Supporting Information

Lee et al. 10.1073/pnas.0906651106

SI Text

Recombinant Plasmids, Adenoviral Constructs, and Virus Production. Plasmids pcDNA3.1-*ckIε-myc*, *Per2-V5* were described in refs. 1 and 2. pcDNA3.1-DN-*ckIε-myc* and *tau ckIε* were generated by PCR using oligonucleotides encoding the point mutations (AAG->AGG for DNε and CGC->TGC for *tau*). The full-length pcDNA3.1-*3XFlag-ckIε* was generated by PCR amplification of the template pcDNA3.1-*ckIε-myc* using the following oligos and cloning into *Xho*I/*Xba*I sites of the pcDNA3.1 V5-His-A plasmid.

Forward:

ATCCCTCGAGGCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGAGTTGCGTGTGGGAAATAAGTAT

Reverse:

TAGGTCTAGATCATTTCCCAAGATGGTCAAATGG.

To construct the full length pcDNA3.1-*3XFlag-ckIδ*, the full-length *ckIδ* with N-terminal Flag tag was obtained by PCR amplification of a full-length *ckIδ*-encoding cDNA (Open Biosystems) using the following primers and cloning the PCR product into *Xho*I/*Xba*I sites of the pcDNA3.1 V5-His-A plasmid.

Forward:

ATCCCTCGAGGCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGAGCTGAGGGTCGGGAACAGGTAC

Reverse:

TAGGTCTAGATCATCGGTGCACGACAGACTGAAG.

Catalytic (aa 1–277 for CKIε/δ) and C-terminal (aa 278–416 for CKIε and aa 278–415 for CKIδ) domains were cloned into pcDNA3.1-V5-His and p3xFlag-CMV10 (Sigma), respectively. First, these domains were amplified using the following oligos and cloned into *Xho*I/*Xba*I sites of pcDNA3.1V5-His-A and *Hin*dII/*Xba*I sites of p3xFlag-CMV10, respectively.

CKIε Catalytic domain:

Forward:

ATCCCTCGAGGCCACCATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGAGTTGCGTGTGGGAAATAAGTAT

Reverse:

TAGGTCTAGATCAAAAGAGATTTCGGAAGAGCTGGCG

CKIε C-terminal domain:

Forward:

ATCCAAGCTTCATCGCCAAGGCTTCTCCTACGAC 3,

Reverse:

TAGGTCTAGATCATTTCCCAAGATGGTCAAATGG 3′

CKIδ Catalytic domain:

Forward:

ATCCCTCGAGGCCACCATGGACTACAAAGACCATGACGGTGATTATAAA GATCATGACATCGATTACAAGGATGACGATGACAAGGAGCTGAGGGTCGGGAACAGGTAC

Reverse:

TAGGTCTAGATCAGAACAGATTTCTGAAGAGCTGTCT

CKIδ C-terminal domain:

Forward:

ATCCAAGCTTCATCGCCAAGGCTTCTCCTACGAC

Reverse:

TAGGTCTAGATCATCGGTGCACGACAGACTGAAG

For adenoviral wt and DN-3xFlag-CKIε, *Xho*I/*Xba*I fragments from the above pcDNA were cloned into the same sites in pAd-Track-CMV (a shuttle vector). The shuttle vector has two CMV promoters; one for GFP and the other for the gene of interest. The cloned pAdTrack-CMV shuttle vectors were subsequently cut with *Pme*I for linearization and then transformed into the *E.coli* BJ5183 strain along with the pAdEasy-1 adenoviral backbone vector to generate a complete adenoviral vector by in vivo recombination, as described by He et al. (3).

For adenoviral wt and DN-3XFlag-CKIδ, *Xho*I/*Xba*I fragments from wt and DN-3XFlag-CkIδ-pcDNA were cloned into the shuttle vector and complete adenoviral vectors were generated as above. DN-3XFlag-CkIδ-pcDNA3.1 was generated from wt template using site-directed mutagenesis as above (AAG->AGG).

CKBD-P2 and P3 adenoviral constructs were generated from Flag-CKBD-P2/P3-pcDNA, which were made by PCR amplification of CKBD-encoding sequence from *Per2*-pcDNA and *Per3*-pcDNA.

Final adenoviral constructs were generated using the following oligos which add the nuclear localization signal from SV40 large T antigen at the N terminus of the proteins.

CKBD-P2:

Forward:

ATCCTCTAGAGCCACC*ATGGATCCAAAAAAGAAGAG-AAAGGTA*GATTACAAG GAT GACGATGACAAG (NLS is italicized)

Reverse:

TAGGGTTTAAACTCAACTCGCCTGGGCTCGGGACCGCTC

CKBD-P3:

Forward:

ATCCTCTAGAGCCACC*ATGGATCCAAAAAAGAAGAG-AAAGGTA*GATTACAAGGATGACGATGACAAG (NLS is italicized)

Reverse:

TAGGGTTTAAACTCAAACACAGGAGTACTGAGCACGGTT

PCR products were cloned into *Xba*I and *Pme*I/*Eco*RV sites in pAd-Track-CMV and final adenoviral vectors were generated as described above. An exogenous NLS was added because CKBD-P2 without NLS was exclusively cytoplasmic, whereas NLS-CKBD-P2 was predominantly nuclear as were endogenous clock proteins. Adenovirus-CKBD-P2 was generated using primers similar to those above but without the NLS sequence. For *Per2-V5* adenovirus, *Per2-V5* were cloned into *Xho*I/*Eco*RV of pAdTrack-CMV and a final adenoviral vector was generated as above.

