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miRNAs are required for generating a time-delay critical for the circadian oscillator

Rongmin Chen[1], **Matthew D'Alessandro**[1], and **Choogon Lee**[1,2]

[1]Department of Biomedical Sciences, Neuroscience program, College of Medicine, Florida State University, 1115 West Call Street, Tallahassee, FL 32306, USA

Abstract

Background—Circadian clocks coordinate an organism's activities and regulate metabolic homeostasis in relation to daily environmental changes, most notably light/dark cycles. As in other organisms, the timekeeping mechanism in mammals depends on a self-sustaining transcriptional negative feedback loop with a built-in time delay in feedback inhibition. Although the time delay is essential for generating a slow, self-sustaining negative feedback loop with a period close to 24 hours, the exact mechanisms underlying the time delay are not known.

Results—We show here that RNA interference mediated by microRNAs (miRNAs) is an essential mechanism in generating the time delay. In *Dicer*-deficient (and thus miRNA-deficient) cells and mice, circadian rhythms were dramatically shortened (by ~2 hours), although the rhythms remained robust. The period shortening was caused by faster PER1 and PER2 translation in the *Dicer*-deficient cells. We also identified three specific miRNAs that regulate *Per* expression, and showed that knockdown of these miRNAs in wild-type cells also shortened the circadian period.

Conclusions—Consistent with the canonical function of miRNAs as translational modulators of target genes and their widespread roles in cell physiology, circadian rhythms are also modulated by miRNA-mediated RNA interference acting on posttranscriptional regulation of key clock genes. Our present study definitively shows that RNA interference is an important modulator of circadian rhythms by controlling the pace of PER synthesis, and presents a novel layer of regulation for the clock.

Introduction

In mammals, a master clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus drives circadian rhythms, adjusts itself according to light input, and synchronizes clocks in peripheral tissues such as liver and kidney [1–3]. It is now known that in addition to the SCN, peripheral tissues and even cultured cells have self-sustaining clocks whose molecular mechanism is very similar to that of the master clock [4–8]. Findings from studying cultured cells are faithfully reproduced in intact animals [6, 8–10].

As in model organisms such as *Neurospora* and *Drosophila*, the circadian clock in mammals depends on interacting transcriptional/translational feedback loops [11–13]. The backbone

[2]Correspondence: Choogon Lee, Department of Biomedical Sciences, Florida State University, 1115 West Call Street, Tallahassee, FL 32306, Ph: 850-645-1478, Fax: 850-644-5781, Choogon.lee@med.fsu.edu.

of the clock is a negative transcriptional feedback loop with delayed feedback inhibition. Within this feedback loop, the heterodimer of CLOCK (or its paralog NPAS2) and BMAL1 activates transcription of the negative elements, *Per* and *Cry*, as well as circadian output genes. As PER1 and PER2 levels rise in the cytoplasm, PER associates with CRY (1 and 2) and is progressively phosphorylated by PER kinases CK1δ/ε. Then the complexes translocate into or accumulate in the nucleus, where PER:CRY binds directly to CLOCK:BMAL1 to inhibit transcription [8, 14–16]. Additional feedback loops are generated by nuclear receptors of the REV-ERB and ROR families, which are responsible for generating *Bmal1* oscillations and may also be involved in direct regulation of other clock genes [17–20]. Although PER, CRY, CLOCK (or NPAS2), and BMAL1 are all essential components of the main feedback loop, PER is of special importance for clock regulation. PER is the rate-limiting component involved in period and phase determination and in phase resetting of the circadian oscillator [21–24]. PER levels vary dramatically over the course of a circadian cycle [18, 24], and the endogenous rhythm in PER levels is required for clock function [8].

The time delay between generation of the transcriptional inhibitors (PER and CRY) and execution of the feedback inhibition is critical for the circadian feedback loop. Although the mechanisms for the time delay remain unclear, the time delay seems to be mainly mediated by slow accumulation of PER in the cytoplasm, as has been shown in flies [24–28]. In both mouse and *Drosophila*, PER is targeted for degradation following phosphorylation by CK1δ/ε and PER becomes more stable in the cytoplasm in the absence of CK1δ/ε [25, 29]. However, it has been shown that mouse PER accumulation in the cytoplasm is only modestly altered in CK1δ/ε-deficient cells, but PER is constitutively cytoplasmic in the cells, suggesting that PER phosphorylation by CK1δ/ε in the cytoplasm may be more important for timing of nuclear entry [29]. Considering that the accumulation of PER proteins is delayed several hours relative to *Per* mRNA, in both liver and SCN [24, 30], other mechanisms must also contribute to the delayed accumulation of PER in the cytoplasm and delayed nuclear entry for feedback inhibition.

MicroRNAs (miRNAs) are small, noncoding RNAs (~22 nucleotides) that normally inhibit translation of target genes by base-pairing with the 3′-untranslated region (3′-UTR) of the target gene mRNAs as part of RNA-induced silencing complexes (RISCs) [31, 32]. Since it has been proposed that more than one third of human genes are regulated by miRNAs in diverse aspects of cell physiology [33, 34], and multiple miRNA species have been implicated in the molecular clock and output pathways in several model organisms [35–41], it is highly likely that RNAi regulation plays critical roles in the mammalian clock. In an extensive in vivo study, Cheng et al. implicated miR-219 and 132 in the time-keeping mechanism: brain-specific miRNA219 was implicated in period determination, while miRNA132 was implicated in photic entrainment [36]. However, direct target genes for these miRNAs have not been identified. Other studies implicated different specific miRNAs in the regulation of clock genes such as *Per* and *Bmal1*, but it has not been shown how these miRNAs are integrated into the circadian system and the function of these miRNAs remains to be tested in vivo systems [38, 39]. In another extensive study searching for clock-relevant miRNAs in *Drosophila*, Kadener et al. demonstrated that a developmental regulator, *bantam*, can affect the circadian clock through translational regulation of *clock* (*clk*) [37]. Although it is imperative to understand how RNAi is involved in the time keeping mechanism, previous studies concerning RNAi in the clock mechanism have focused on specific miRNA species, and their roles in the clock mechanism were as subtle modulators rather than significant regulators. To systematically study how the miRNA-mediated regulation is integrated with the timekeeping mechanism, we employed a decisive and fundamental approach using *Dicer* mutant mice in which miRNA processing is globally compromised. Our current studies using *Dicer* mutant cells demonstrate that RNA interference mediated by

miRNAs primarily affects the clock through translational control of *Per* in the cytoplasm, which delays cytoplasmic PER accumulation and thus generates a time delay in the circadian feedback inhibition. Our in vivo and in vitro studies have identified three miRNAs, miR-24, 29a and 30a, that affect the circadian clock through regulation of *Per1* and *2* mRNA stability and translation. Thus, our studies exposed a novel mechanism for regulation of the pacemaker genes, *Per1* and *Per2,* and for generating the time delay crucial for the circadian feedback loop.

