analysis as factors correlated with differentiation-coupled circadian clock development. Among them, we find the misregulation of *Kpna2* (*Importin-α2*) during the differentiation of the *c-Myc*-overexpressed and *Dnmt1*$^{-/-}$ ESCs, in which sustained cytoplasmic accumulation of PER proteins is observed. Moreover, constitutive expression of *Kpna2* during the differentiation culture of ESCs significantly impairs clock development, and KPNA2 facilitates cytoplasmic localization of PER1/2. These results suggest that the programmed gene expression network regulates the differentiation-coupled circadian clock development in mammalian cells, at least in part via posttranscriptional regulation of clock proteins.

circadian clock | cellular differentiation | c-Myc | Dnmt1 | Kpna2 (Importin-α2)

The circadian clock is an intrinsic time-keeping system that regulates essential physiological functions such as sleep/wake cycles, body temperature, and metabolism (1–3). In the mammalian clock system the central pacemaker resides in the suprachiasmatic nucleus of the hypothalamus, coordinating cell-autonomous molecular oscillators throughout the body to perform tissue-specific functions. The molecular oscillator consists of transcriptional/translational feedback loops of clock genes. Two transcription factors, CLOCK and BMAL1, heterodimerize and transactivate core clock genes such as the *Period* (*Per1, 2, 3*), *Cryptochrome* (*Cry1, 2*), and *Rev-Erbα* genes via E-box regulatory elements. PER and CRY proteins, in turn, translocate into the nucleus to suppress CLOCK/BMAL1 activity, leading to cyclic expression of these clock genes (4–9). Furthermore, REV-ERBα negatively regulates *Bmal1* transcription via the RORE promoter element, thus driving antiphasic expression patterns of *Bmal1* (10, 11).

Despite uncovering the emergence of the circadian rhythms that occur during development (12–14), the precise mechanism of circadian clock development in mammalian cells remains unclear. Recently it has been found that pluripotent ES cells (ESCs) do not display discernible circadian molecular oscillations, whereas in vitro-differentiated ESCs displayed robust circadian oscillations of reporter expression (15–17). Moreover, we also have shown that circadian oscillations were abolished when differentiated cells were reprogrammed to regain pluripotency by expression of reprogramming factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) (15). These results suggested that the emergence of the cell-autonomous circadian oscillator is coupled with cellular differentiation. Cellular differentiation, as well as reprogramming, results in global alterations of the transcriptional program and epigenetic modifications such as DNA methylation (18, 19). On the other hand, misregulated differentiation can lead to aberrant transdifferentiation and abnormal cellular states, such as cancer (20–22).

In this study our established in vitro circadian clock formation assay clearly shows that the constitutive expression of *c-Myc* and *Dnmt1*$^{-/-}$ disrupted the development of the circadian clock. Global gene expression analysis reveals that 484 genes are identified as candidate factors correlating with emergence of circadian clock oscillation. In failure of clock development, a significant increase of *Kpna2*, one of the candidate factors, encoding Importin-α2 and aberrant subcellular localization of PER proteins are identified as shared events. Moreover, the doxycycline (Dox)-dependent overexpression of *Kpna2* during ESC differentiation results in the significant impairment of clock

Significance

The emergence of the cell-autonomous circadian oscillator is coupled with cellular differentiation. Cellular differentiation, as well as reprogramming, results in global alterations of the transcriptional program via epigenetic modification such as DNA methylation. We here demonstrate that *c-Myc* constitutive expression and *Dnmt1* ablation disrupt the differentiation-coupled emergence of the clock from mouse ES cells (ESCs). Using these model ESCs, 484 genes were identified by global gene expression analysis as factors correlated with circadian clock development. Among them, we find that misregulation of *Kpna2* (*Importin-α2*) during the differentiation culture of ESCs significantly impairs clock development, and KPNA2 facilitates cytoplasmic localization of PER1/2. These results suggest that the programmed gene expression network regulates the differentiation-coupled circadian clock development in mammalian cells.

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Data deposition: The microarray and RNA-sequencing data reported in this paper have been deposited in Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession nos. GSE61096 and GSE61184).

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development. In addition, *Kpna2* expression facilitates cytoplasmic accumulation of PER proteins. These observations suggest that the differentiation-coupled transcriptional program of certain genes, including *Kpna2*, may critically regulate circadian clock development in mammalian cells.

Results

In Vitro Assay System Investigating Differentiation-Coupled Circadian Clock Development Using Mouse ESCs. We recently described an in vitro differentiation method to observe the development of the circadian clock from ESCs and its utility for evaluating the effect of genetic mutations on circadian clock development (15, 17). Here we use ESCs expressing *mBmal1:luc* (15) and *PER2::Luc* knock-in ($PER2^{Luc}$) (23, 24) circadian reporters to investigate the developmental mechanisms of the cellular circadian clock. Although no circadian oscillation of reporter bioluminescence was detected in these ESC lines as we previously reported in other cell lines, weak circadian bioluminescence rhythms first appeared in day-14 cultures (Fig. S1). Oscillation of bioluminescence became robust on day 21 and attained maximum amplitude on day 28 (Fig. S1). *Bmal1:luc* and $PER2^{Luc}$ ESC-derived differentiated cells showed the emergence of circadian bioluminescence at almost the same time, with nearly antiphasic rhythms, concordant with their endogenous phase relationship in the mammalian clock system.

To elucidate the molecular mechanisms underlying the observed differentiation-coupled clock development, we tried to analyze two model systems in which we perturbed mouse ESC differentiation and tested their effects on the development of circadian rhythmicity using the in vitro circadian clock formation assay: (*i*) ESCs with Dox-conditional expression of *c-Myc*, and (*ii*) ESCs deficient in DNA metyltransferases, such as $Dnmt1^{-/-}$, $Dnmt3a^{-/-}$, $Dnmt3b^{-/-}$, $Dnmt3a^{-/-}3b^{-/-}$, and $Dnmt1^{-/-}3a^{-/-}3b^{-/-}$ (triple knockout, TKO).

Dox-Inducible c-Myc Overexpression ESCs as a Perturbation Model for Differentiation-Coupled Transcriptional Program. Previous studies have shown that the MYC affects global gene expression and can induce misregulation of the transcriptional program in various cell types (21, 25, 26). Therefore, overexpression of *c-Myc* during ESC differentiation could perturb the regulatory network of the cellular differentiation process. To test the role of *c-Myc* in differentiation-coupled circadian clock development, we used Dox-inducible *c-Myc* expression in ESCs with either *mBmal1:luc* or $PER2^{Luc}$ reporters (*tetO:c-myc mBmal1:luc* ESC and *tetO:c-myc* $PER2^{Luc}$ ESC) (Fig. 1*A*). Dox treatment of these ESCs showed constitutive expression of *c-Myc* mRNA and nuclear accumulation of c-MYC protein in both ESCs (Fig. 1*B* and Fig. S2).

To investigate the development of circadian clock oscillation, the in vitro differentiation culture was performed for 28 d using these *c-Myc* inducible ESCs with (Dox^+) or without Dox (Dox^-) (Fig. 1*C*). In this condition, differentiated-cell-like morphology (Fig. 1*D*) and loss of *Nanog*, *Oct3/4*, and *Sox2* gene expression examined by quantitative PCR using the primers indicated in Table S1 (Fig. 1*E*) were observed after the differentiation under both Dox^+ and Dox^- conditions. In the in vitro circadian clock formation assay, real-time bioluminescence analysis revealed that the *mBmal1:luc*- and *PER2::Luc*-driven circadian oscillations were abolished even after 28-d differentiation culture in Dox^+ condition, whereas robust circadian oscillations were present in the Dox^- condition (Fig. 1*F*). Likewise, at the single-cell level, constitutive expression of *c-Myc* also led to significant impairment of cellular clock formation both in *mBmal1:luc* and $PER2^{Luc}$ ESCs (Fig. 1 *G–J*). These results revealed that the sustained expression of *c-Myc* during ESC differentiation resulted in the disruption of circadian clock formation.