Generation and purification of recombinant adenovirus in packaging cells (HEK293) was also performed according to methods described by He et al. (3).

Transfection. In Fig. 1*B*, NIH 3T3 cells were transfected with 50 ng PER2, 100 ng wt, DN, or tau CKI expression plasmid. pcDNA3.1 vector was used to normalize DNA concentration. In Fig. 1*C*, HEK293 cells were transfected with 50 ng PER2 and 100 ng of the full-length CKIδ/ε, or 100 ng N-terminal (*Cat*alytic domain; amino acids 1–277) + 100 ng C-terminal (aa 278–416 for CKIε and aa 278–415 for CKIδ). In Fig. 1*D*, NIH 3T3 cells were transfected with 25 ng PER-V5 and empty pcDNA3.1, 50

ng wt-CKI, or 50 ng wt + 500 ng DN-CKI. pcDNA3.1 vector was used to normalize DNA concentration. In Fig. 1*E*, NIH 3T3 cells were transfected with 25 ng PER-V5 and empty pcDNA3.1, 50 ng wt-CKIε, 50 ng wt CKIε + 500 ng DN-CKIδ, or 50 ng wt CKIδ + 500 ng DN-CKIε. pcDNA3.1 vector was used as above to normalize total DNA concentration.

Animals, Cells, and Antibodies. *ckIδ* hetero and homozygote mutant MEFs in Figs. 3 and 4 were heterozygote for $Per2^{Luc}$. For immunoblots from these MEFs, PER2 is shown since PER2's smaller size results in better resolution than PER2:Luc. Both *ckIδ* mutant and $Per2^{Luc}$ mice had been backcrossed to C57BL/6J mice for several generations before we received the mice. Antibodies to clock proteins were described in refs. 2 and 4. Rabbit anti-ACTIN antibody was purchased from Sigma. Anti-MYC and FLAG were from Roche and Sigma, respectively. Cycloheximide (40 μg/mL) and proteasome inhibitors [40 μM MG132 + 40 μM Z-Ile-Glu(OBu^t)-Ala-Leu-H (PSI); BIOMOL International] were added 24 h after serum shock, when PER levels are high.

Immunoblotting and Immunoprecipitation (IP). Immunoblotting and IP were performed as described in ref. 5.

Monitoring of Bioluminescence Rhythms. MEFs were grown in DMEM supplemented with 10% FBS. To measure bioluminescence rhythms from MEFs expressing GFP and clock proteins, MEFs in 35-mm dishes were infected with adenovirus or PBS (mock infection) for 2 h, and then serum-shocked with 50% horse serum for 2 h followed by serum-free DMEM without phenol red, supplemented with bicarbonate (350 mg/L), 10 mM HEPES (pH 7.2), and 0.1 mM luciferin, and placed into a Lumicycle luminometer (Actimetrics). At least three samples (dishes) per condition were monitored. A bioluminescence trace representative of the three (or more) samples was presented for each experiment. For Fig. 4*A*, additional traces are shown in Fig. S4*D*. For period calculation using the ClockLab software, only the first three peaks were considered. The half-life of bioluminescence in Fig. S4 *B* and *C* was calculated as described by Meng et al. and Etchegaray et al. using the GraphPad Prism program. Significance levels (*P* value) were calculated using a *t* test in Microsoft Office Excel or GraphPad Prism.

Quantitative Real Time RT-PCR. Total RNA was extracted from MEFs using TRIzol Reagent according to the manufacturer's protocol (Invitrogen). Total RNA (1 μg) was reverse transcribed using Bio-Rad iScript first cDNA synthesis kit. Quantitation of mRNA levels was performed by real-time PCR using an iCycler iQ PCR System (Bio-Rad). Analyses were performed using the standard curve method with *β-Actin* as the normalizing endogenous control; the following primer sequences were used:

Per1 sense 5′-TCCCTGTTTCGTCCTCCACT-3′,
antisense5′-CTTGAGCCATTGCTGTTTGC-3′,
Per2 sense 5′- GGCGAAGCGCTTATTCCAG-3′,
antisense5′-TGGAGCAGTTCTCGTTTCCG-3′
Dbp sense 5′-GCAGGCTTGACATCTAGGGAC-3′
antisense5′-GCTGCTTCATTGTTCTTGTAC-3′
β-Actin sense 5′-ATGGGTCAGAAGGACTCCTATGTGGG-3′
antisense5′-GGCCACACGCAGCTCATTGTAGAAGG-3′

1. Kume K, et al. (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* 98:193–205.
2. Lee C, Weaver DR, Reppert SM (2004) Direct association between mouse PERIOD and CKIepsilon is critical for a functioning circadian clock. *Mol Cell Biol* 24:584–594.
3. He TC, et al. (1998) A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA* 95:2509–2514.
4. Lee C, Etchegaray JP, Cagampang FR, Loudon AS, Reppert SM (2001) Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* 107:855–867.
5. Shearman LP, Jin X, Lee C, Reppert SM, Weaver DR (2000) Targeted disruption of the mPer3 gene: Subtle effects on circadian clock function. *Mol Cell Biol* 20:6269–6275.