Results

Period of the circadian clock is shortened in the absence of Dicer

To begin to determine if RNA interference mediated by microRNAs is involved in the time-keeping mechanism, we measured the circadian clock in a *Dicer* mutant mouse in which biogenesis of mature miRNAs is globally disturbed. A conditional *Dicer* mutant mouse was generated by Harfe et al. [42] so that *Dicer* could be retained during early development, when it is essential, and disrupted later to enable researchers to study its roles in later development and adulthood. While we were generating mice combining the floxed *Dicer* mutation with different *cre* drivers, to study miRNA involvement in the clock in whole animals, we tested the roles of miRNA in the clock mechanism in a simpler system, $Dicer^{flox/flox}$; $Per2^{Luc}$ mouse embryonic fibroblasts (MEFs). The $Per2^{Luc}$ allele involves a knockin of luciferase into the 3′ end of the *Per2* gene coding sequence [5]. The resulting *Per2:Luciferase* fusion RNA retains the full length 3′-UTR of *Per2* (which may be a target of miRNA), and the fusion protein retains the circadian function of PER2 while serving as a bioluminescent reporter. We deleted the floxed *Dicer* in the MEFs by introducing *cre* using an adenoviral vector. This method of transgene delivery is highly efficient (>95% delivery) [8, 29]. The adenovirus-*cre*-infected cells were grown for 5–6 days with two passages to deplete miRNAs in the *Dicer*-deleted cells. During this time, cells grew a little slower than adenovirus-*gfp*-infected control cells, but remained viable and morphologically normal as described previously by Harfe et al [42]. *Dicer* deletion was verified by PCR-genotyping and immunoblotting of these cells (Fig 1A, 1B, S1A).

Depletion of most miRNAs was confirmed by quantitative real-time PCR using an miRNA PCR array which includes 88 of the most abundantly expressed mouse miRNAs (Fig 1C, Table S1). In cells collected 7–8 days after adenovirus-*cre* infection, most miRNA levels were reduced more than 10-fold, while small control RNAs (like Rnu6) were unaffected by loss of *Dicer*.

In terms of circadian rhythms in bioluminescence, CRE-expressing (i.e., *Dicer*-deficient) cells exhibited robust rhythms but a dramatically shorter period (by ~2 hrs) than GFP (*Dicer*-intact) cells (Fig 2A, S1B). These data suggest that miRNA-mediated RNAi may not be essential for the circadian feedback loop, but does strongly regulate the pace of the clock. The baseline of the bioluminescence rhythms was higher in *Dicer* mutant cells compared to control cells (Fig 2A), which is striking considering that there were fewer cells per dish due to the slow growth rate of *Dicer* mutant cells (Fig S1C). Clearly, PER2-LUC expression levels per cell were substantially increased when *Dicer* was disrupted.

The shortened period must be due to *Dicer* deletion by CRE, not by CRE itself, because CRE-expressing wild-type (wt) cells do not show a significant difference in circadian period compared to GFP-expressing wt cells, as shown previously [29]. Comparable period shortening was also observed in *Dicer*-deficient MEFs without the $Per2^{Luc}$ knockin but infected with adenovirus-*Per2* promoter-*Luc* (a transient transgenic reporter) (Fig 2B), confirming that the effect is not due to abnormalities caused by insertion of luciferase into the *Per2* locus.

Using mice with a transgenic *cre* driver, we determined whether *Dicer* deficiency shortens the circadian period not just in dispersed cells, but also in whole animals. *Dicer*$^{flox/flox}$/*CAG-cre-Esr1* mice were given mouse chow mixed with tamoxifen to trigger *Dicer* deletion [43], while their daily behavior was being monitored. The circadian period gradually shortened, and the mice died within 1–5 weeks after receiving tamoxifen (Fig 2C, S2A). However, the same treatment did not affect viability or circadian period in control mice with only the *Dicer*$^{flox/flox}$ allele or only the *CAG-cre-Esr1* transgene, demonstrating that *Dicer* deletion produced the effects. The degree of period shortening— calculated by phase angle change in the last several days —was ~1 hr, half of that observed in *Dicer*-mutant MEFs. This modest effect is probably due to incomplete deletion of floxed *Dicer* in the SCN, because deletion of floxed genes in vivo was less efficient than in vitro. This is supported by genotyping PCR of brain tissue, performed when we tested two different *cre* drivers before selecting *CAG-cre-Esr1*. The *CAG-cre-Esr1* transgenic mouse produced greater period shortening and more efficient deletion of the floxed *Dicer* allele than *Scg2:tTA/tetO:cre* transgenic mice based on PCR-genotyping of brain tissue, but deletion of *Dicer* in the brain occurred in only about half of the cells (Fig S2B). The period shortening is not a non-specific consequence of deleting essential genes: deletion of *CK1δ* affected viability but induced period lengthening, not shortening [29, 44].

The period shortening is caused by faster accumulation of PER in cytoplasm

Since the circadian oscillator runs substantially faster in the *Dicer* mutant cells, some of the core clock components must have been affected by disrupted miRNA biogenesis in the mutant cells. We hypothesized that protein levels of the affected core clock components would be elevated in the *Dicer* mutant cells if they are direct targets of certain miRNAs. Among all the core clock proteins (CLOCK, BMAL1, PER1, PER2, CRY1, CRY2, CK1δ, and CK1ε), we found that only PER1 and PER2 levels were significantly elevated in the mutant cells (Fig 3A, 3B, S3A), suggesting that the mRNA of these two genes may be the direct targets of the miRNAs whose biogenesis is disrupted in the absence of Dicer. *Per1* mRNA levels were not affected while *Per2* mRNA levels were only slightly elevated in the *Dicer* mutant cells (Fig 3C). Since *Per* mRNA levels are down-regulated when PER protein is overexpressed [8], these data (high or unchanged mRNA levels in the face of higher levels of inhibitory protein) suggest that miRNAs probably affect *Per1* and *Per2* mRNA through both translational inhibition and mRNA degradation. Consistent with elevated levels of the limiting circadian inhibitors, mRNA levels of clock-controlled genes such as *Dbp* and *Rev-Erbα* were significantly reduced (Fig 3C).

To test if mRNAs of *Per1/2 genes* are direct targets of miRNAs, we indirectly assessed the translation rate of *Per2-Luc* by measuring real-time accumulation of PER2-LUC between control and *Dicer*-deleted cells, after depletion of existing PER2-LUC by cycloheximide (CHX) treatment. PER2-LUC accumulates faster and peaks earlier in *Dicer*-deficient cells (Fig 3D), suggesting that the pace of PER2 cytoplasmic accumulation is regulated by miRNA. In Fig 3D, while the upswing of the bioluminescence rhythms was shortened in the *Dicer* mutant cells, the downswing was not significantly affected, suggesting that the period shortening in *Dicer* mutant cells is generated almost exclusively from the accumulation phase of the inhibitor in the feedback loop. This is consistent with the asymmetric bioluminescence traces for *Dicer* mutant cells in Fig 2A and S1B. In all of the traces for *Dicer* mutant cells, the upswing is shorter than the downswing, while the traces for the control cells are symmetric (Fig 3E).