Next we compared global transcriptional profiles by microarray analysis in cells after in vitro differentiation culture from the *tetO:c-myc* ESCs with (Dox^+) or without Dox (Dox^-). In the cells differentiated in Dox^+ condition, the expression profiles of 2,782 genes [893 up-regulated (3.8%), 1,889 down-regulated (8.0%)] in *tetO:c-myc mBmal1:luc* ESC-derived cells and 3,777 genes [2,030 up-regulated (8.6%), 1,747 down-regulated (7.4%)] in *tetO:c-myc* $PER2^{Luc}$ ESC-derived cells were changed more than twofold (Fig. 2 *A* and *B* and Dataset S1). Interestingly, core clock gene expression remained largely unchanged, whereas the expression levels of thousands of other genes were more strongly (>twofold) affected by *c-Myc* during differentiation (Fig. 2 *A* and *B*). Because these assays were performed in the absence of synchronizing agents, the levels are indicative of the mean expression levels of oscillating clock genes in these cells. Therefore, the lack of dramatic changes in the expression level of core clock genes suggests that they were not responsible for the disruption of circadian clock development during ESC differentiation.

Next we examined the effect of acute overexpression of *c-Myc* in differentiated and clock-oscillating cells. In contrast to the developing condition, acute induction of the *c-Myc* gene did not abolish circadian oscillations in the differentiated condition (Fig. S3 *A–C*). Moreover, even after an additional 28 d with Dox (a total of 56 d), the circadian rhythm in c-*Myc*-expressing cells remained robust, suggesting that after the completion of circadian clock differentiation, *c-Myc* expression does not affect the rhythm (Fig. S3 *D* and *E*). In addition, $PER2^{Luc}$ mouse embryonic fibroblasts (MEFs) expressing *tetO:c-Myc* and *CAG:rtTA* showed that acute *c-Myc* expression did not disrupt circadian oscillations but altered the phase of $PER2^{Luc}$ bioluminescence in MEFs (Fig. S3 *F* and *G*). Because induction of *c-Myc* resulted in a 1.3- to twofold increase of expression levels in *Per1*, *Per2*, *Clock*, *Rev-erbα*, and *Cry1* genes but a slight down-regulation in *Bmal1* and *Dbp* genes (Fig. S3*H*), acute over-expression of *c-Myc* affects clock gene expression patterns but does not abolish the circadian oscillation of a preexisting clock system.

Dnmt-Deficient ESCs as Perturbation Models for Differentiation-Coupled Epigenetic Regulation. Next, because it is known that cellular differentiation-coupled transition of the transcriptional network is regulated by programmed DNA methylation (18, 19), mouse ESCs lacking DNA methyltransferase(s) [$Dnmt1^{-/-}$, $Dnmt3a^{-/-}$, $Dnmt3b^{-/-}$, $Dnmt3a^{-/-}3b^{-/-}$, and $Dnmt1^{-/-}3a^{-/-}3b^{-/-}$ (TKO)] (27, 28) were used as models for perturbing epigenetic regulation during differentiation.

We initially observed morphological changes during the differentiation culture of WT, $Dnmt1^{-/-}$, $Dnmt3a^{-/-}$, $Dnmt3b^{-/-}$, $Dnmt3a^{-/-}3b^{-/-}$, and TKO ESCs (Fig. 3*A*). To evaluate the differentiation, ESC markers such as *Nanog*, *Sox2*, and *Oct3/4* were examined by quantitative PCR. Other than TKO cells, the expression of these ESC markers was dramatically reduced or lost after the 28-d differentiation culture (Fig. 3*B*). Weak expression of ESC markers was still detected in differentiated $Dnmt1^{-/-}$ cells (Fig. 3*B*), suggesting that *Dnmt1* deficiency may result in abnormal or partial differentiation.

Next, in vitro circadian clock formation was assessed in $Dnmt1^{-/-}$, $Dnmt3a^{-/-}$, $Dnmt3b^{-/-}$, $Dnmt3a^{-/-}3b^{-/-}$, and TKO mutant ESCs stably transfected with *Bmal1:luc* bioluminescence reporter. $Dnmt1^{-/-}$ ESCs failed to show normal circadian clock development, whereas robust circadian oscillations were observed in $Dnmt3a^{-/-}$ and $Dnmt3b^{-/-}$ cells after the differentiation (Fig. 3*C*). Double mutant $Dnmt3a^{-/-}3b^{-/-}$ ESCs could also develop circadian oscillations, indicating that $Dnmt3a^{-/-}$ and $Dnmt3b^{-/-}$ are not essential for circadian clock development. Single-cell analysis of 28-d differentiated $Dnmt1^{-/-}$ cells confirmed that the circadian clock was abolished at the single-cell level (Fig. 3 *D* and *E*).

To investigate the mechanism underlying clock development, we determined global gene expression profiles using next-generation sequencing comparing rhythmic cells (28-d differentiation of WT, $PER2^{Luc}$, $Dnmt3a^{-/-}$, $Dnmt3b^{-/-}$, and $Dnmt3a^{-/-}3b^{-/-}$) and