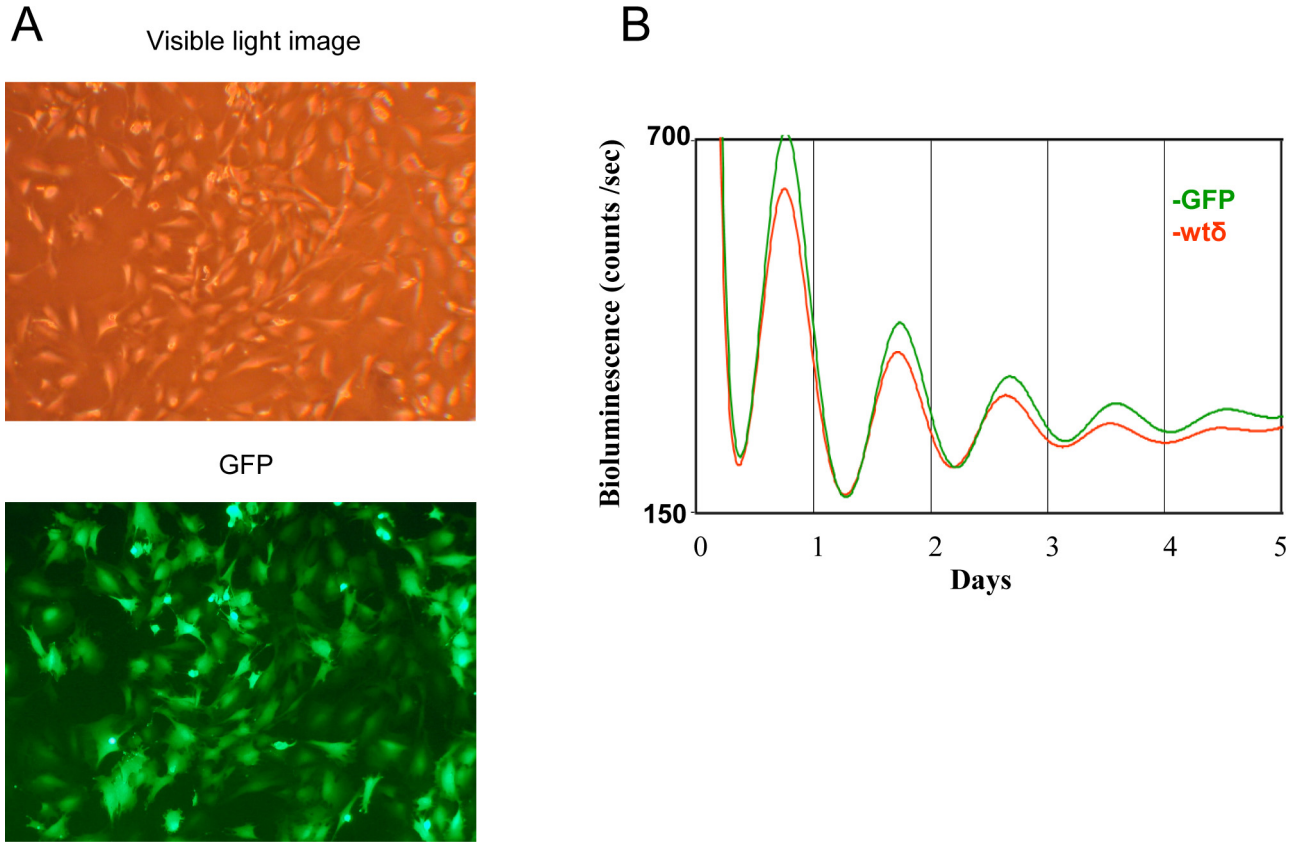


Fig. S1. Overexpression of wt-CKIδ slightly shortens circadian period. (*A*) The adenovirus efficiently infects MEFs. MEFs were infected with GFP-adenovirus and fixed 24 h later, and a visible light image and a fluorescence (GFP) image were taken. (*B*) *PerLuc* MEFs were infected with adenovirus, for expression of wt-CKIδ or GFP, for 2 h, serum shocked for 2 h, and placed into the real-time luminometer for luciferase monitoring.