If the period shortening is indeed caused by a reduced time delay between transcription and feedback inhibition by the protein product, faster accumulation of the protein product and resulting earlier feedback inhibition should be observed in *Dicer* mutant cells. We measured *Per* mRNA and protein after CHX treatment, under the same conditions used for Fig 3D;

this approach creates similar starting points for comparing control and Dicer mutant cells: PER and its inhibitory action on *Per* transcription have been depleted, and thus when CHX is removed, the pace of PER accumulation and *Per* inhibition can be readily compared. In *Dicer*-mutant cells, PER1 and PER2 protein accumulated and peaked faster (Fig 4A, B), and feedback inhibition was accelerated based on earlier down-regulation of mRNA levels in *Dicer* mutant cells (Fig 4C). However, consistent with the bioluminescence rhythms, PER degradation rates were not significantly affected in *Dicer* mutant cells (Fig 4D). These results further support that the period shortening is caused by faster accumulation of the limiting clock component PER rather than even compression of the whole cycle or faster relief of feedback inhibition through accelerated degradation of PER.

Overexpression of 3′-untranslated regions (3′-UTR) of *Per1* and *Per2* mRNA causes period shortening

Target sites for miRNAs are highly conserved across different species and generally found in the 3′-UTR of mRNAs [33, 45, 46]. If certain miRNAs interfere with translation of *Per1* and *Per2* genes through their interaction with the 3′-UTR of these genes, and disruption of this mechanism caused the period shortening in the *Dicer* mutant cells, as suggested by the data above, then, we hypothesized, overexpression of the full-length 3′-UTRs of the *Per1* and *Per2* genes would produce similar results as in *Dicer* mutant cells. Since miRNAs bind target mRNAs stably through the protein complex, RISC, the overexpressed *Per1* or *Per2* 3′-UTR would deplete endogenous miRNA molecules specific to the UTRs, similar to dominant negative approaches, which have been successfully used to study the function of many clock genes [8, 47–49].

When *Per1* or *Per2* 3′-UTR was overexpressed in wt $Per2^{Luc}$ MEFs, we observed bioluminescence rhythms with a shortened period, as seen in *Dicer* mutant cells, suggesting that these UTRs indeed contain target sites for miRNA-induced translational regulation of *Per1* and *Per2* (Fig 5). The period shortening caused by overexpression of either *Per1* or *Per2* 3′-UTRs was a little less than that in *Dicer* mutant cells. Overexpression of both UTRs also did not cause as much period shortening as in *Dicer* mutant cells, probably because the dominant negative approach is less efficient in disrupting the relevant miRNAs than the loss of Dicer function. However, we cannot rule out the possibilities that clock-relevant miRNA target sites may also reside in the coding region of *Per1/2* or in the mRNA of other clock genes. Due to high transduction rates of the adenoviral vector, the levels of exogenous 3′-UTRs were hundreds of times higher than those of endogenous 3′-UTR and remained high 7 days after the infection (Fig S3B).

miR-24, 29a and 30a regulate the circadian clock by interfering with translation of *Per1* and *Per2*

Since our data strongly indicate that *Per1* and *Per2* genes are regulated by miRNAs at the posttranscriptional level, we sought to determine which miRNAs are specifically involved in the timekeeping mechanism through interaction with the 3′-UTRs of *Per1* and *Per2.* To identify *Per*-targeting miRNAs, we used the TargetScanMouse algorithm developed by the Bartel laboratory, which predicts miRNA:mRNA pairs by searching 3′-UTRs with conserved complementary sequence to the seed sequence (nucleotides 2–7) of the miRNAs [33]. The accuracy is greatly improved if the Watson-Crick base-pairing is extended immediately downstream (nucleotide 1) or upstream (nucleotide 8) of the miRNAs beyond the seed match. Using the algorithm, we identified several matching miRNAs, of which the three most promising species are miR-24, which could target both *Per1* 3′-UTR and *Per2* 3′-UTR; miR-29a, which could target *Per1* 3′UTR; and miR-30a, which could target *Per2* 3′-UTR (Fig 6A). All of these miRNAs exhibit perfect 7mer or 8mer matches to the target sites in the 3′UTR of *Per1*/2, and the target sites in the 3′UTRs were conserved across most

mammalian species (Fig S4). These miRNAs were included in the miRNA PCR array described above, and their levels were reduced by >10-fold in *Dicer* knockout cells (see Table S1).

The first functional tests were luciferase reporter assays in transiently transfected NIH 3T3 cells. To test if these sites are regulated by miRNAs, these sites were mutated in the UTRs and the effect of the mutations on expression of the luciferase reporter was measured. Mutations of either miR-24 or miR-29a target sites in *Per1* 3′-UTR only increased luciferase activity slightly, but mutation of both target sites dramatically increased the luciferase activity, suggesting that *Per1* is regulated by these two miRNAs in a combinatorial manner (Fig 6B). Similar combinatorial regulation was also observed for *Per2* 3′-UTR and the corresponding miRNAs (Fig 6B). The increase in expression of luciferase associated with mutant *Per* 3′-UTR was greater in NIH 3T3 cells compared to MEFs, probably because endogenous levels of the relevant miRNAs are higher in NIH 3T3 cells (Fig S5A).

To test further if these sites are major target sites, we overexpressed *Per1* 3′-UTR with mutated miR-24 and 29a sites and *Per2* 3′-UTR with mutated miR-24 and 30a sites in wt $Per2^{Luc}$ MEFs. Our data showed that overexpression of *Per1* or *Per2* wt 3′-UTR induces significant period shortening (Fig 5); if the UTRs mediate this effect by sequestering endogenous *Per*-targeting miRNAs, then overexpression of the mutant 3′-UTRs that cannot bind the specific miRNAs would not induce period shortening. Indeed, although expression levels of these mutant 3′-UTR were comparable to those of wt 3′-UTRs in the cells, the period was not significantly altered (Fig 6C, D) indicating that these sites are functional and clock-relevant miRNA target sites in vivo.

Finally, to test if miR-24, 29a and 30a are involved in the period determination, we knocked down these miRNAs in wt $Per2^{Luc}$ cells using Locked Nucleic Acid (LNA)-antisense oligonucleotides [50] and measured the period of the bioluminescence rhythms. As in *Dicer* mutant cells, the period of the circadian clock was significantly shortened in wt $Per2^{Luc}$ cells treated with the antisense oligonucleotides to the miRNAs (Fig 6E), demonstrating that these miRNAs are indeed involved in the timekeeping mechanism. The antisense oligonucleotide-treated cells, like *Dicer* mutant cells, had elevated basal levels of PER2-LUC, consistent with a role for the miRNAs in modulating PER synthesis. miR-24, 29a and 30a are likely to be involved in the timekeeping mechanism in most, if not all tissues, since they are widely expressed in fibroblasts, U2OS cells, and liver (liver data are shown in Fig S5B). These miRNAs did not exhibit significant circadian oscillations in liver, suggesting that miRNA-induced *Per* regulation is not time-gated (Fig S5B).

