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Crystal Structure of the Heterodimeric CLOCK:BMAL1 Transcriptional Activator Complex

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Material and Methods

Protein expresion, purification, and crystallization

6xHis tagged mouse CLOCK bHLH-PAS domains (residues 26-384) and native mouse BMAL1 bHLH-PAS domains (residues 68-453) were cloned into pFastBac HTb and pFastBac1 vectors, respectively, and coexpressed in Sf9 insect cells. Frozen cell pellets were lysed by sonication in a lysis buffer containing 50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 15 mM imidazole, 5 mM β-mercaptoethanol, 10% v/v glycerol, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 1% v/v Brij-5. The clarified cell lysate was applied onto a Ni Sepharose column (GE Healthcare) equilibrated with the lysis buffer (without PMSF) and the bound protein was eluted with a gradient of 15-500 mM imidazole. The pooled fraction was buffer exchanged into a buffer containing 20 mM Tris pH 8.0, 200 mM NaCl, 10% v/v glycerol, 1 mM DTT, and the 6xHis tag was removed by treatment with TEV protease overnight at 4°C. The CLOCK:BMAL1 complex was further purified using a heparin column followed by a Superdex200 gel filtration column equilibrated in 20 mM HEPES pH 7.5, 300 mM NaCl, 5% glycerol, and 1 mM DTT. The protein was concentrated to 5.0 mg/ml, aliquoted, and flash frozen in liquid nitrogen and stored in -80°C.

CLOCK:BMAL1 crystals were grown at 20°C in a hanging drop vapor diffusion setup. The reservoir solution consisted of 100 mM HEPES pH 8.0, 6% PEG 3350, and 75 mM NaF and was mixed with the protein in a 1:1 ratio. Crystals reached maximum size after two days and were harvested at that time. Crystals were transferred stepwise to cryoprotectant solutions consisting of 50 mM HEPES pH 7.5, 8% PEG3350 and increasing concentrations of xylitol up to 36% (w/v), and flash frozen in liquid nitrogen. Selenomethionine (SeMet) labeled CLOCK:BMAL1 was expressed in Sf9 insect cells in a procedure similar to that described in (*44*). The crystals were grown from the similar conditions as the native protein. The native

CLOCK:BMAL1 crystals diffracted to about 2.6Å resolution while SeMet protein crystals diffracted to about 2.3Å. Therefore, data from SeMet CLOCK:BMAL1 crystals were used in the *ab initio* phase determination and final structure refinement (Supplementary Table S1).

X-ray Data collection, structure determination and analysis

Single-wavelength anomalous dispersion (SAD) data from a selenomethionine labeled CLOCK:BMAL1 crystal were collected to a resolution of 2.44Å at beamline 8.2.1 of Advance Light Source (ALS), Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA. The diffraction images were indexed, merged and scaled using HKL2000 (*45*). Selenium site determination, initial phasing, and density modification were performed in AutoSharp suite (*46-48*). Initial model building was performed using ArpWarp (*49*) and Buccaneer (*50*). The rest of the model was manually built using Coot (*51*). Further structure refinements were performed using REFMACS (*52, 53*) and phenix.refine (*54*). Diffraction data from a second selenomethionine labeled CLOCK:BMAL1 crystal was later collected at the Advance Photon Source (APS), Argonne National Laboratory to a resolution of 2.28Å and was used in the refinement of the final model. The crystal data and final refinement statistics are summarized in Supplementary Table S1.

The electrostatic potentials of CLOCK:BMAL1 were calculated using program ABPS (*55*). Full charges were assigned to protein atoms. The dielectic constants used were 2 for the protein and 80 for solvent. The calculation was performed using a 0.15M ionic strength solvent. All structural figures were drawn using the program PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC).

Electrophoresis mobility shift assays (EMSA) and fluorescence anisotropy assays

For EMSA, a 20 nucleotide DNA containing *mPer2* E2-box sequence (*40*) 5'-GCGCGGTCACGTTTTCCACT was synthesized (Sigma) and annealed with a 5' fluorescein labeled complementary DNA strand by heating at 94 °C for 5 min and slowly cooling down to room temperature. The annealed double strand DNA probe was incubated with increasing concentrations of CLOCK:BMAL1 in the reaction buffer (50 mM Tris pH 7.4, 45 mM NaCl) supplemented with 1.25 nM ssDNA competitor (GCCGGATCCGATGTCAGCCAAAATTAGAGCGGTG) for 10 min at room temperature. The samples were then loaded on an 8% non-denaturing polyacrylamide gels and run at constant voltage of 10 V cm^{-1} in 0.5X TBE buffer at room temperature. The gels were directly scanned on a Typhoon 9410 image system (GE Healthcare) using excitation filter of 488 nm and emission filter of 526 nm.