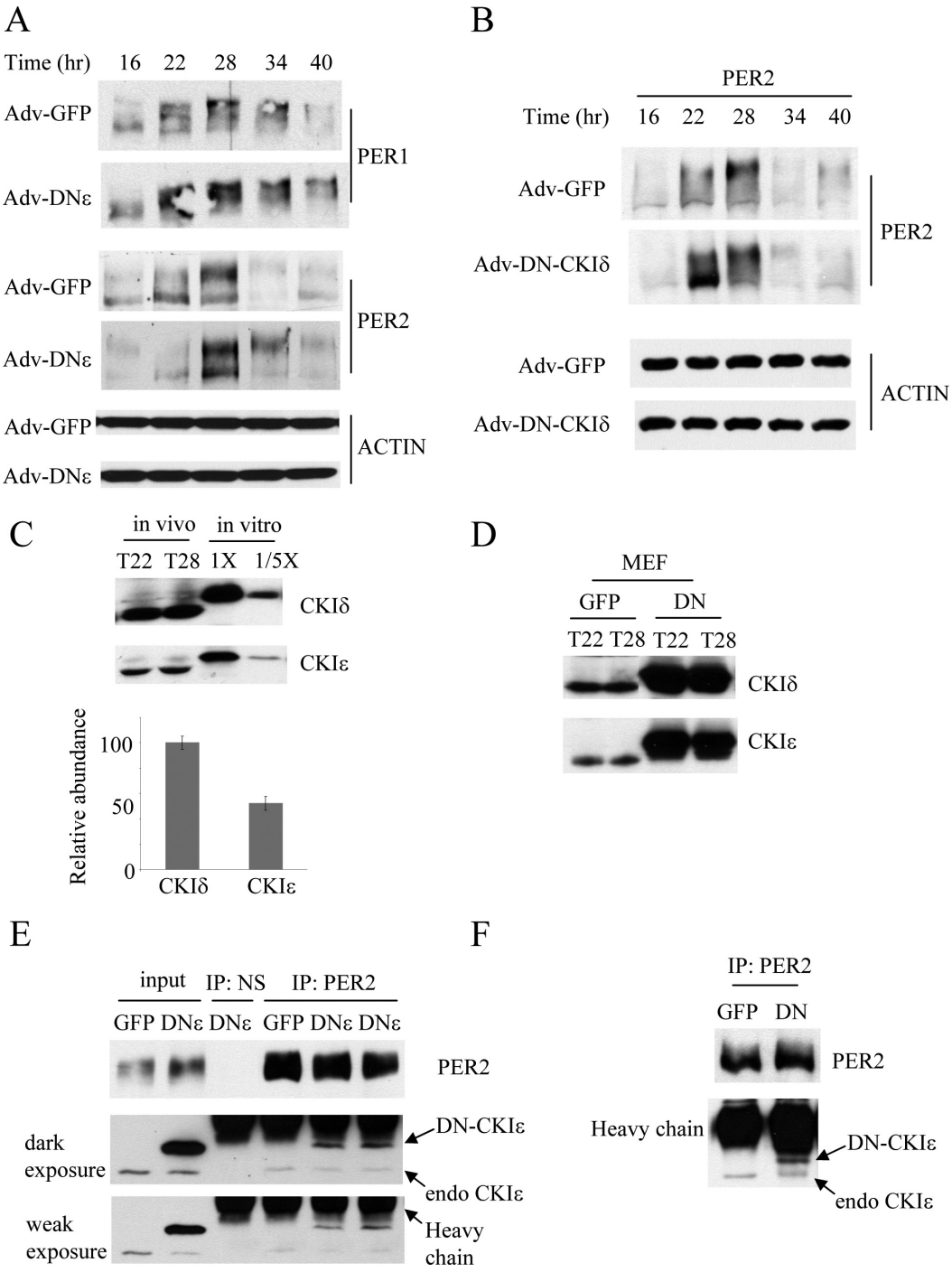


Fig. S2. The exogenous DN-CKIδ or ε does not eliminate circadian rhythms, even though it is more abundant than combined levels of endogenous CKIδ/ε in DN-CKIδ/ε-overexpressing MEFs. (*A*) GFP or DN-CKIε MEFs were collected at the indicated times after adenoviral infection and serum shock, and subjected to immunoblotting to show PER1 and 2 rhythms (relative to constitutive Actin levels). T16 indicates 16 h after serum shock. Immunoblots are representative of at least three experiments. (*B*) GFP and DN-CKIδ MEFs were collected at the indicated times after adenoviral infection (2 h) and serum shock (2 h), and subjected to immunoblotting for PER2 and Actin as above. Blots are representative of two experiments. (*C*) Levels of endogenous CKIδ and ε in GFP MEF extracts were compared with known amounts of in vitro translated CKIδ and ε by immunoblotting. 1X and 1/5X is roughly equal to 1 fmol and 0.2 fmol in vitro translated protein. Molar amounts of endogenous kinases were calculated relative to these in vitro translated proteins. The molar amounts of endogenous kinases were converted into a relative ratio between them and shown in the graph as mean ± SEM of three experiments. (*D*) CKIδ/ε levels were compared between GFP and DN-CKIδ/ε MEFs. (*E*) GFP and DN-CKIε MEFs were harvested 24 h after serum shock and the extracts were subjected to IP with a nonspecific (NS) or anti-PER2 antibody. Note that the ratio of DN:endogenous CKIε in the immunocomplex is less than in the input extracts. Another experiment is shown in *F*.