Discussion

The time delay, between transcription of *Per* and *Cry* and PER:CRY-mediated feedback inhibition, is one of the main features of the circadian feedback loop, but the underlying mechanism is not fully understood. In mammals and flies, it has been proposed that accumulation of the limiting negative regulator, PER, is somehow delayed because protein oscillations are delayed several hours relative to those of mRNA oscillations [24–26]. Here we reveal that RNAi is a crucial mechanism contributing to the time delay by interfering with translation of *Per1* and *Per2*. We provide multiple lines of evidence supporting the mechanism. For example, the asymmetric shortening of the bioluminescence rhythms in *Dicer* mutant cells shows that the period of the rhythm is shortened mostly by accelerated upswing of PER (or PER2-LUC) synthesis, with little effect on the downswing, or PER turnover. This is further supported by our analysis of bioluminescence (PER2-LUC) and PER accumulation data after existing PER proteins are removed by CHX treatment, by the shortening of circadian period by overexpression of *Per* 3′-UTR, and by period shortening in

wt cells by knockdown of *Per*-targeting miRNAs. The direct inhibition of PER accumulation in the cytoplasm by miRNAs provides a very intuitive mechanism for how miRNAs are integrated into the timekeeping mechanism, since most miRNAs function in the cytoplasm by inhibiting the translation of specific mRNA species.

The behavioral analysis of our conditional *Dicer* mutant mice confirmed that RNAi is important for circadian period in the SCN in vivo, not only in cultured MEFs. However, the mice showed milder period shortening compared to MEFs. We attribute this finding to the fact that *Dicer* was deleted only in half of brain cells and thus probably in only a subset of SCN cells, and behavioral phenotype is determined by the combined activities of all the SCN cells, as shown previously [51]. Kadener et al. previously studied how the *Drosophila* clock is affected by the knockdown of *dicer-1* expression and found that *dicer-1* knockdown flies did not show any significant circadian phenotype [37]. Furthermore, in *Drosophila,* TIMELESS (TIM) is critical for accumulation of dPER in the cytoplasm and delayed nuclear entry, whereas in mammals, mTIMELESS is irrelevant to the mammalian clock mechanism [52]. We believe that RNAi is a novel and alternative means of generating the time gap in mammals that is not conserved in flies.

In *Dicer* mutant cells and mice, where basic cell physiology is sufficiently disrupted to affect viability and/or cell growth, the circadian clock shows robust oscillations albeit with altered pace. The same is true of *CK1δ* mutant cells [29]. These data suggest that the clock mechanism is independent of the basic physiology related to cell survival, which may explain the resilience of the clock to physiological fluctuations in cells under different growth conditions and throughout the cell cycle.

Although our data implicate three specific *Per*-targeting miRNAs in the mammalian clock mechanism, we cannot rule out the possibility that other miRNAs are also important. First, our *Dicer* deletion did not eliminate all small, noncoding RNAs, so there may be Dicer-independent, RNA species involved in the clock mechanism. Second, we may have missed subtle dysregulation of other clock genes besides *Per1* and *Per2* in the *Dicer* mutant cells. As with the general function of miRNAs in gene expression, modulation of clock gene expression by miRNAs may not be dramatic. Even with *Per1/2*, mRNA and protein expression levels were only mildly affected in *Dicer* mutant cells. We believe that these small changes were able to produce a robust phenotype because they only had to alter the phase of PER oscillations, rather than absolute levels of *Per* expression. As long as PER shows normal oscillations, PER levels have only a subtle effect on period, as shown in *Per* transgenic mice and cells [8, 53]. Third, there may be additional miRNAs targeting *Per1/2*. Each miRNA may have multiple target genes and each gene can be targeted by multiple miRNAs. It is possible that miRNAs other than miR-24, 29a, and 30a can target *Per1/2* genes. Because the seed pairing does not have to match perfectly and allows wobbles— as shown in let-7:lin41 and miR-196:HoxB8 pairings [54, 55]—there may be more clock-relevant miRNAs that cannot be detected by the current prediction algorithms. However, even if other miRNAs contribute to the circadian feedback loop's time delay, our data clearly demonstrated that *Per* expression is modulated by miRNA interaction with the 3′-UTR of *Per1/2* genes.

Experimental Procedures

Dicer mutant, cre driver transgenic mice

The floxed *Dicer* mutant (*Dicer* $^{fl/fl}$) and *Per2*^{Luc} mice were described previously [5, 42]. The conditional transgenic mice expressing the brain-specific Tetracycline Activator (*Scg2-tTA*) were described previously [56]. When these mice are mated with a second transgenic mouse that carries transgenic *cre*, controlled by a tetracycline-responsive promoter element

(TRE or *tetO*), expression of *cre* is conditionally regulated by the presence or absence of doxycycline (Dox) in the drinking water [57]. In parallel, $Dicer^{flox}$ was combined with the *CAG-cre-Esr1* transgene to activate CRE recombinase in the presence of tamoxifen [43]. This *cre* driver mouse was also used by the Evans lab recently to delete *Rev-erbα* and *β* genes and study the behavioral rhythms of the mutant mice [17]. Both *tetO-cre* and *CAG-cre-Esr1* transgenic mice are commercially available from The Jackson Laboratory (stock # 006234 and 004682). All of these mice are in or close to the genetic background of the C57BL/6J strain, which exhibits robust locomotor activity rhythms. All animals were maintained in a climate-controlled room and used according to the FSU Animal Care and Use Committee's guidelines.

RT-qPCR analysis for miRNAs

miRNAs were extracted from the samples by miRNeasy Mini kit (Qiagen, Germantown, MD, USA). For Fig 1C, first-strand cDNA was generated with the RT^2miRNA First Stand Kit (Qiagen) and subjected to RT-PCR using the mouse miRNA Finder kit, which includes the 88 most abundant mouse miRNAs (Qiagen). Data were calculated according to the manufacturer's protocol and presented in Table S1. For individual miRNAs, the same primers used in Fig 1C were ordered from Qiagen and used for RT-qPCR.

Bioluminescence and behavioral rhythms

For *Dicer* mutant cells, bioluminescence rhythms were measured 5 days after adenoviral *cre* or *GFP* infection as described previously [29]. For Fig 6E, miR-24, 29a and 30a were inhibited by commercial antisense oligonucleotides (miRCURY power inhibitors; #426983-08 for miR-24, #460039-1 for miR-29a and #460026-1 for miR-30a; Exiqon; Woburn, MA). The oligonucleotides were mixed at 50 nM (final concentration) each and introduced using Lipofectamine RNAi Max (Invitrogen, Grand Island, NY) into wt $Per2^{Luc}$ cells grown in 24-well plates. Bioluminescence rhythms were measured by a real-time luminometer that can accommodate 24-well plates (Actimetrics, Wilmette, IL). To confirm high transfection efficiency, miRCURY-24 antisense oligonucleotide was labeled with TexasRed and the efficiency was estimated by counting fluorescent cells. Knockdown efficiency for the miRNAs was measured by RT-qPCR. In two separate experiments, miR-24, 29a and 30a were knocked-down more than 90% compared to control cells transfected with the same concentration of negative control miRCURY power oligonucleotide (#199004-08). Period was calculated by the periodogram function in the Clocklab software using the 2^{nd} and 3^{rd} peaks.

Locomotor activity rhythms were measured as described previously [58] using the Stanford Software System (Santa Cruz, CA, USA). The mice were entrained in 12-hr light and 12-hr dark (LD) cycles for 2 weeks before being released into constant darkness (DD). After 2 weeks in DD, regular mouse chow was switched to tamoxifen-containing food (400 mg tamoxifen + 0.8% sucrose/kg food; Harlan laboratories, Inc.) for *CAG-cre-Esr1* mice, and Dox water was switched to regular water for *Scg2-tTA;tetO mice.* When judged by genotyping PCR of brain tissue, the *CAG-cre-Esr1* transgene was much more efficient in deleting *Dicer* in vivo and caused larger period shortening. Period was calculated using the χ^2-periodogram in the Stanford Software System. In *Dicer* mutant mice ($Dicer^{flox/flox}$/*CAG-cre-Esr1*) after the tamoxifen treatment, the last several days of behavioral data were used to calculate period by hand drawing a best-fit line across several activity onsets.