The same fluorescein labeled 20 nucleotide double strand DNA was used for the measurement of K_d by fluorescence anisotropy assays. Before the experiment, the CLOCK:BMAL1 complex was pre-equilibrated in the assay buffer containing 50 mM HEPES, pH 7.5, 300 mM NaCl and 1.0 mM DTT by gel filtration. The direct binding assay was performed by titrating 5 nM fluorescein labeled probe DNA with increasing amounts of CLOCK:BMAL1. Anisotropy data were collected on a QuantaMaster Spectrofluorometer (Photon Technology International). The data were fitted to the equation $\theta = ((B_U - B_L)\times\frac{[P_T]}{[D_T]} + B_L$ using program SigmaPlot. θ is the fraction of DNA bound; D_T is the total DNA concentration; P_T is the total protein concentration; B_U is the upper baseline and B_L is the lower baseline (*56*).

The K_d's of an unlabeled 18-nt *mPer1* E1-box containing DNA (5'-AGCCTGCACGTGTTCCCT) (*57*) and an unlabeled 18-nt *mPer2* E2-box DNA were measured by competition with the fluorescein labeled 20-nt *mPer2* E2 box probe. In the competition binding assay, 5 nM fluorescein labeled DNA probe and 144 nM CLOCK:BMAL1 mixture was titrated with increasing amount of unlabeled probes. The anisotropy data were fitted to the equation

$$F = F_0 + (F_{max} - F_0)\frac{\left\{2\sqrt{(a^2-3b)}\cos\left(\frac{\theta}{3}\right)-a\right\}}{3K_A+\left\{2\sqrt{(a^2-3b)}\cos\left(\frac{\theta}{3}\right)-a\right\}}$$

where $a = K_A + K_B + [A]_0 + [B]_0 - [P]_0$,

$b = K_B([A]_0 - [P]_0) + K_A([B]_0 - [P]_0) + K_A K_B$,

$c = -K_A K_B [P]_0$,

K_A was kept as constant 59.32 nM (*58*).

Site-directed Mutagenesis

For the transactivation assays and co-immunoprecipitation experiments, full-length mouse *Clock* cDNA was cloned into p3xFlag-CMV vector (Sigma #E4401) between the Not1 and BglII restriction sites. Full-length mouse BMAL1 was cloned into pcDNA3.1 vector with an N-terminal HA tag. Site-directed mutagenesis was performed as described in (*59*) using the primer sets listed in Table S2.

Co-immunoprecipitation

HEK293T cells (American Type Culture Collection) were seeded in 60 mm dishes at an initial density of $2X10^6$ in DMEM supplemented with 10% FBS containing penicillin/streptomycin (Invitrogen). The following day, cells were transfected using a standard calcium phosphate method with total 10μg of DNA (6μg *Clock* and 4 μg of *Bmal1*). Cells were harvested 48 hours post transfection and were resuspended in 250 μL lysis buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100,10% Glycerol, 0.5 mM Na_3VO_4, 0.1 mM PMSF, 1 mM DTT, 10 mM NaF, 1X Protease inhibitor complete EDTA-free cocktail (Roche #11873580001)), incubated on ice for 10 minutes followed by sonication on ice. After sonication, 750 µL wash buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.05% Triton X-100, 0.5 mM Na_3VO_4, 0.1 mM PMSF, 1 mM DTT, 10 mM NaF, 1X Protease inhibitor complete EDTA-free cocktail) was added to the lysed cells. Tubes were spun at 13,000 rpm in a bench top centrifuge. Approximately 50 µL of supernatant was saved for Input gels and rest were added to 10 µL of ANTI-FLAG M2 affinity gel (Sigma #A2220) and incubated at 4°C for 2 hours. Beads were washed twice with 1 mL of wash buffer. Approporiate amount of 1X SDS loading buffer were then added so that the total protein concentration is approximately 100 µg/µL.

Input samples (10 µg) were loaded on 8% polyacrylamide gels and Western blots were performed using a mixture of Monoclonal ANTI-FLAG M2-HRP antibody (Sigma #A8592) and HA-3F10 HRP antibody (Roche #1867431) at a dilution of 1:7000 and 1:1000 respectively. Gels with immuno-precipitated samples were loaded with 500 µg equivalent total protein and Western blots were performed using the HA-3F10 antibody at 1:1000 dilution. Blots were incubated with antibody at room temperature for two hours and were developed using supersignal West Pico Chemiluminescent substrate method (Thermo scientific # 34080) according to manufacturer's instructions.

Transactivation Assay

HEK293T cells were grown in DMEM/10% FBS/1% Penicillin-Streptomycin.. Cells were plated the day before transfection at 2×10^5 cells per well in twelve-well plates. Cells were transfected with 25 ng of Per2-luciferase reporter plasmid (pE2, (*40*)), 25 ng of pCMV-β-galactosidase (Promega) for normalization, and 75 ng each of mouse *Clock* and mouse *Bmal1* constructs using Effectene kit (Qiagen) according to the manufacturer's protocol. 36 hours after transfection, cells were lysed and luminescence was measured from 20 μl of lysate using the Luciferase Assay System (Promega) on a luminometer (AutoLumet Plus; Berthold).