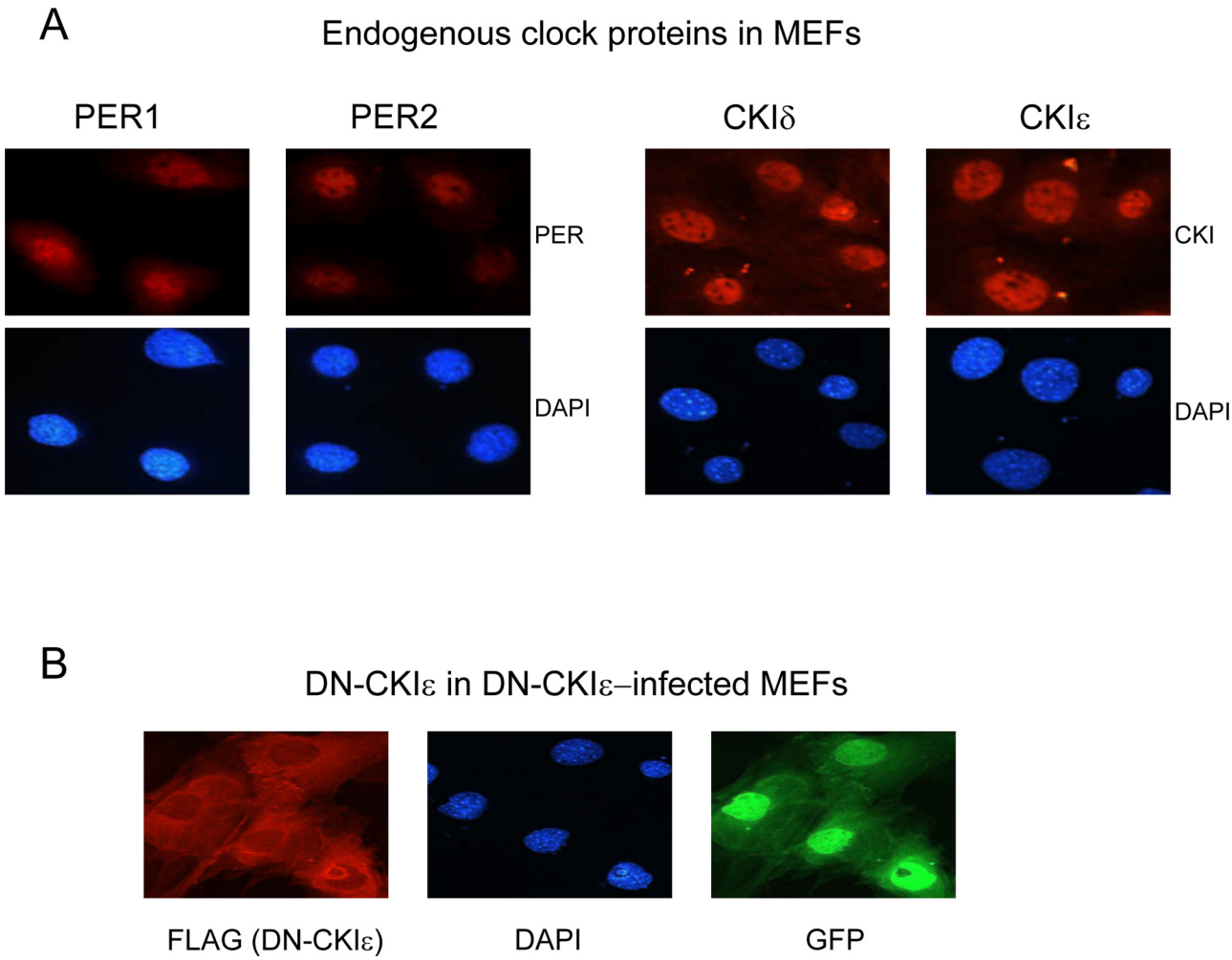


Fig. S3. Endogenous PER and CKIδ/ε are nuclear, whereas exogenous DN-CKIε is both cytoplasmic and nuclear. (*A*) Subcellular localization of endogenous PER1, 2, CKIδ, and ε in MEFs. Images are representative of two experiments. (*B*) Subcellular localization of overexpressed DN-CKIε in MEFs. Images are representative of two experiments. The adenoviral vector has dual CMV promoters, one default CMV promoter for GFP and the other one for the gene of interest. In this case DN-CKIε. Thus, GFP signal (right most image) demonstrates that GFP-positive cells are indeed infected. The DN-CKIε signal is specific, as it is not detected in GFP-negative cells.