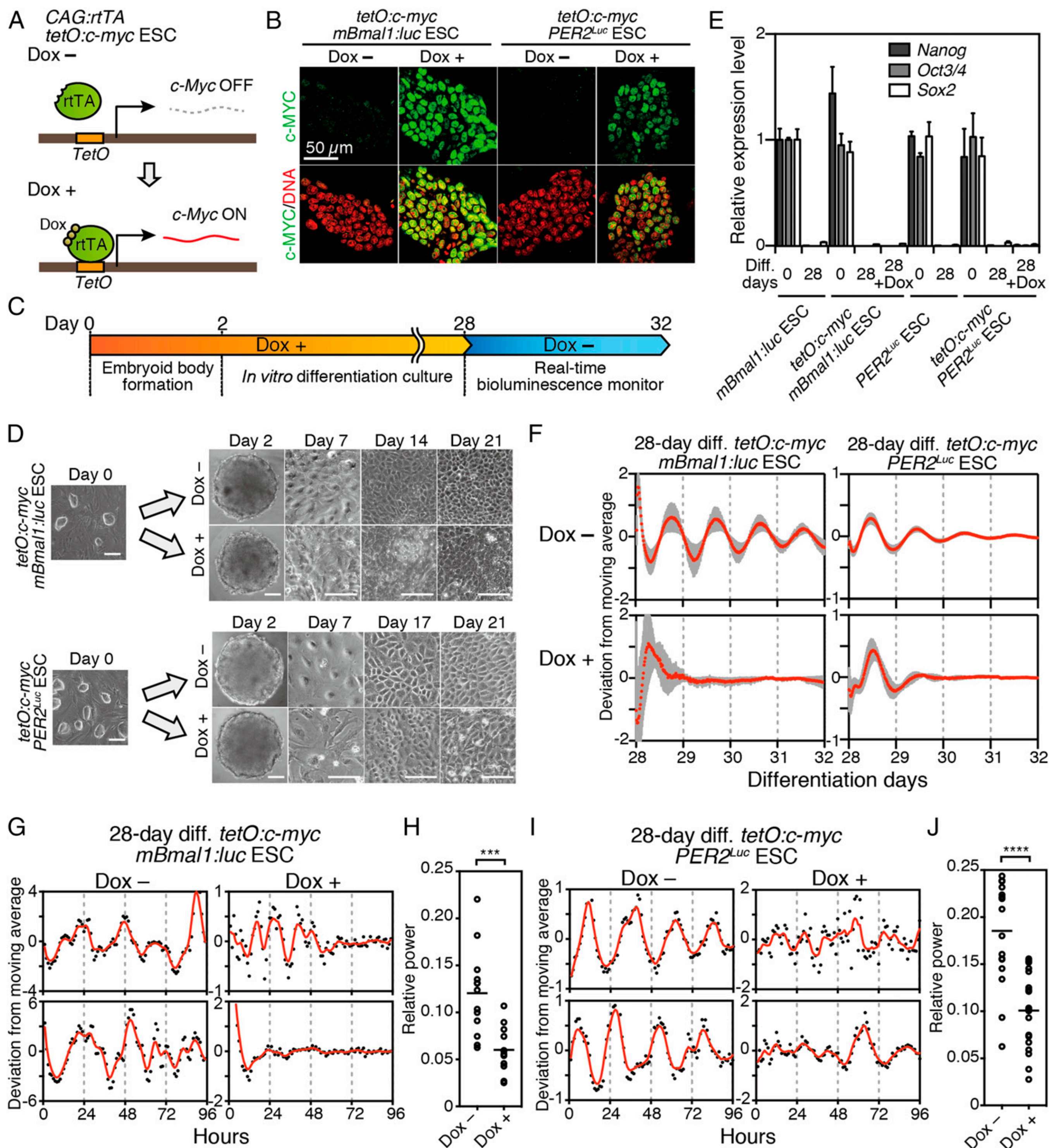

Fig. 1. Constitutive *c-Myc* expression across cellular differentiation prevents the development of circadian clock oscillation. (*A*) *mBmal1:luc* ESCs or *PER2^{Luc}* ESCs carrying Dox-inducible *c-Myc* (*tetO:c-myc mBmal1:luc* ESCs or *tetO:c-myc PER2^{Luc}* ESCs) were established using PiggyBac transposon vectors with a Dox-inducible *c-Myc*. (*B*) Immunofluorescent staining of *tetO:c-myc* ESCs using anti-c-MYC antibody. *c-Myc* was induced by addition of 40 ng/mL Dox (*Left*, *tetO:c-myc mBmal1:luc* ESCs) or 500 ng/mL Dox (*Right*, *tetO:c-myc PER2^{Luc}* ESCs) for 1 d. (*C*) Schematic of method to express *c-Myc* constitutively. During in vitro differentiation, culture medium was changed with EFM containing 0, 40 (for *tetO:c-myc mBmal1:luc* ESCs), or 500 ng/mL Dox (for *tetO:c-myc PER2^{Luc}* ESCs). (*D*) Morphological observation of in vitro 2-, 7-, 14-, and 21-d differentiated *tetO:c-myc mBmal1:luc* ESCs (*Upper*) or *tetO:c-myc PER2^{Luc}* ESCs (*Lower*) in the absence or presence of Dox. (Scale bars, 100 μm.) (*E*) Quantitative RT-PCR analysis of differentiation markers, *Nanog*, *Oct3/4* (*Pou5f1*), and *Sox2*, in ESCs or in vitro-differentiated ESCs. Data are mean ± SD ($n = 3$). (*F*) Bioluminescence rhythms of *tetO:c-myc mBmal1:luc* ESCs (*Left*) and *tetO:c-myc PER2^{Luc}* ESCs (*Right*) after 28-d differentiation culture with or without Dox. (mean with SD, $n = 12$). (*G*) Representative single-cell bioluminescence traces of in vitro 28-d-differentiated *tetO:c-myc mBmal1:luc* ESCs in the absence or presence of Dox. The red line is the LOWESS curve of the fitted values. (*H*) FFT (Fast Fourier Transform) spectral power analysis of bioluminescences of in vitro 28-d-differentiated *tetO:c-myc mBmal1:luc* ESCs in the absence or presence of Dox. Bars are mean ($n = 12$). Student *t* test, ***$P < 0.001$. (*I*) Representative single-cell bioluminescence traces of in vitro 28-d-differentiated *tetO:c-myc PER2^{Luc}* ESCs in the absence or presence of Dox. (*J*) FFT spectral power analysis of bioluminescence of in vitro 28-d-differentiated *tetO:c-myc PER2^{Luc}* ESCs in the absence or presence of Dox. Bars are mean ($n = 15–19$). Student *t* test, ****$P < 0.0001$.

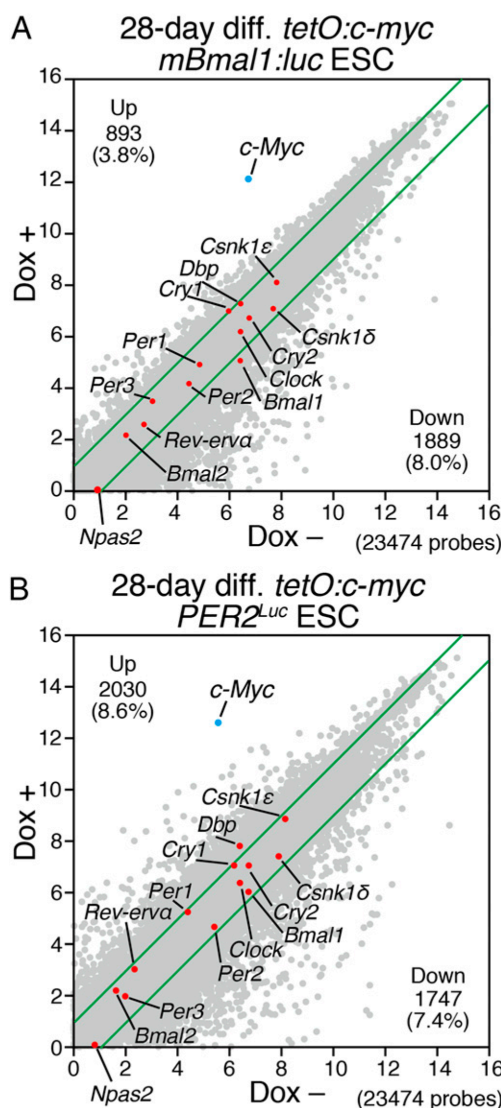

Fig. 2. Global gene expression analysis of in vitro 28-d-differentiated ESCs with *c-Myc* expression. (*A* and *B*) Microarray analysis of in vitro 28-d-differentiated *tetO:c-myc mBmal1:luc* ESCs (*A*) or *tetO:c-myc PER2^{Luc}* ESCs with/without Dox (*B*). Scatter plots of all examined gene expressions (*gray*), a set of core clock gene expressions (red), and *c-Myc* (blue) of in vitro 28-d-differentiated *tetO:c-myc mBmal1:luc* ESCs (*A*) or *tetO:c-myc PER2^{Luc}* ESCs (*B*) compared with with/without Dox. Green lines indicate twofold up- or down-changes. All data were transformed to the log 2 base scale.

nonrhythmic cells (all ESCs; 7-d differentiation of WT, *PER2^{Luc}*, *Dnmt1$^{-/-}$*, and TKO; 28-d differentiation of *Dnmt1$^{-/-}$* and TKO). First we examined the expression profiles of core clock genes in these samples (Fig. S4). Because the gene expression analysis was performed in the absence of synchronizing agents, the obtained results represented the mean expression levels of oscillating clock genes in these cells. Interestingly, critical impairment of core clock gene expression explaining the loss of circadian clock oscillation was not observed in the nonrhythmic cells.