Bimolecular Fluorescence Complementation Assay:

Truncated Venus fragments in the pEGFP-C1 (Clontech) backbone were created as VenN (residues 1-155) and VenC (residues 156-239) by site directed mutagenesis PCR as described in previous reports of BiFC analysis (*60, 61*). Full-length Venus (Ex515/Em528) was described previously (*62*). The VenN and VenC sequences were fused in frame at the C-termini of truncated *Bmal1* (residues 1-465) and *Clock* (residues 1-400), respectively.

Twenty-five ng histone H2B-mRFP1 (Ex584/Em607) (*63*) as a nuclear marker and 25ng *mPer2*(-279~+112)-Eluc-PEST (gift from Yoshihiro NAKAJIMA, IAST, Japan) as an E-box transactivation marker, were mixed with 100 ng each of *Bmal1* and *Clock* BiFC plasmids. $3x10^5$ HEK293A cells were suspended in DMEM supplemented with 10% FBS when transfected with 250 ng DNA by Effectene kit (Qiagen), and then plated in a well of 24-well black Visiplate (Perkin-Elmer) and incubated overnight at 37°C, 5% CO_2 followed by a medium change. 48 hrs after transfection, the plate was washed with PBS once and fixed with 4% paraformaldehyde in PBS for 15 min, washed twice, immersed with PBS, and sealed with top-seal A membrane (Perkin-Elmer).

Fluorescence images were acquired on a Deltavision personal DV imaging system (Applied Precision), equipped with an inverted 20x 0.45NA UPLFL objective and a Microtiter stage for Olympus IX71 microscope. Four fields of view for each well were picked and autofocused with RFP1 fluorescence, and the points were scanned with RFP (Ex575/25; Em632/60) followed by Venus (Ex513/17; Em559/34) filter sets. Image files were imported to and organized by ImageJ (NIH) and exported image sequences were analyzed through a custom pipeline run by Cellprofiler (Broad Institute). Briefly, the nuclei were first recognized based on RFP fluorescence, then inverse-masked in the Venus channel. The strongly fluorescent aggregates were also masked to be excluded from the image analysis. The mean Venus intensity value of each masked nucleus was measured. Between 40-70 cells were identified and measured in each image, and the average value of cells in one image was collected for further normalization and statistical analysis. Each experiment was performed 3 times independently.

Real-Time Monitoring of Circadian Rhythms

Wild-type and mutant Cerulean-*Bmal1* and Venus-*Clock*, under the control of the CMV promoter, were cloned into the pFUW Lentivirus vector. This vector and accompanying plasmids were gifts from Dr. Xueliang Zhu (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). Lentivirus vectors containing the full-length *Bmal1* and *Clock* constructs were cotransfected with packaging vectors into HEK293T cells (ATCC) using the calcium phosphate method (CalPhos kit from Clontech) as described in (*64*). A total of 10 μg of DNA was used, including 6 μg of the pFUW vectors containing *Clock* or *Bmal1*, 3 μg of Δ8.9 and 1 μg of V-SVG. Virus particles were harvested twice after transfection, at 48 and 72 hrs, passed through a 0.45 μm syringe filter, and added to 6 well plates containing a clonal line of immortalized fibroblasts derived from mPER2::LUC-SV40 knock-in mice ($2x10^5$ fibroblasts/well) (*65*). Forty-eight hours following the second addition of virus, the transduced fibroblasts were subcultured into 35 mm dishes and propagated for 24 hrs. For each *Bmal1* or *Clock* mutant, two separate cultures were prepared from each of two virus packaging and transduction procedures. These 4 cultures were used as replicates in the luminescence recording and statistical period analysis.

The determination of circadian period by luminescence recording was performed as previously reported (*32, 65, 66*). Briefly, lentivirus transduced fibroblasts were synchronized with regular culture medium supplemented with 100 nM dexamethasone for 2 hrs, then washed once with warm PBS, and immersed with 2 ml warm recording medium (phenol-red free DMEM, 2% FBS, 10 mM HEPES, 0.035% Sodium Bicarbonate, 0.1 mM Luciferin, pH 7.2). The plate was sealed with vacuum grease and round coverslips and mounted on a Lumicycle machine (Actimetrics) maintained at 37^{o}C. Raw luminescence data was analyzed with LumiCycle Analysis software (Actimetrics). The baseline was determined by the running average method using 6 days of data. The baseline-corrected data were fit using LM-Fit (damped sine) to determine circadian period.