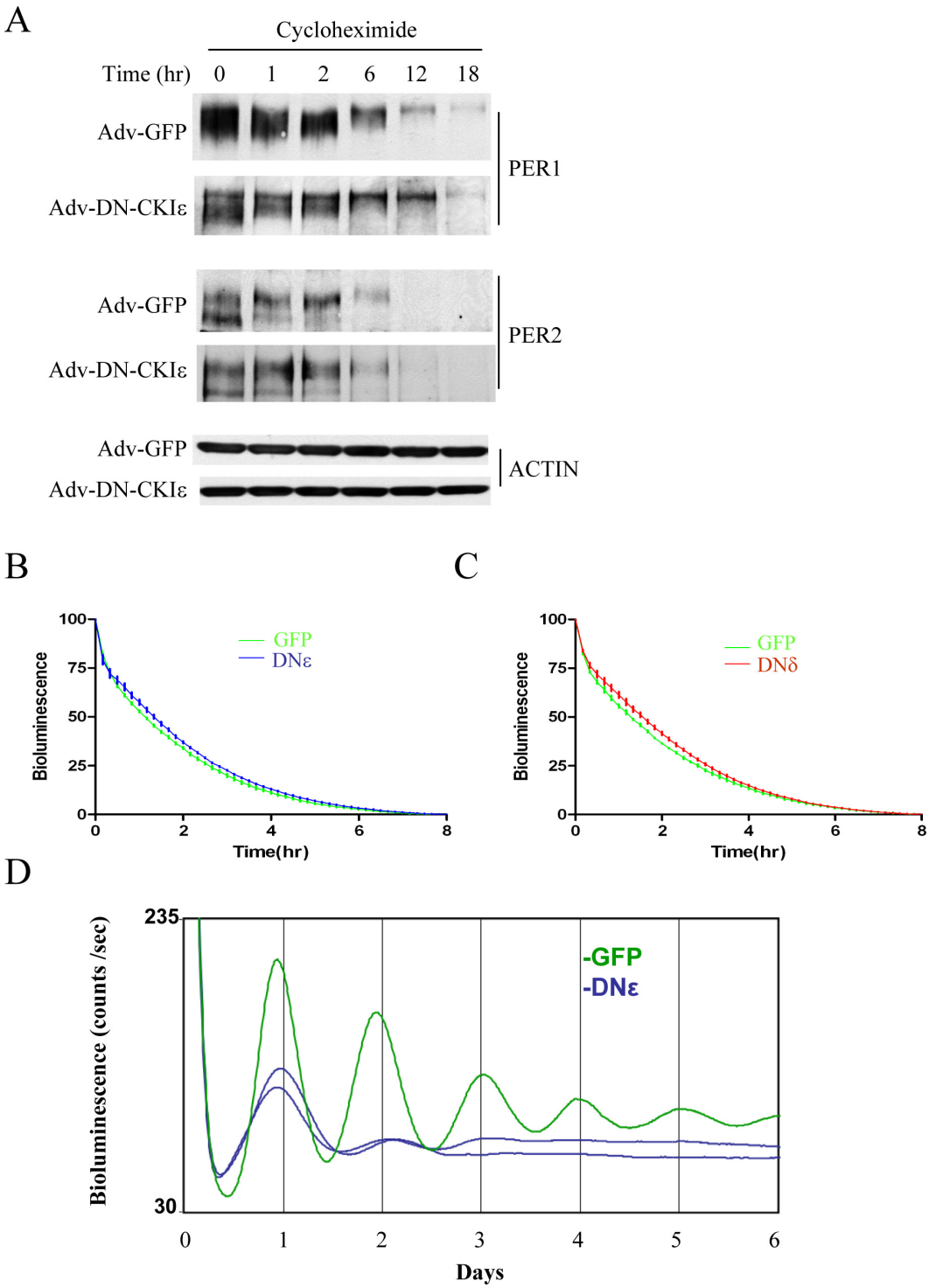


Fig. S4. Half-life of PER2:Luc is significantly lengthened in DN-CKIδ/ε-expressing MEFs compared to control MEFs. (*A*) MEFs were treated with cycloheximide (CHX) 24 h after serum shock and harvested at indicated times after the treatment. (*B* and *C*) PER2:Luc half-lives are lengthened in DN-CKIδ/ε overexpressing MEFs. The decline in bioluminescence was monitored for 8 h after cells were treated with CHX at the time of peak bioluminescence. Half-lives in bioluminescence were lengthened 14.5 min ($P < 0.01$; $n = 4$ for GFP and DN-CKIδ MEFs) and 11.9 min ($P < 0.01$; $n = 6$ for both GFP and DN-CKIε MEFs) in DN-CKIδ MEFs and DN-CKIε MEFs, respectively. The results are representative of three experiments. (*D*) Bioluminescence rhythms in Adv-DN-CKIε-infected CKIδ-deficient MEFs. This is a replicate of the experiment shown in Fig. 4*A*.