DNA constructs

Adenoviral *GFP, Per2 promoter-luciferase* and *cre* constructs have been described previously [8, 29]. The full-length 3′-UTRs of *Per1* and *Per2* were amplified from the *pCMV-sport6-Per1* and *Per2* plasmids (Addgene #4133009 and #3964321) using the

following primers. The fragments were cloned into XbaI/SalI (for the *Per1 3′-UTR*) and XbaI/BamHI sites (*Per2 3′-UTR*) of the pGL3 basic plasmid.

Per1-3′UTR Fwd

5- ATC CTC TAG AACTCC ATT TTG GGG CCG CTT ACA GCA G -3

Per1-3′UTR Rev

5- ATC CGT CGA CCG AGG GAT ACA CTA GAG CGG CCG C -3

Per2-3′UTR Fwd

5- ATC CTC TAG ACC CTG TCC CCC AGC CAG AGGTC -3

Per2-3′UTR Rev

5- ATC CGG ATC CCG AGG GAT ACA CTA GAG CGG CCG C -3

The following mutations for the miR target sites were introduced into the constructs using the Quickchange II XL Site-Directed Mutagenesis kit (Agilent technologies, Santa Clara, CA, USA).

Per1 miR-24: CUGAGCC → GTCGCAG

Per1 miR-29a: UGGUGCU → GGTACCT

Per2 miR-24: CUGAGCCU → CCGCGCCU

Per2 miR-30a: UGUUUACA → CGCUUACA

To generate *Flag tag-partial length luciferase-Per 3′-UTR* in an adenoviral vector, the following primers were used to amplify the UTRs from the pGL3-Per 3′-UTR plasmids.

pGL3-basic-luc Fwd (with Flag tag)

5′-ATC CCT CGA GGC CAC CAT GGA TTA CAA GGA TGA CGA TGA CAA GTC CGG TTA TGT AAA CAA TCC GGA AGC G -3′

pGL3-basic-luc_Rev

5′-TAG GGA TAT CGG AAG CGG AAG AGC GCT CCC GGC A -3′

Lentiviruses expressing miR-24, 29a and 30a were purchased from the Open Biosystem (miRIDIAN shMIMIC microRNAs). Expression of these miRs was confirmed by RT-PCR as described above.

Real-time qPCR for clock genes, luciferase reporter assays and immunoblotting

Real time qPCR for clock genes was performed as described previously using the same primers [8]. Immunoblotting was performed using the antibodies described previously [14, 24]. PER1-1-R and PER2-1-R were used for PER1 and 2 immunoblotting, respectively. Novel anti-Dicer antibodies were generated using the N-terminal region of Dicer protein (amino acids 241–426) by Cocalico Biologicals, Inc (Reamstown, PA) and thoroughly characterized using in vitro and in vivo samples. Dicer GP47 was used for this study. Luciferase reporter assays were performed as described previously [8].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The period of the circadian clock is shortened in *Dicer*-mutant cells and mice.
2. The time delay in the feedback loop is compromised in the *Dicer*-mutant cells.
3. RNAi contributes to the time delay in the clock by targeting PER translation.
4. Three miRNAs regulate the accumulation of PER1 and PER2 in the cytoplasm.

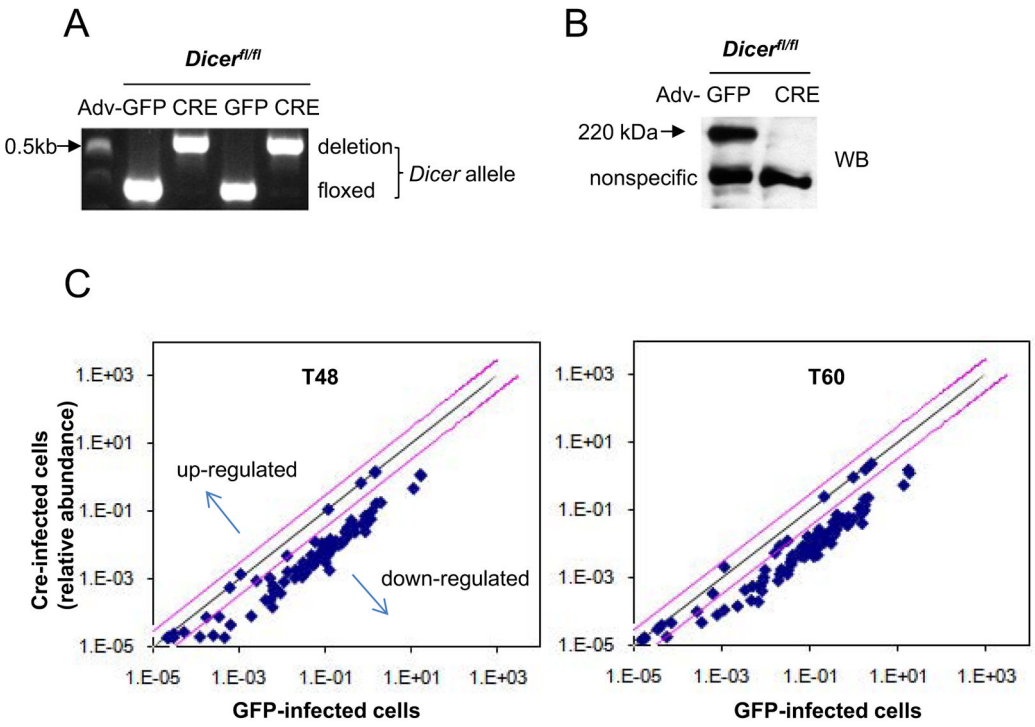

Fig 1.

Deletion of *Dicer* in *Dicer*$^{flox/flox}$ MEFs by adenovirus-*cre*. Cells were infected with adenovirus expressing GFP or CRE and harvested 5 days later. (A) PCR-genotyping was performed using three primers described in Harfe et al. [42]. Two independent experiments are shown. (B) Representative immunoblots of the cell extracts. Dicer, indicated by the arrow, was eliminated in >95% of CRE-expressing cells, based on comparison to GFP cells. More immunoblots from different experiments are shown in Fig S1A. (C) Depletion of miRNAs in *Dicer*-deficient cells. Cells were infected with CRE- or GFP-expressing adenovirus, maintained for 5 days, given a 2-hr serum shock, and harvested 48 or 60 hours later to mimic the conditions for recording bioluminescence rhythms as we have done previously [8, 29]. The miRNA levels at T48 and T60 were normalized to control RNAs (Rnu6 and snoRNA202) according to the manufacturer's protocol, and each point on the graph shows the average of 6 samples (duplicate samples in 3 independent experiments). The black line indicates no difference between GFP- and CRE-expressing cells. The pink lines indicate a 3-fold difference. Levels of all miRNAs except 8 species were significantly reduced between GFP and CRE cells ($p<0.05$). See also Table S1.