Identification of Candidate Genes Correlating with Differentiation-Coupled Circadian Clock Development. To identify potential factors commonly altered in the clock disrupted cells and ESCs, RNA-sequencing (RNA-seq) data obtained from *Dnmt*-deficient cells and microarray data obtained from *tetO:c-myc* cells were compared. First, using the RNA-seq data of *Dnmt*-deficient cells, 2,606 overlapping genes altered under all clock-disrupted conditions (all ESCs, differentiated *Dnmt1$^{-/-}$* cells, differentiated TKO cells, and 7-d differentiated WT and *PER2^{Luc}* ESCs) were identified from the comparison with the clock-oscillating cells (Fig. 4*A* and Dataset S2). Next, genes showing similar expression changes in the differentiated cells in the Dox^{+} of *tetO:c-myc Bmal1:luc* ESCs and *tetO:c-myc PER2^{Luc}* ESCs were extracted. Compared with the Dox^{-} condition, expression of 648 genes was increased in the Dox^{+} (*c-Myc* over-expressed) condition for both cell lines, and 1,078 genes were decreased in the Dox^{+} condition (Fig. 4*B* and Dataset S3). Then, these extracted gene sets were compared with the up- or down-regulated gene sets from the RNA-seq of the clock-disrupted cells. This analysis revealed 98 up-regulated and 386 down-regulated genes shared between both clock-disrupted *Dnmt*-deficient cells and *c-Myc*-overexpressed cells (Fig. 4*C* and Dataset S4). Heat map representation shows the different patterns of expression levels between clock-oscillating (rhythmic) cells and clock-disrupted (nonrhythmic) cells (Fig. 4*D*). Because the identified 484 genes (98 genes up-regulated and 386 genes down-regulated in "Non-rhythmic cells") are candidate genes for regulating circadian clock development in mammalian cells, we next tried to validate the biological significance of this gene set as clock-development regulators. As described above, expression profiles of essential core clock genes were not predictive indicators of clock-oscillating cells. Moreover, the identified gene set did not include core clock genes (Dataset S4). Therefore, the mechanism to generate cell-autonomous circadian cycling includes additional gene networks other than the clock genes.

A previous study reported that the gene expression signature of ESCs was dissected into three functional modules: core-pluripotency factors (ESC-Core), polycomb repressive complex factors (ESC-PRC), and Myc-related factors (ESC-Myc) (25). Because circadian clock development is also closely correlated with cellular differentiation from ESCs, we compared our identified gene set with these ESC-related modules. Interestingly, the identified gene set did not overlap greatly with the factors of the ESC-Core module, ESC-PRC module, and even ESC-Myc module (Fig. S5). These results suggest that circadian clock development-related factors may form an independent functional module from other ESC-related modules.

Abnormal Cytoplasmic Accumulation of PER Proteins in Clock-Disrupted Cells and Undifferentiated ESCs. To survey the underlying mechanism for the loss of oscillation in ESCs as well as *Dnmt1$^{-/-}$* and *c-Myc*-overexpressed ESC-derived cells, immunofluorescence staining of endogenous circadian clock proteins was performed. Interestingly, in ESCs, PER1 protein was located exclusively in the cytoplasm, although a nuclear dominant expression pattern was observed in MEFs (Fig. 5*A* and Fig. S6). PER2 signals were weak, consistent with low-level expression of *Per2* mRNA, as described above. To rule out the circadian variation of PER1 subcellular localization, we investigated the temporal profile of the PER1 expression pattern. Immunofluorescence analysis revealed that the PER1 protein in ESCs was expressed exclusively in the cytoplasm throughout the day (Fig. 5*B*). Because temporal accumulation of PER proteins in the nucleus is believed to be essential for circadian clock oscillation, the cytoplasmic expression of PER1 is a possible reason why circadian clock oscillation is not exhibited in ESCs.

Next, we also examined the expression patterns of PER proteins in *Dnmt*-deficient ESCs and 28-d differentiated cells. All *Dnmt*-deficient ESCs also showed cytoplasmic accumulation of PER proteins, which was essentially identical to the expression pattern of WT ESCs (Fig. 5*C*). Clock-oscillating differentiated cells exhibited a predominant nuclear localization of PER proteins (Fig. 5*D*). However, clock-disrupted cells such as *Dnmt1$^{-/-}$* and TKO cells showed cytoplasmic localization of PERs even after the 28-d differentiation culture, similar to ESCs (Fig. 5*D*).