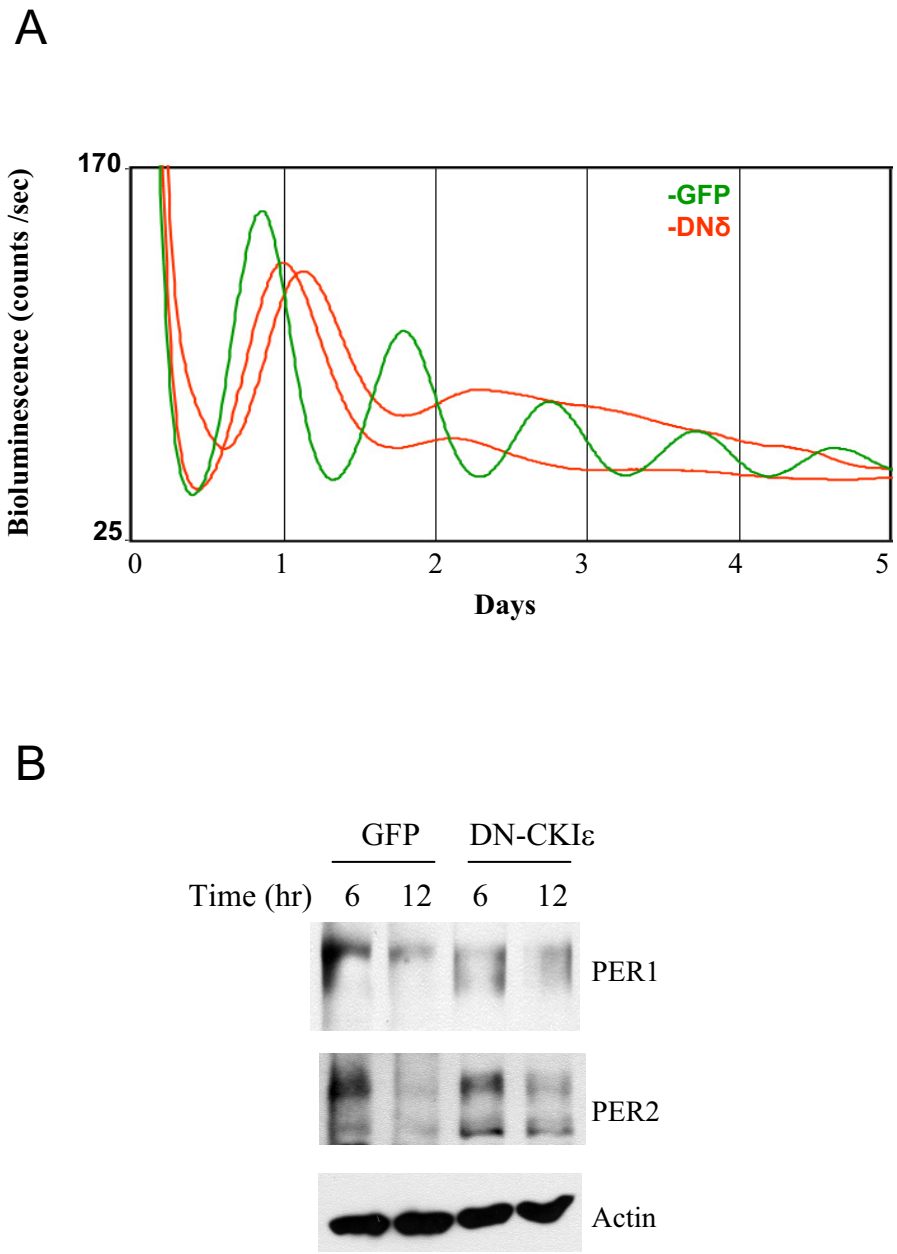


Fig. S5. Overexpression of DN-CKIδ also severely disrupts the bioluminescence rhythms. (*A*) Bioluminescence rhythms in Adv-DN-CKIδ-infected CKIδ-deficient MEFs. Traces are representative of three experiments. (*B*) Side-by-side comparison of T6 and T12 samples in the cycloheximide experiment shown in Fig. 4*C*.

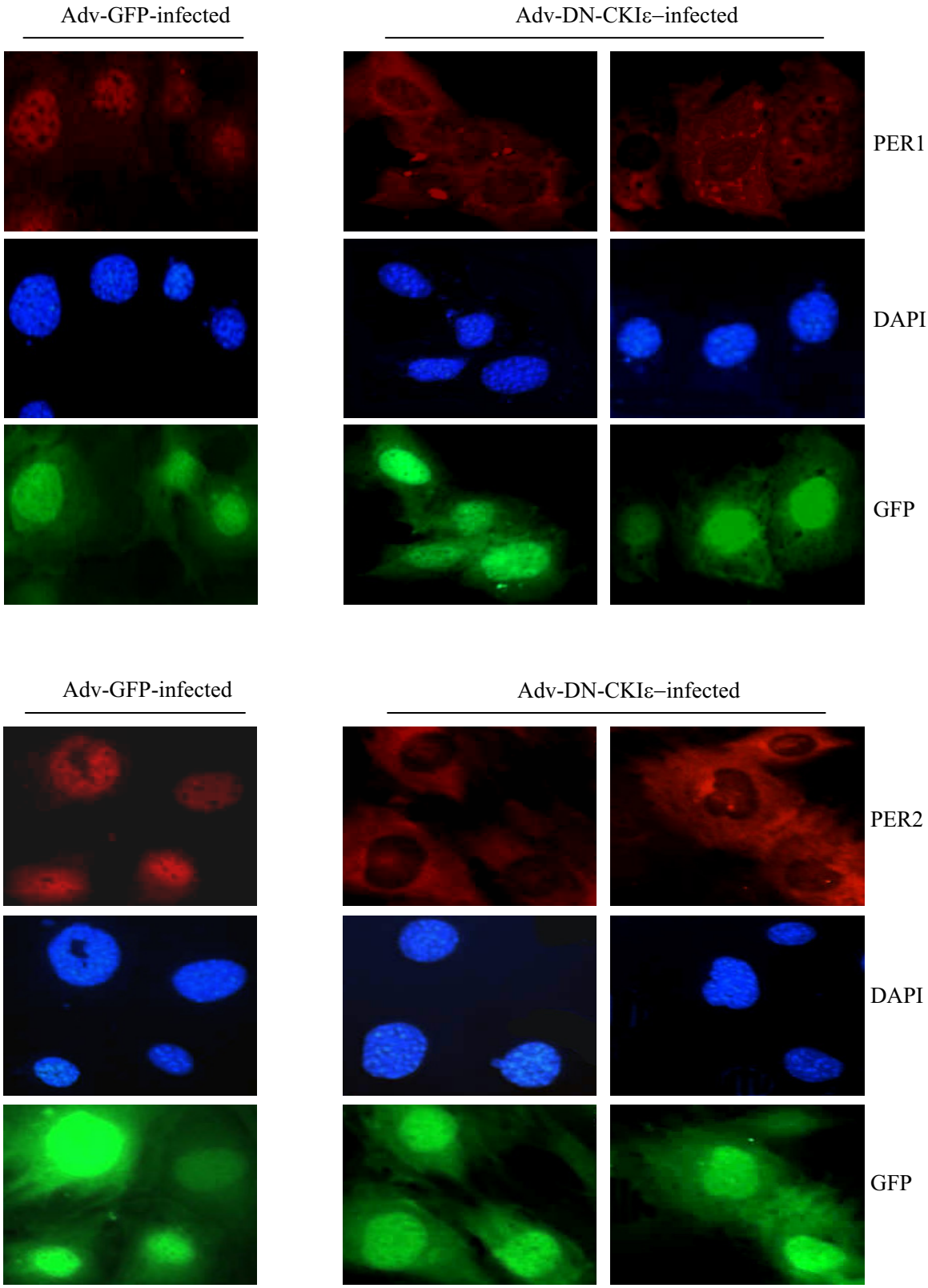
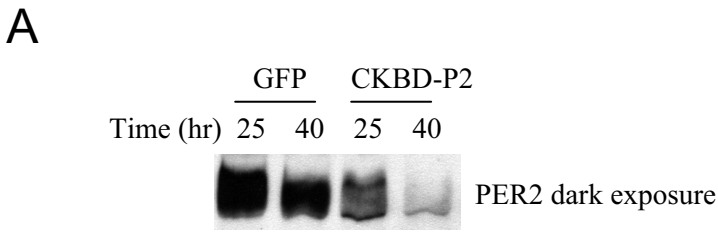
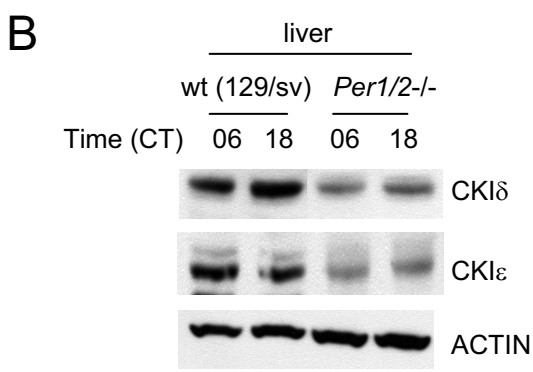
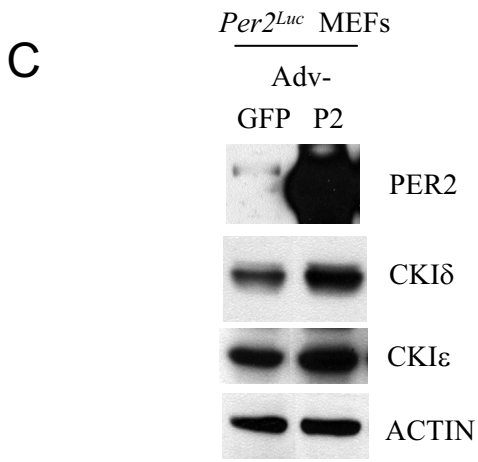