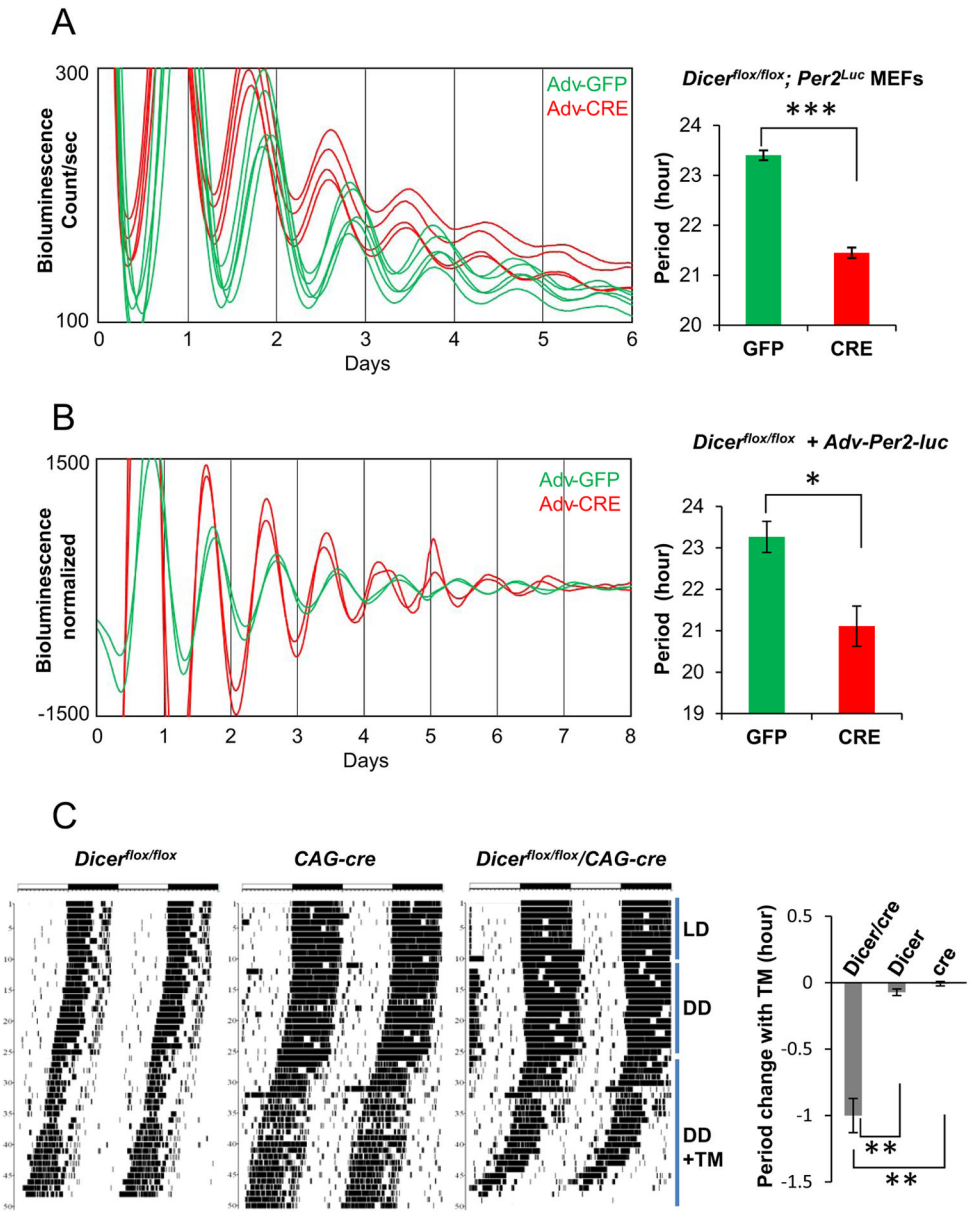

Fig 2.

Circadian period is dramatically shortened in *Dicer*-deficient cells and mice. (A) The period of bioluminescence rhythms in CRE-expressing (Adv-CRE) *Dicer*$^{flox/flox}$;*mPer2*^{Luc} MEFs is shorter than that in control cells expressing GFP (Adv-GFP). Note that the basal lines are higher in CRE-MEFs than in GFP-MEFs. Quantitation of circadian period is shown in the graph. Mean+/−SEM; n=14 for each sample; p<0.001. See also Fig S1B and S1C. (B) Period is similarly shortened in Adv-CRE *Dicer*$^{flox/flox}$ MEFs without *Per2*^{Luc}. Bioluminescence was produced from an exogenous *Per2* promoter-driven luciferase reporter introduced into the MEFs using the adenoviral vector (*Adv-Per2-Luc*). Mean+/−SEM; n=5 for GFP, n=3 for CRE; p<0.05. (C) Period is also shortened in whole mice when *Dicer* is deleted using tamoxifen-dependent activation of CRE. Representative double-plotted actograms are shown for three different genotypes: *Dicer*$^{flox/flox}$, *CAG-cre-Esr1* and *Dicer*$^{flox/flox}$/*CAG-cre-Esr1* mice (n=5 each). Black markings indicate wheel running, and recordings from consecutive days are stacked in rows. Mice were entrained in a 12:12 LD

cycle (12 hrs light and 12 hrs dark) for 2 weeks followed by 2 weeks in constant darkness (DD). On the 15th day, the food was switched to tamoxifen (TM)-containing food at 0.4mg/g food. All *Dicer*^{flox}/*CAG-cre-Esr1* mice were found dead at the end of the recording. In these *Dicer*-deficient mice, the period was ~1 hr shorter in the last 7 days before death when compared to the period in DD before tamoxifen treatment. However, there was no significant difference in period before and after tamoxifen treatment in control (*Dicer*$^{flox/flox}$ or *CAG-cre-Esr1*) mice. See also Fig S2.