Supplementary Figures and Tables

Figure S1

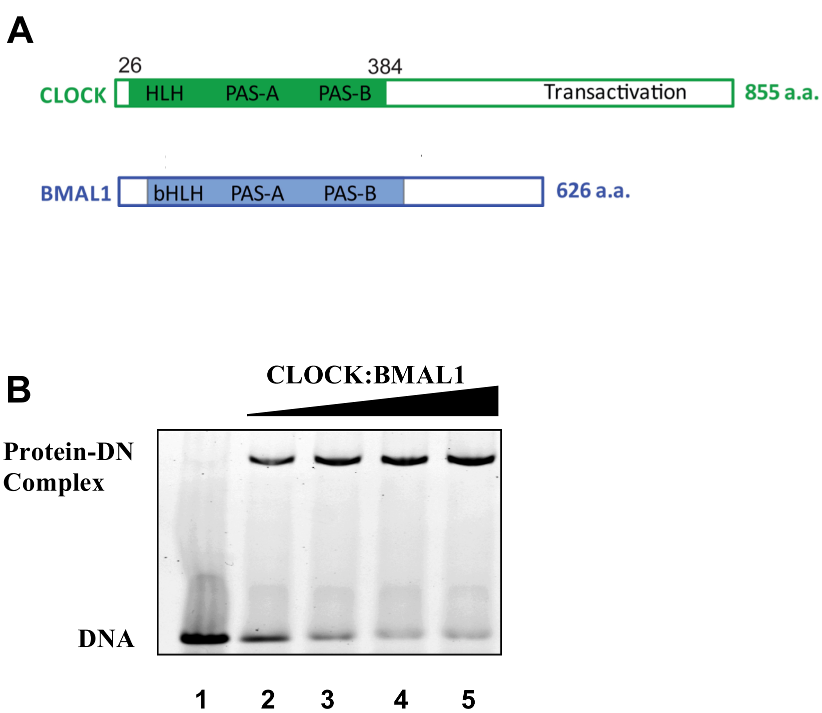


Fig. S1. The bHLH-PAS-AB construct of CLOCK:BMAL1 heterodimer binds E-box DNA with high affinity. (A) Domain organization of CLOCK and BMAL1. The constructs used in the present structural study encompassing the bHLH-PAS-AB domains are color shaded. (**B**) EMSA assay using an 8% non-denaturing polyacrylamide gel. 12.5 nM 5'-fluorescein-GCGCGGTCACGTTTTCCACT (E2 box of *mPer2* promoter (*40*)) was incubated with increasing amounts of purified recombinant CLOCK:BMAL1 (0, 72.5 nM, 145 nM, 290 nM, 435 nM for lane 1-5).

Figure S2

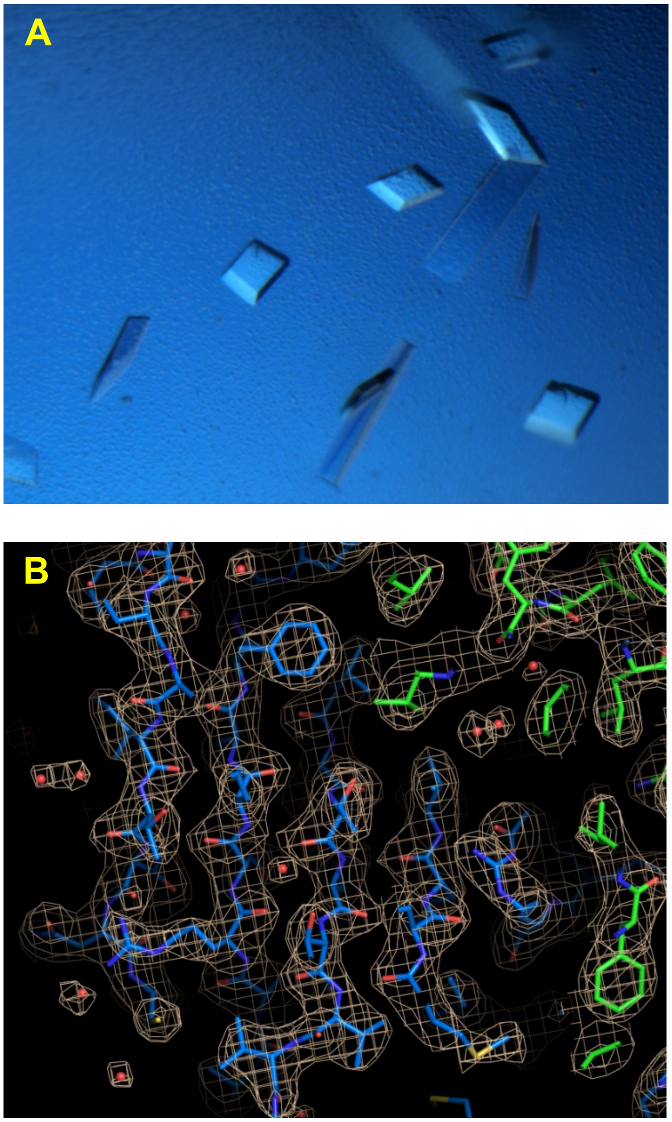
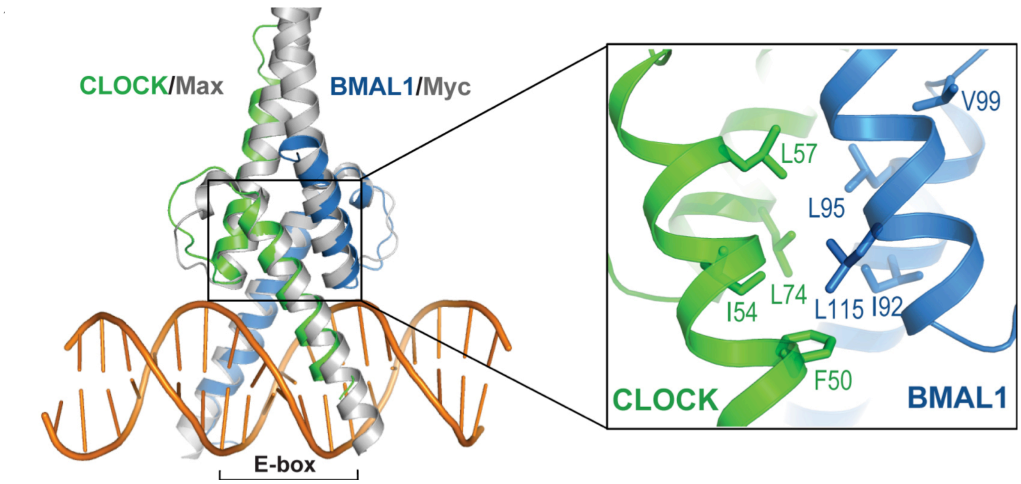