Fig. S6. Subcellular localization of PER1 and PER2 in Adv-GFP or Adv-DN-CKIε-infected cells.







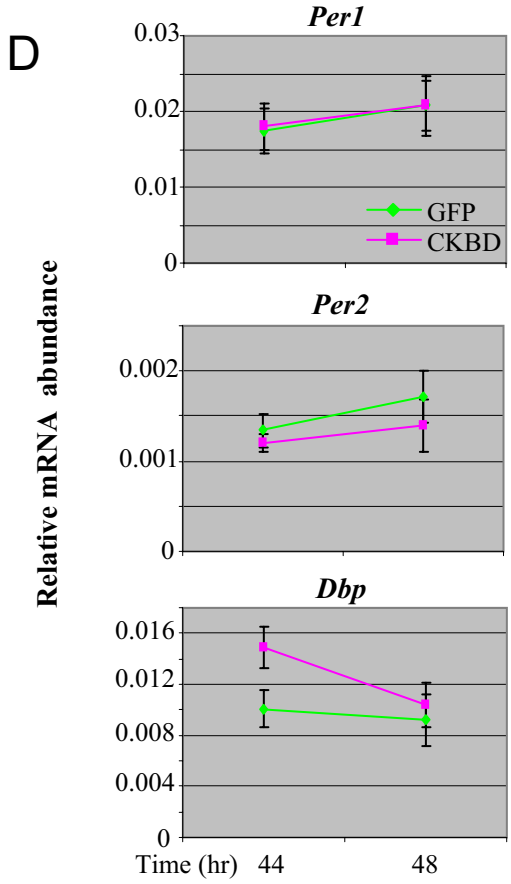


Fig. S7. Physical interaction between PER and CKIδ/ε stabilzes each other. (*A*) A dark exposure of the PER2 blot shown in Fig. 5*D*. (*B*) *Per1/2*−/− and matching wt liver tissue extracts were subjected to immunoblotting, showing that PER deficiency results in decreased CKIδ/ε levels relative to ACTIN. (*C*) GFP or PER2 was overexpressed in MEFs and the extracts were immunblotted, showing that PER2 overexpression results in increased CKIδ/ε levels relative to ACTIN. Data are representative of several experiments. (*D*) *Per1*, *2*, and *Dbp* mRNA levels were compared between GFP and CKBD-P2 MEFs by real-time quantitative PCR.