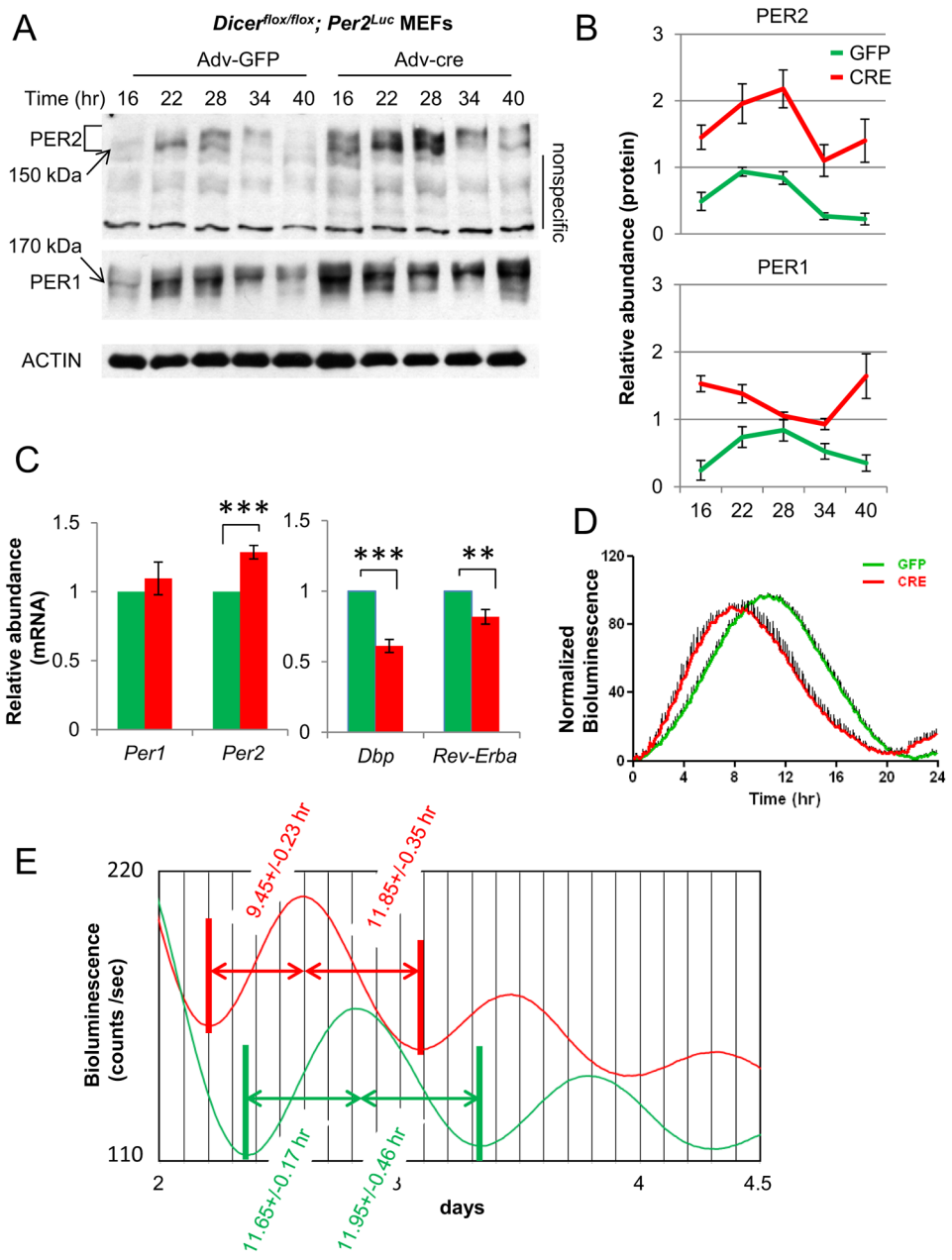

Fig 3.

PER protein levels are elevated and bioluminescence (PER2-LUC) rhythms accumulate faster and peak earlier in *Dicer*-deficient cells (CRE) compared to *Dicer*-intact cells (GFP). (A) PER protein levels are higher in *Dicer*-deficient cells than control cells. The samples were harvested at the indicated times after a 2-hr serum shock. See also Fig S3A. (B) Quantitation is shown as mean+/−SEM of three independent experiments. (C) mRNA levels of *Per1* and *Per2* are only modestly increased in *Dicer*-deficient cells, less substantially than their protein levels. mRNA levels of clock-controlled genes *Dbp* and *Rev-erbα* are significantly lowered. mRNA levels were measured by quantitative RT-PCR from 6 samples harvested at several times over one circadian cycle. The data are shown as mean+/−SEM. (D) In the circadian rhythm of PER2-LUC expression, the upswing is shortened while the downswing is not affected. *Dicer*$^{flox/flox}$;*Per2*^{Luc} MEFs were treated with 40 ug/ml

cycloheximide (CHX) for 10 hrs followed by washoff, and then bioluminescence was measured. We showed that endogenous PER1/2 can be almost completely depleted by CHX treatment for 10 hrs [29]. The peak of bioluminescence was set at 100 in each case, as has been done by Meng et al. and Etchegaray et al. [44, 59]. Note that the ~2-hr period shortening mostly comes from the accumulation (upswing) phase. The results are shown as mean+/−SEM of 10 samples. (E) Similar asymmetrical bioluminescence profiles are observed in Fig 2A. Single representative traces for GFP and Cre-MEFs are shown, but the numbers in mean+/−SEM were calculated from 10 samples each. Note that the period difference resulted from the shortened rising phase of the curves ($p<0.01$). Times from trough to peak and from peak to trough were calculated using Clocklab software after applying smoothing.

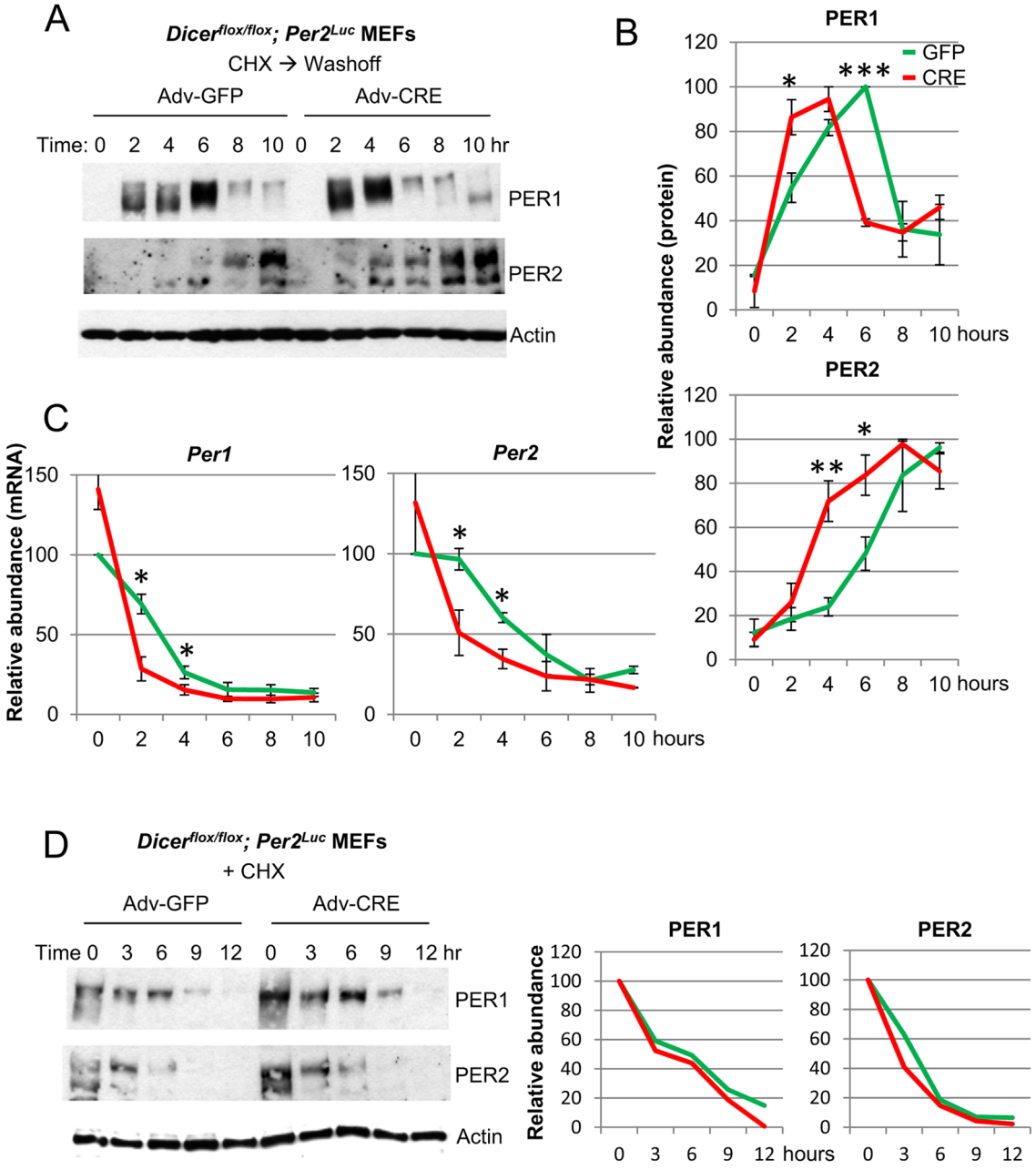

Fig 4.