Fig. S2. Crystal structure determination of CLOCK:BMAL1. (A) Crystals of CLOCK:BMAL1 complex obtained from selenomethione labeled protein. **(B)** Electron densities of CLOCK:BMAL1. The 2Fo-Fc map is contoured at 1.2σ. A representative area of the map is shown. The CLOCK subunit is colored green, BMAL1 is colored blue, and water molecules are shown as red spheres.

Figure S3

A



B

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CLOCK_MOUSE     33   KAKRVSRNKSEKKRRDQFNVLIKELGSMLPGN------ARKMDKSTVLQKSIDFLRKHKETTAQSDAS------  94
NPAS2_MOUSE      8   RAKRASRNKSEKKRRDQFNVLIKELSSMLPGN------TRKMDKTTVLEKVIGFLQKHNEVSAQTEIC------  69
CLOCK_DROME     14   FLCRKSRNLSEKKRRDQFNSLVNDLSALISTS------SRKMDKSTVLKSTIAFLKNHNEATDRSKVF------  75
AHR_MOUSE       36   PAEGIKSNPSKRHRDRLNTEL-DRLASLLPFP---QDVINKLDKLSVLRLSVSYLRAKSFFDVALKSTPADRNG  95
HIF1A_MOUSE     16   ERRKEKSRDAARSRRSKESEVFYELAHQLPLP---HNVSSHLDKASVMRLTISYLRVRKLLDAGG---------  77
EPAS1_MOUSE     13   ELRKEKSRDAARCRRSKETEVFYELAHELPLP---HSVSSHLDKASIMRLAI-FLRTHKLLSSVC---------  74
HIF3A_MOUSE     11   ELRKEKSRDAARSRRSQETEVLYQLAHTLPFA---RGVSAHLDKASIMRLTISYLRMHRLCAAGE---------  72
SIM1_MOUSE       1   --MKEKSKNAARTRREKENSEFYELAKLLPLP---SAITSQLDKASIIRLTTSYLKMRVVFPEGLGEAW-----  64
SIM_DROME       33   CAMKEKSKNAARTRREKENTEFCELAKLLPLP---AAITSQLDKASVIRLTTSYLKMRQVFPDGLGEAW-----  88
BMAL1 Mouse     71   KNAREAHSQIEKRRRDKMNSFIDELASLVPTC---NAMSRKLDKLTVLRMAVQHMKTLRGATNPYTE------- 134
BMAL2_MOUSE     47   SGIREAHSQMEKRRRDKMNHLIQKLSSMIPPH---IPTAHKLDKLSVLRRAVQYLRSLRGMTELYLG------- 110
CYCL_DROME      29   ENRKQNHSEIEKRRRDKMNTYINELSSMIPMC---FAMQRKLDKLTVLRMAVQHLRGIRGSGSLHPFNG-----  94
ARNT_MOUSE      88   RLARENHSEIERRRRNKMTAYITELSDMVPTC---SALARKPDKLTILRMAVSHMKSLRGTGNTSTD------- 151
ARNT_DROME      12   FASRENHCEIERRRRNKMTAYITELSDMVPTC---SALARKPDKLTILRMAVAHMKALRGTGNTSSD-------  75