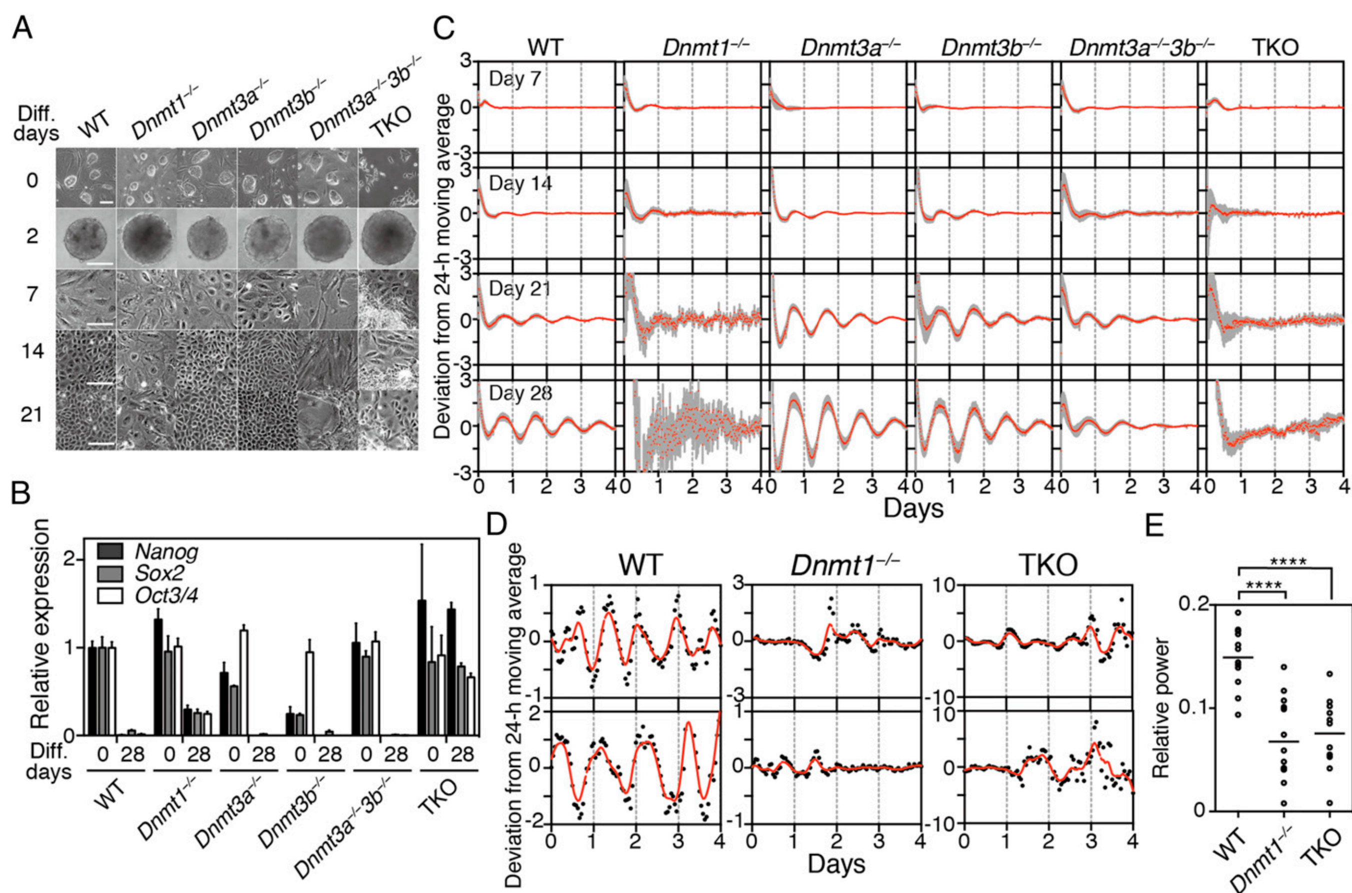

Fig. 3. Disruption of differentiation-coupled circadian clock development in *Dnmt1*$^{-/-}$ ESCs. (*A*) Morphological changes after differentiation culture of WT, *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$, *Dnmt3a*$^{-/-}$*3b*$^{-/-}$, and *Dnmt1*$^{-/-}$*3a*$^{-/-}$*3b*$^{-/-}$ (TKO) ESCs. (*B*) Quantitative RT-PCR analysis of pluripotent markers, *Nanog*, *Oct3/4* (*Pou5f1*), and *Sox2*, in ESCs or in vitro-differentiated ESCs. Data are mean ± SD (n = 3). (*C*) Averaged bioluminescence traces after in vitro 7-, 14-, 21-, or 28-d differentiation of indicated ESCs carrying *mBmal1:luc* reporters. Data, detrended by subtracting a 24-h moving average, are means with SD (n = 24). (*D* and *E*) Single-cell bioluminescence observations and FFT spectral power analysis of in vitro 28-d-differentiated indicated ESCs. The red line is the LOWESS curve of the fitted values. Each circle represents a single cell from in vitro-differentiated ESCs. Bars are mean (n = 11 or 12, one-way ANOVA followed by Bonferroni's post hoc comparisons tests, ****P < 0.0001).

Intriguingly, immunofluorescence staining of PER2 in *tetO:c-myc mBmal1:luc* ESCs after the differentiation culture also exhibited the cytoplasmic accumulation of PER2 in the Dox^{+} condition, whereas nuclear localization occurred in the Dox^{-} condition (Fig. 5*E*) In *tetO:c-myc PER2*^{Luc} ESCs, we found that *PER2::Luc* fusion proteins localized exclusively in the cytoplasm when the cells were differentiated in the Dox^{+} condition, whereas *PER2::Luc* was predominantly nuclear in localization in the Dox^{-} condition (Fig. 5*F*). Moreover, we investigated the expression of PER1 and CRY1 in 7-d differentiated WT ESCs. Notably, CRY1 protein already expressed in the nuclei of 7-d differentiated ESCs, but PER1 was still predominantly accumulated in the cytoplasm (Fig. 5*G*). These findings strongly indicate that the regulatory mechanisms of cytoplasmic accumulation of PERs occur in the clock-less conditions.

Kpna2 Functions as a Critical Factor Regulating Differentiation-Coupled Circadian Clock Development. Within the gene set correlating with circadian clock development, the *Kpna2* gene was identified as an up-regulated gene in all of the clock-disrupted cells, including ESCs (Fig. 6*A*). The *Kpna2* gene encodes the Importin-α2 protein known to regulate the nuclear translocation of transcription factors, and the switching of the expression of the Importin-α subtype from α2 to α1 is known to play an important role in ESC differentiation (29, 30). Therefore, we first analyzed the ratio of *Kpna* expression in differentiated cells against ESCs. In WT ESCs, the ratio of *Kpna2* expression in differentiated cells was dramatically reduced, whereas the expression level of *Kpna2* remained higher in *Dnmt1*$^{-/-}$ and TKO cells even after 28-d differentiation culture conditions (Fig. 6*B*). Immunofluorescence of endogenous KPNA2 protein confirmed that the strong expression of KPNA2 was observed in WT and all *Dnmt*-deficient ESCs. Strikingly, strong expression of KPNA2 protein was also seen in 28-d differentiated *Dnmt1*$^{-/-}$ and *Dnmt* TKO cells (Fig. S7 *A* and *B*). These results suggest that sustained high expression of *Kpna2* may be a causal factor blocking circadian clock development.

Next we examined subcellular localization of transiently expressed PER2 with or without EGFP-KPNA2 coexpression in MEFs. Coexpression of EGFP-KPNA2 significantly accelerated cytoplasmic accumulation of PER2 protein (Fig. S8 *A* and *B*), whereas CRY2 coexpressed with EGFP-KPNA2 was exclusively localized in the nucleus (Fig. S8 *C* and *D*). Thus, the observed immunofluorescence indicated an increase of cytoplasmic localization of PER2 by coexpression of EGFP-KPNA2 and supported the involvement of *Kpna2* in the mechanism of circadian clock development.

Next, to test the effect of *Kpna2* expression on the development of the circadian clock during ESC differentiation, we created Dox-inducible *Kpna2* overexpression *PER2*^{Luc} (*tetO:Kpna2 PER2*^{Luc}) ESCs. The expression of *Kpna2* after Dox treatment was observed in a dose-dependent fashion in these ESCs (Fig. 6*C*). We subsequently performed in vitro circadian clock formation