Accumulation of PER proteins and feedback inhibition of *Per* are accelerated in *Dicer*-deficient cells. (A), (B) PER accumulates faster in Dicer-deficient cells. Cells were harvested after CHX treatment followed by washoff as described for Fig 3D and subjected to imunoblotting for PER1 and 2. The mean+/−SEM of 3 and 4 experiments is shown for PER1 and 2, respectively. (C) Feedback occurs earlier in Dicer mutant cells. Cells were also harvested for mRNA analysis under the same condition. Data are shown as mean+/−SEM of three experiments. (D) PER degradation rates are comparable between control and *Dicer* mutant cells. As has been done previously [29], cells were treated with CHX and harvested at the indicated times. Results are representative of two experiments.

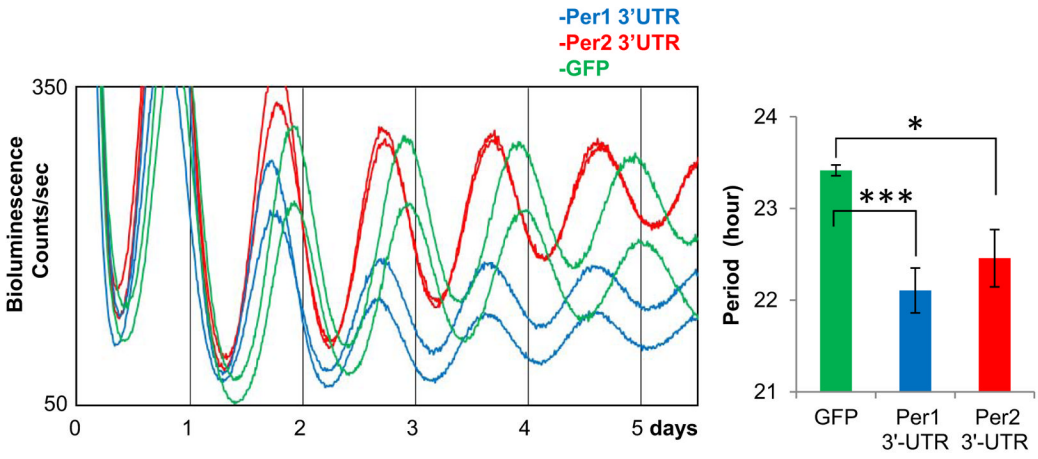

Fig 5.

Overexpression of *Per1* or *Per2* 3′-UTR in wt *Per2*^{Luc} MEFs induces period shortening similar to *Dicer*-deficient cells. The 3′-UTR adenoviral constructs include a partial luciferase coding sequence (to increase stability of mRNA) followed by a full-length *Per1* or *Per2* 3′-UTR. Data are shown as mean+/−SEM. n=6 for GFP; 8 for Per1 3′-UTR; 8 for Per2 3′-UTR. See also Fig S3B.

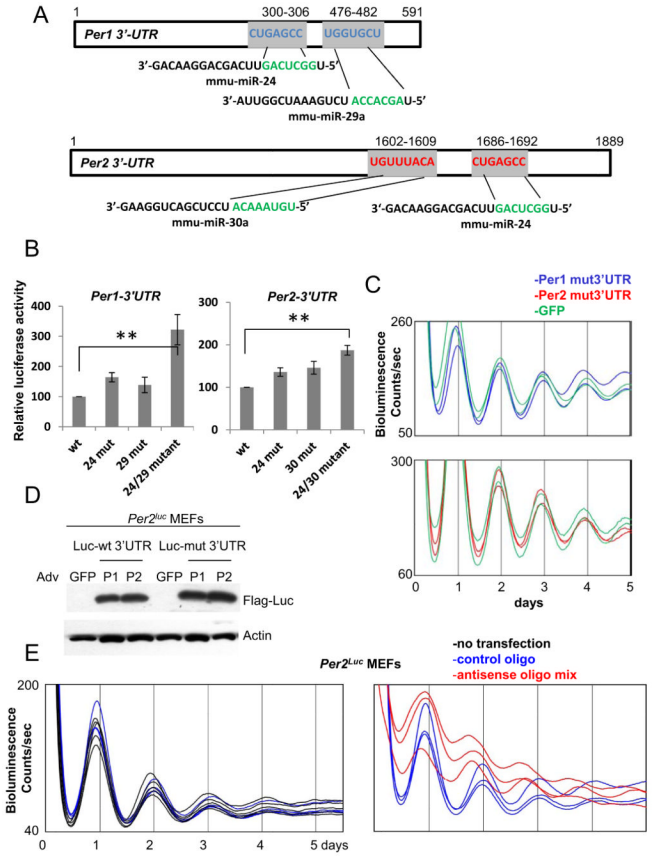

Fig 6.

miR-24/29a and 24/30a post-transcriptionally regulate *Per1* and *Per2*, respectively. (A) *Per1* and *Per2* 3′-UTRs have conserved target sites for miR-24/29a and 24/30a, respectively. The target sites were identified by TargetScanMouse 5.2. Note that these target sites have perfect 7 or 8 nucleotide matches. See also Fig S4. (B) Luciferase expression is higher when the target sites are mutated in transient transfection-based reporter assays. The mutant 3′-UTRs in the pGL3 basic plasmid were expressed in NIH3T3 cells and their expression levels were compared. Single mutations only modestly increased luciferase expression, but double mutations increased the luciferase expression significantly higher compared to wt UTRs. See also Fig S5A. (C) Overexpression of mutant miR-24/29a *Per1* 3′-UTR or mutant miR-24/30a *Per2* 3′-UTR does not induce period shortening. The mutations in the miRNA target sites were introduced into the adenoviral 3′-UTR constructs used in Fig 4. (D) Expression levels of these mutant UTRs were comparable to those of wt UTRs when expression levels of the partial luciferase attached to the UTRs were compared. (E) Circadian period is shortened in wt $Per2^{Luc}$ cells treated with antisense oligonucleotides to miR-24, 29a and 30a. A negative control oligonucleotide did not affect circadian period compared to non-treated control cells (left panel). The periods of bioluminescence rhythms in control and specific oligonucleotide-treated cells were 24.25+/−0.08 and 22.88+/−0.10, respectively (mean+/−SEM of 8 traces each; $p<0.001$). See also Fig S5B.