1HLO_A (Max)    12   ADKRAHHNALERKRRDHIKDSFHSLRDSVPS----LQG-EKASRAQILDKATEYIQYMRRKN
1NKP_A (Myc)   353   NVKRRTHNVLERQRRNELKRSFFALRDQIPE----LENNEKAPKVVILKKATAYILSVQAEEQ
1NLW_A (Mad)    58   ---RSTHNEMEKNRRAHLRLSLEKLKGLVPL----GPDSSRHTTLSLLTKAKLHIKKLEDSD
1AN4_A (USF)   198   EKRRAQHNEVERRRRDKINNWIVQLSKIIPD-SSMESTKSGQSKGGILSKASDYIQELRQSNH
1A0A_A (PHO4)    1   --KRESHKHAEQARRNRLAVALHELASLIPAEWKQQNVSAAPSKATTVEAACRYIRHLQQNGS
1MDY_A (MyoD)  108   ADRRKAATMRERRRLSKVNEAFETLKRSTSS-----NPNQRLPKVEILRNAIRYIEGLQALLRD

                        DNA-binding         4-helical             4-helical
                                            Bundle                Bundle
```

Fig. S3. Structure of the bHLH DNA-binding domain of CLOCK:BMAL1.
A). Superposition of CLOCK:BMAL1 bHLH domain with the bHLH Myc:Max-DNA complex (pdb: 1NKP). The Myc:Max are colored gray and DNA orange. The blown-up inset shows the details of the hydrophobic core of the bHLH domain. **B**). Multiple sequence alignment of the bHLH domains of bHLH-PAS family proteins (*upper block*) with other bHLH-containing proteins of known structure (*lower block*). The Swiss-Prot or PDB identification codes of the sequences are used except for mouse BMAL1, which has GeneBank accession code NP_031515. The conserved residues involved in DNA binding are highlighted in cyan;, while the conserved hydrophobic core residues are in yellow. The secondary structure elements of the domain are shown at the top. Residues that were mutated in our mutagenesis analyses are highlighted in magenta.

Figure S4

PAS-A domain

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                                   A'α       Aβ         Bβ    Cα      Dα      Eα        Fα
ss:             hhhhhhhhhh  eeeeeee     eeee   hhhhhh   hhhh    hhhh    hhhhhhhhhhh
CLOCK_MOUSE     -EIRQDWKPTFLSNEEFTQLMLEALDGFFLAIMTD-GSIIYVSESVTSLLEHLPSDLVDQSIFNFIPEGEHSEVYKILSTHLLES-DSLTPEY------------------LKSKN----  189
NPAS2_MOUSE     -DIQQDWKPSFLSNEEFTQLMLEALDGFVIVVTTD-GSIIYVSDSITPLLGHLPADVMDQNLLNFLPEQEHSEVYKILSSHMLVT-DSPSPEF------------------LKSDN----  164
CLOCK_DROME     -EIQQDWKPAFLSNDEYTHLMLESLDGFMMVFSSM-GSIFYASESITSQLGYLPQDLYNMTIYDLAYEMDHEALLNIFMNPTPVIEPRQTD--------------------ISSSN----  169
AHR_MOUSE       GQDQCRAQIRDWQDLQEGEFLLQALNGFVLVVTAD-ALVFYASSTIQDYLGFQQSDVIHQSVYELIHTEDRAEFQRQLHWALNPDSAQGVDEAHGPPQAAVYYTPDQLPPENASFM----  210
HIF1A_MOUSE     ------LDSEDEMKAQMDCFYLKALDGFVMVLTDD-GDMVYISDNVNKYMGLTQFELTGHSVFDFTHPCDHEEMREMLTHRNGPVRKGK-----------------------ELNT----  163
EPAS1_MOUSE     ----SENESEAEADQQMDNLYLKALEGFIAVVTQD-GDMIFLSENISKFMGLTQVELTGHSIFDFTHPCDHEEIRENLTLKNGSGFGKKSK---------------------DVST----  164
HIF3A_MOUSE     ------WNQVEKGGEPLDACYLKALEGFVMVLTAE-GDMAYLSENVSKHLGLSQLELIGHSIFDFIHPCDQEELQDALTPRPNLSKKKL-----------------------EAPT----  158
SIM1_MOUSE      -GHTSRTSPLDNVGRELGSHLLQTLDGFIFVVAPD-GKIMYISETASVHLGLSQVELTGNSIYEYIHPADHDEMTAVLTAHQPYHSHFVQ----------------------EYEI----  156
SIM2_MOUSE      -GQPSRTGPLDSVAKELGSHLLQTLDGFVFVVASD-GKIMYISETASVHLGLSQVELTGNSIYEYIHPSDHDEMTAVLTAHPPLHHHLLQ----------------------EYEI----  156
SIM_DROME       --GSSPAMQRGATIKELGSHLLQTLDGFIFVVAPD-GKIMYISETASVHLGLSQVELTGNSIFEYIHNYDQDEMNAILSLHPHINQHPLAQTHTPIGSPNGVQHPSAYDHDRGSHTIEI-  204
BMAL1 Mouse     ----ANYKPTFLSDDELKHLILRAADGFLFVVGCDRGKILFVSESVFKILNYSQNDLIGQSLFDYLHPKDIAKVKEQLSSSDTAPRERLIDAKTGL---PVKTDITPGPSRLCSGA----  243
BMAL2_MOUSE     ----ENSKPSFIQDKELSHLILKAAEGFLFVVGCERGRIFYVSKSVSKTLRYDQASLIGQNLFDFLHPKDVAKVKEQLS-CDGSPREKPIDTKTS----QVYSHPYTGRPRMHSGS----  217
CYCL_DROME      ----SDYRPSFLSDQELKMIILQASEGFLFVVGCDRGRILYVSDSVSSVLNSTQADLLGQSWFDVLHPKDIGKVKEQLSSLEQCPRERLIDAKTML---PVKTDVPQSLCRLCPGA----  203
ARNT2_MOUSE     ----GAYKPSFLTEQELKHLILEAADGFLFVVAAETGRVIYVSDSVTPVLNQPQSEWFGSTLYEQVHPDDVEKLREQLCTSENSMTGRILDLKTGT----VKKEGQQSSMRMCMGS----  233
ARNT_MOUSE      ----GSYKPSFLTDQELKHLILEAADGFLFIVSCETGRVVYVSDSVTPVLNQPQSEWFGSTLYDQVHPDDVDKLREQLSTSENALTGRVLDLKTGT----VKKEGQQSSMRMCMGS----  259
ARNT_DROME      ----GTYKPSFLTDQELKHLILEAADGFLFVVSCDSGRVIYVSDSVTPVLNYTQSDWYGTSLYEHIHPDDREKIREQLSTQESQNAGRILDLKSGT----VKKEGHQSSMRLSMGA----  183
PER2_MOUSE  170 ------SYSMEQVEGITSEYIVKNADMFAVAVSLVSGKILYISNQVASIFHCKKDAFSDAKFVEFLAPHDVSVFHSYTTPYKLPPWSVCSG-------------------LDSFTQECME  264
PER1_MOUSE  195 --MDMSTYTLEELEHITSEYTLRNQDTFSVAVSFLTGRIVYISEQAGVLLRCKRDVFRGARFSELLAPQDVGVFYGSTTPSRLPTWGTGTSAGSG----------------LKDFTQ----  293
PER_DROME   223 ----------AAAAGTGQRGERVKEDSFCCVISMHDGIVLYTTPSITDVLGYPRDMWLGRSFIDFVHLKDRATFASQITTGIPIAESRGSV-----------------------PKDA------  307
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PAS-A dimerization