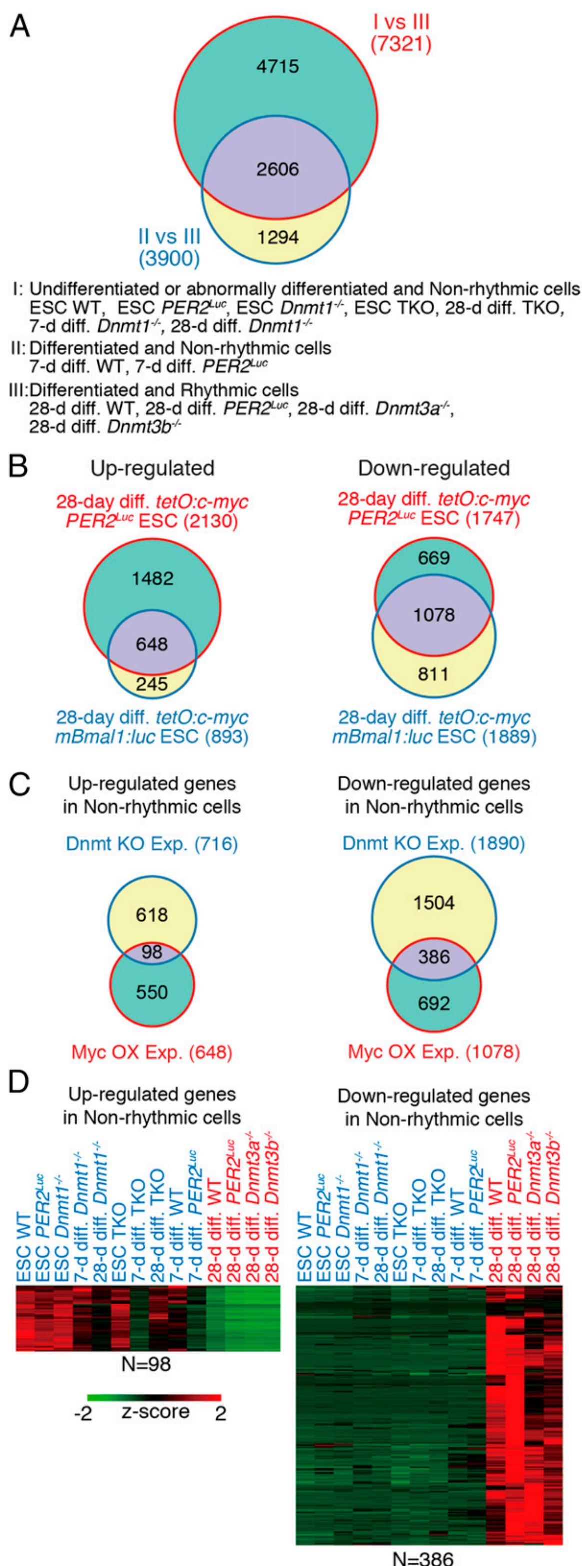

Fig. 4. Identification of correlated genes with differentiation-coupled circadian clock development. (*A*) Venn diagrams extracting common factors correlating with disruption of circadian clock development using RNA-seq data obtained from the *Dnmt*-deficient ESC model systems. Groups I, II, and III indicate condition of cells as described. (*B*) Venn diagrams extracting up- or down-regulated genes of in vitro 28-d-differentiated *tetO:c-myc mBmal1:luc* ESCs with Dox against Dox-untreated conditions. (*C*) Venn diagrams extracting the up- or down-regulated genes in clock-disrupted conditions of *Dnmt*-deficient ESCs derived cells and *c-Myc* overexpression experiments. (*D*) Heat maps of the extracted "clock development correlating-module" gene set correlating with differentiation-coupled circadian clock development.

assays using differentiation cultures with (Dox$^+$) or without Dox (Dox$^-$). Bioluminescence analysis revealed significant reduction of the *PER2^{Luc}* oscillatory amplitude in the 28-d differentiated cells with *Kpna2* overexpression (Dox$^+$) (Fig. 6 *D* and *E*). A similar tendency was also observed in a *mBmal1:luc* reporter read-out system. *TetO:Kpna2 mBmal1:luc* ESCs differentiated with or without Dox revealed that the constitutive overexpression of *Kpna2* severely suppressed the *mBmal1:luc*-driven circadian clock oscillation (Fig. 6 *F–H*), suggesting that high-level expression of *Kpna2* during ESC differentiation actually obstructed the circadian clock development. Importantly, cytoplasmic accumulation of endogenous PER1 and PER2 proteins was dramatically increased in the Dox$^+$ condition compared with the Dox$^-$ condition (Fig. 6*I*).

However, as seen by the still-remaining nuclear staining of PER2 immunofluorescence in part of the cells of Dox$^+$ condition (Fig. 6*I*), the effect of KPNA2 on the subcellular localization of PER proteins may be incomplete. Because the physical interaction between KPNA2 and PER2 was very weak (Fig. S9), abnormal cytoplasmic accumulation of PER proteins in clockless cells, including ESCs, may be an indirect effect of KPNA2. In addition, although the amplitude of the circadian clock was dramatically reduced, the differentiation-coupled *PER2^{Luc}* oscillation was still detectable even in cells of the Dox$^+$ condition (Fig. 6*D*). This suggests that other factors for circadian clock development in addition to *Kpna2* remain to be elucidated. Altogether, our results reveal that the misregulation of *Kpna2*, which was identified as a candidate factor through global gene expression analysis, resulted in the abnormal cytoplasmic accumulation of PER proteins and impairment of differentiation-coupled circadian clock development.

Discussion

We recently showed that circadian clock development is closely linked with the cellular differentiation processes (15). Because cellular differentiation is regulated by global epigenetic and transcriptional programs (31), we examined *c-Myc*-overexpressing ESCs and *Dnmt*-deficient ESCs as model systems perturbing the cellular differentiation program to understand the molecular mechanisms for circadian clock development. Cellular differentiation with constitutive expression of *c-Myc* disrupted the circadian clock development from mouse ESCs. Moreover, *Dnmt1* was found to be essential for circadian clock development during in vitro differentiation of ESCs. Through global gene expression analysis using the *c-Myc*-overexpressed and *Dnmt*-deficient ESC models, we identified a gene set correlating cellular differentiation-coupled circadian clock development in mammalian cells. Among the identified genes, we discovered that *Kpna2* encoding Importin-α2 protein functioned as a critical factor regulating the differentiation-coupled switching of subcellular localization patterns of PER proteins and circadian clock development in mammalian cells.

In this study, our model system revealed that the ectopic expression of *c-Myc* critically affected the developmental process of the circadian clock and abolished molecular oscillations even after 28 d of culture differentiation of ES cells. One possible mechanism is the direct effect of c-MYC on E-box enhancer elements competing with BMAL1/CLOCK. Previously it has been reported that MYC can recognize the BMAL1/CLOCK E-box enhancer and up-regulate E-box-driven clock genes (32, 33). In this study we examined global gene expression profiles with or without *c-Myc* expression during ESC differentiation. Interestingly, clock gene expression profiles were not dramatically