```
                    Gβ                                           Hβ                                                Iβ
ss:            eeeeeeeeee                                 eeeeeeeeeeeee                                       eeeeeeeee
CLOCK_MOUSE    QLEFCCHMLRGT-----------------------IDPKEPSTYEYVRFIGNFKSLTS-VSTSTHNGF-----------EGTIQRTHRPSYEDRVCFVATVRL----ATPQF-IKEMCTV  269
NPAS2_MOUSE    DLEFYCHLLRGS-----------------------LNPKEFPTYEYIKFVGNFRSYNN-VPSPSCNGF-----------DNTLSRPCHVPLGKDVCFIATVRL----ATPQF-LKEMCVA  244
CLOCK_DROME    QITFYTHLRRGG-----------------------MEKVDANAYELVKFVGYFRNDTN-TSTGSSSEVSNGSNGQPAVLPRIFQQNPNAEVDKKLVFVGTGRV----QNPQL-IREMSII  250
AHR_MOUSE      ERCFRCRLRCLL--------------------------DNSSGFLAMNFQGRLKYLHG-QNKKG---------------------KDGALLPPQLALFAIATP----LQP----PSILEI  274
HIF1A_MOUSE    QRSFFLRMKCTLTSR------------------GRTMNIKSATWKVLHCTGHIHVYDT-NSNQP---------------------QCGYKKPPMTCLVLICEP----IPH----PSNIEI  235
EPAS1_MOUSE    ERDFFMRMKCTVTNR------------------GRTVNLKSATWKVLHCTGQVRVYNNCPPHSS---------------------LCGSKEPLLSCLIIMCEP----IQH----PSHMDI  237
HIF3A_MOUSE    ERHFSLRMKSTLTSR------------------GRTLNLKAATWKVLHCSGHMRAYKP-PAQTS-------------------PAGSPRSEPPLQCLVLICEA----IPH----PASLEP  232
SIM1_MOUSE     ERSFFLRMKCVLAKR--------------------NAGLTCGGYKVIHCSGYLKIRQY-SLDMS----------------------PFDGCYQNVGLVAVGHS----LPP----SAVTEI  225
SIM2_MOUSE     ERSFFLRMKCVLAKR--------------------NAGLTCSGYKVIHCSGYLKIRQY-MLDMS----------------------LYDSCYQIVGLVAVGQS----LPP----SAITEI  225
SIM_DROME      EKTFFLRMKCVLAKR--------------------NAGLTTSGFKVIHCSGYLKARIY-PDRGD----------------------GQGSLIQNLGLVAVGHS----LPS----SAITEI  273
BMAL1 Mouse    RRSFFCRMKCNRPSVKVEDKDFA--------STCSKKKADRKSFCTIHSTGYLKSWPP-TKMGLD-----------------EDNEPDNEGCNLSCLVAIGRL----HSHMVPQPANGEI  333
BMAL2_MOUSE    RRSFFFRMKSCTVPVKEEQPC----------SSCSKKK-DHRKFHTVHCTGYLRSWPL-NVVGME-----------------KESGGGKDSGPLTCLVAMGRL----HPYIVPQ-KSGKI  303
CYCL_DROME     RRSFFCRMKLRTASNNQIKEESDTSSSSRSSTKRKSRLTTGHKYRVIQCTGYLKSWTP--IKDED-----------------QDADSDEQTTNLSCLVAIGRIPPNVRNSTVPASLDNHP  304
ARNT2_MOUSE    RRSFICRMRCGNAPLDHLPLNRITTMRKRFRNGLGPVKEGEAQYAVVHCTGYIKAWPP-AGMTIP------------------EEDADVGQGSKYCLVAIGRL----QVTSSPVCMDMSG  330
ARNT_MOUSE     RRSFICRMRCGTSSVDPVSMNRLSFLRNRCRNGLGSVKEGEPHFVVVHCTGYIKAWPP-AGVSLP------------------DDDPEAGQGSKFCLVAIGRL----QVTSSPNCTDMSN  356
ARNT_DROME     RRGFICRMRVGNVNPESMVSGHLNRLK--QRNSLGPSRDGT-NYAVVHCTGYIKNWPP-TDMFPNM---------------HMERDVD-DMSSHCCLVAIGRL----QVTST-AANDMSG  278
PER2_MOUSE     EKSFFCRVSVGKH------------------------HENEIRYQPFRMTPYLVKVQE-QQG---------------------------AESQLCCLLLAERV--------HSGYEAPRI  324
PER1_MOUSE     EKSVFCRIRGGPD------------------------RDPGPRYQPFRLTPYVTKIRV-SDG---------------------------APAQPCCLLIAERI--------HSGYEAPRI  353
PER_DROME      KSTFCVMLRRYRGLK----------------SGGFGVIGRPVSYEPFRLGLTFREAPE-EARPDN---------------------YMVSNGTNMLLVICATP----IKSSYKV-PDEIL  384
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linker