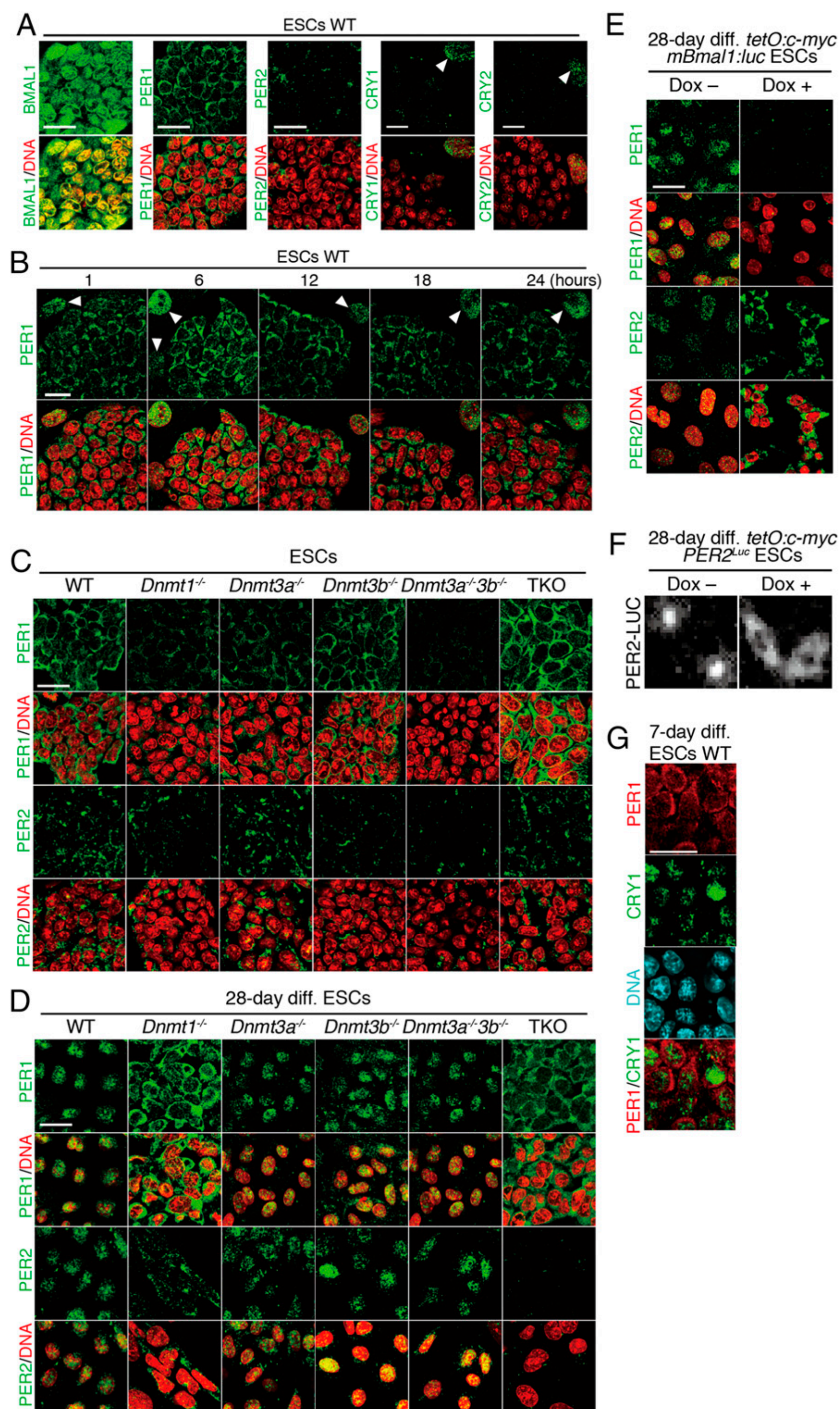

Fig. 5. Cytoplasmic PERs in clock-disrupted conditions. (*A*) Immunofluorescence study of endogenous BMAL1, PER1, PER2, CRY1, and CRY2 proteins in WT ESCs. (*B*) Temporal expression pattern of PER1 protein in WT ESCs after medium change. Arrowheads indicate nucleus of feeder cells. (*C*) Immunofluorescence of PER1 and PER2 proteins in *Dnmt*-deficient ESCs (WT, *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$, *Dnmt3a*$^{-/-}$*3b*$^{-/-}$, and TKO). (*D*) Immunofluorescence of PER1 and PER2 proteins in in vitro 28-d-differentiated *Dnmt*-deficient ESCs. (*E*) Immunofluorescence of PER1 and PER2 proteins in in vitro 28-d-differentiated *tetO:c-myc mBmal1:luc* ESCs with/without Dox. (*F*) Bioluminescence of PER2-Luciferase in in vitro 28-d-differentiated *tetO:c-myc PER2*^{Luc} ESCs with/without Dox. (*G*) Immunofluorescence double staining of PER1 and CRY1 in vitro 7-d-differentiated WT ESCs. (Scale bars, 25 μm.)

changed even in the *c-Myc*-overexpressed condition (Fig. 2). On the other hand, MYC is known to play a fundamental role in global gene expression and can induce misregulation of the transcriptional program in various cell types (21, 25, 26). Consistently, the expression of thousands of genes was dramatically changed in the differentiated cells with *c-Myc* overexpression. Thus, our investigation using *c-Myc* inducible mouse ESCs indicates an indirect role of *c-Myc* in preventing circadian clock development as opposed to directly abolishing clock gene expression.

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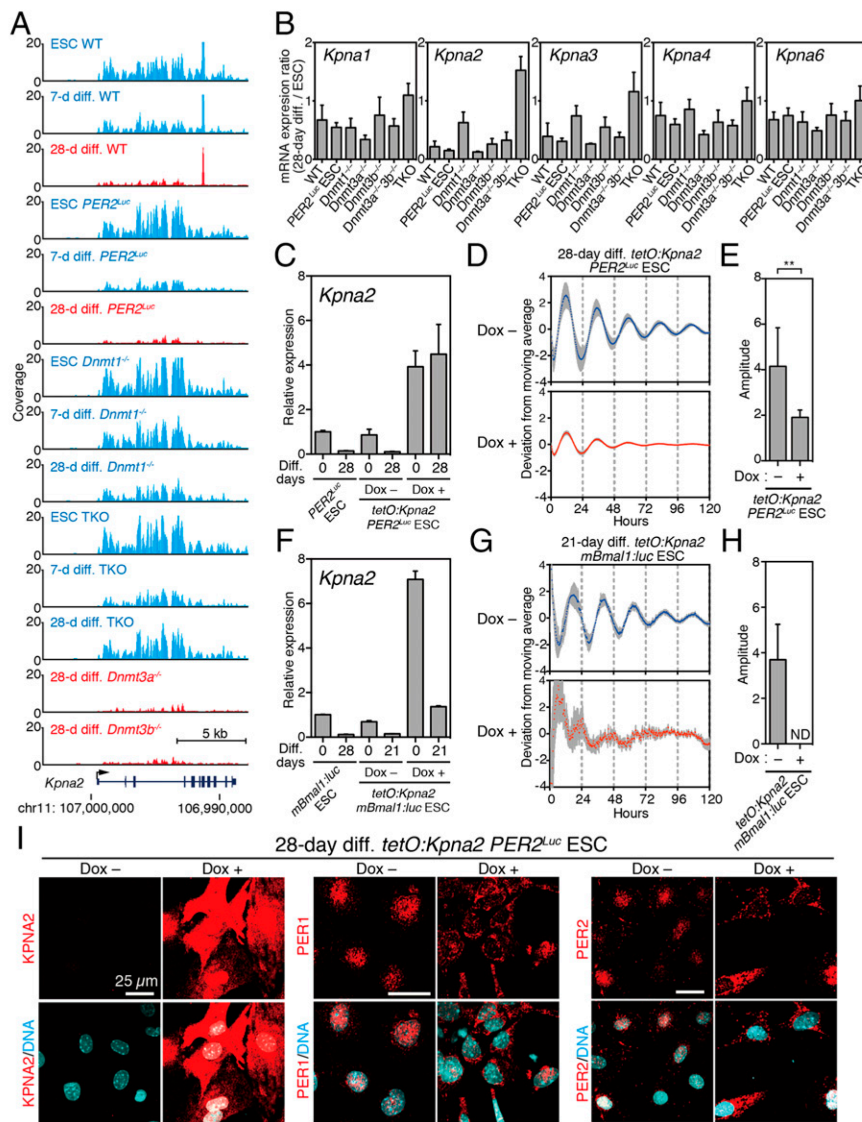

Fig. 6. Constitutive *Kpna2* expression during ESC differentiation inhibits the development of circadian rhythm. (*A*) University of California, Santa Cruz (UCSC) genome browser view of *Kpna2* locus detected as a candidate factor is shown in nonrhythmic cells (blue) and rhythmic cells (red). (*B*) Quantitative RT-PCR analysis of *Kpna1*, *Kpna2*, *Kpna3*, *Kpna4*, and *Kpna6* in in vitro 28-d-differentiated ESCs relative to undifferentiated ESCs. Data are mean ± SD ($n = 3$). (*C*) Relative gene expression of *Kpna2* in *tetO:Kpna2* ESCs or in vitro 28-d differentiation with/without Dox (500 ng/mL). *PER2^{Luc}* ESC represents a control ESC. Data are mean ± SD ($n = 3$). (*D*) Averaged bioluminescence traces of in vitro 28-d-differentiated *tetO:Kpna2* ESCs with (red) or without (blue) Dox treatment. Data detrended by subtracting a 24-h moving average are mean with SD ($n = 6$). (*E*) *PER2^{Luc}*-driven bioluminescence of in vitro 28-d-differentiated *tetO:Kpna2* ESCs with or without Dox (Student *t* test, $^{**}P < 0.01$). (*F*) Relative gene expression of *Kpna2* in *tetO:Kpna2 Bmal1:luc* ESCs or in vitro 28-d differentiation with/without Dox (500 ng/mL). Data are mean ± SD ($n = 3$). (*G*) Averaged bioluminescence traces of in vitro 28-d-differentiated *tetO:Kpna2 Bmal1:luc* ESCs with (red) or without (blue) Dox treatment. Data detrended by subtracting a 24-h moving average are mean with SD ($n = 6$). (*H*) *Bmal1:luc*-driven bioluminescence of in vitro 28-d-differentiated *tetO:Kpna2* ESCs with or without Dox (Student *t* test, $^{**}P < 0.01$). (*I*) Immunofluorescence study against KPNA2, endogenous PER1, and endogenous PER2 in 28-d-differentiated *tetO:Kpna2 PER2^{Luc}* ESCs. DNA was stained by Hoechst 33342.

In this study we show that *Dnmt1* is essential for the differentiation-coupled development of circadian clock oscillations. In contrast, even *Dnmt3a$^{-/-}$3b$^{-/-}$* ESCs were able to exhibit circadian clock oscillations after differentiation culture, suggesting that *Dnmt3a* and *Dnmt3b* are not essential for circadian clock development. *Dnmt3a* was recently reported to affect age-related alteration of circadian behavioral rhythms (34), suggesting that *Dnmt3a* and/or *Dnmt3b* may modulate circadian rhythms under certain conditions. In comparison, DNMT1 plays an essential role in the differentiation of mammalian cells by maintaining the epigenetic landscape of DNA methylation, and disturbance of its function during the cellular differentiation critically affects global transcriptional programs and following alteration of cell states (19, 22). Thus, the findings shown in this study indicate that circadian clock development likely shares fundamental mechanisms with cellular differentiation.

Through the analysis of our mutant ESC models, we found the misregulation of *Kpna2* expression, reported as one of the key factors regulating ESC differentiation (30), as a common factor affecting circadian clock development. In ESCs as well as the differentiated cells that failed to generate clock oscillation, significantly higher expression of *Kpna2* gene was observed compared with cells with functional clock. Our investigation also reveals that sustained ectopic expression of *Kpna2* during ESC differentiation suppressed circadian clock development. Recently it has been reported that subtype switching of Importin-α2

(KPNA2) expression to α1 (KPNA1) is observed during differentiation of ESCs, and Importin-α2 negatively regulates the nuclear import of Oct6 to inhibit ESC differentiation (29, 30). Our study revealed that KPNA2 acts as a key factor controlling circadian clock development. This mechanistic link between ESC differentiation and clock development strongly suggests that circadian clock development and cellular differentiation share a common pathway and may mutually regulate their process in mammalian cells.

In ESCs as well as differentiated *c-Myc*-overexpressed and *Dnmt1*$^{-/-}$ ESCs, we found that the PER proteins did not localize in the nucleus; rather they exclusively accumulated in the cytoplasm. Because PER translocation to the nucleus in critical for the generation of circadian oscillations in mammals, this defect may underlie the lack of a functional clock in these cells (6, 9, 35–37). We also demonstrate that the overexpression of *Kpna2* in differentiating ESCs makes the subcellular localization pattern of PER2 shift much more cytoplasmic. These findings are compatible with the results obtained through the immunofluorescence studies showing exclusively cytoplasmic accumulation of PER proteins as a common feature in clock-disrupted conditions and ESCs. Thus, it is conceivable that the misregulation of *Kpna2* expression via the disturbance of epigenetic and transcriptional programs during differentiation plays a distinct role in blocking circadian clock development. On the other hand, overexpression of *Kpna2* does not completely abolish the clock development or PER2 nuclear localization. These results therefore indicate that additional mechanisms also regulate clock development.

Taken together, our studies using *c-Myc*-expressing ESCs as well as *Dnmt1*$^{-/-}$ ESCs show that the misregulation of the differentiation-coupled transcriptional program may critically affect not only the conversion of the cell state but also the development of the cellular circadian clock during cellular differentiation processes. Our findings suggest that *Kpna2* functions as a key factor for clock development by modulating subcellular localization patterns of PER proteins. Of course, because there are hundreds of misregulated genes other than *Kpna2*, multiple pathways or mechanisms are likely to contribute to circadian clock development during differentiation. Our study highlights the importance of "clock development regulating factors" regulated by the differentiation-coupled transcriptional program in the emergence of the circadian clock in developing mammalian cells.

Materials and Methods

Cell Culture. Two ESC lines (KY1.1 mentioned as WT in text and *PER2*^{Luc} ESC) were used (15, 23, 24). In the *PER2*^{Luc} ESCs the endogenous *PER2* gene is replaced by a fusion of *PER2* and firefly luciferase gene, and an additional SV40 polyadenylation site was inserted downstream of the luciferase (23). *Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, *Dnmt3b*$^{-/-}$, *Dnmt3a*$^{-/-}$*3b*$^{-/-}$, and TKO ESCs (27, 38) were kindly provided from Dr. Masaki Okano (RIKEN, Kobe, Japan). These ESC lines were cultured on a feeder layer of mitomycin C-treated primary MEFs in an ES medium containing Glasgow Minimum Essential Medium (Wako) supplemented with 15% (vol/vol) FBS (HyClone), 0.1 mM MEM nonessential amino acids (Nacalai Tesque), 0.1 mM 2-mercaptoethanol (Sigma), 1,000 U/mL of leukemia inhibitory factor (LIF), and 100 U/mL of penicillin–streptomycin (Nacalai Tesque). For preparation of *PER2*^{Luc} knock-in MEFs (24) or WT MEFs, embryos were collected at embryonic day 15.5 (E15.5). After removal of the head and visceral tissues, the remaining bodies were washed in a fresh PBS and minced, and the isolated cells were maintained in embryonic fibroblast medium (EFM) as mentioned below.

COS-7 cells were cultured in high-glucose DMEM supplemented with 10% (vol/vol) FBS.

In Vitro Differentiation. In vitro differentiation of ESCs was performed as described recently (17). Briefly, after ESCs were trypsinized and feeder cells were removed, embryoid bodies (EBs) were generated by harvesting 2,000 cells and seeding them onto low-attachment 96-well plates (Lipidure Coat, NOF) in a differentiating medium without LIF supplementation (EFM), which was composed of a high-glucose DMEM (Nacalai Tesque) containing 10% FBS, 1 mM sodium pyruvate (Nacalai Tesque), 0.1 mM nonessential amino acids, GlutaMax-I (Invitrogen), 100 μM β-mercaptoethanol, and 100 U/mL penicillin–streptomycin. Two day later, EBs were plated onto gelatin-coated tissue culture 24-well plates and grown for several additional weeks. EFM was changed every 1–2 d.

For constitutive expression of *c-Myc* or *Kpna2* during in vitro differentiation of ESCs carrying Dox-inducible *c-Myc* or *Kpna2*, EFM containing Dox (Invitrogen) was changed every 1 to 2 d. For a real-time monitoring analysis, Dox was removed by washing three times with EFM.

Detailed methods are provided in *SI Materials and Methods*.

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