Figure S4 (*Cont.*)

PAS-B domain

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                   Aβ          Bβ    Cα      Dα       Eα          Fα                Gβ               Hβ                Iβ
ss:                eeeeeeee      eeee  hhhhh   hhhh     hhhh    hhhhhhhhhhhhhh        eeeeeeeee    eeeeeeeeeeeee       eeeeeeeeee
CLOCK_MOUSE  EEPNEEFTSRHSLEWKFLFLDHRAPPIIGYLPFEVLGT-SGYDYYHVDDLENLAKCHEHLMQYG-----KGKSCYYRFLTKGQQWIWLQTHYYITYHQWNSRPEFIVCTHTVVSYAEVRAERRR  387 (855)
NPAS2_MOUSE  DEPLEEFTSRHSLEWKFLFLDHRAPPIIGYLPFEVLGT-SGYNYYHIDDLELLARCHQHLMQFG-----KGKSCCYRFLTKGQQWIWLQTHYYITYHQWNSKPEFIVCTHSVVSYADVRVERRQ  362 (816)
CLOCK_DROME  DPTSNEFTSKHSMEWKFLFLDHRAPPIIGYMPFEVLGT-SGYDYYHFDDLDSIVACHEELRQTG-----EGKSCYYRFLTKGQQWIWLQTDYYVSYHQFNSKPDYVVCTHKVVSYAEVLKDSRK  378 (1027)
AHR_MOUSE    RTKNFIFRTKHKLDFTPIGCDAKGQLILGYTEVELCTRGSGYQFIHAADMLHCAESHIRMIKTG-----ESGMTVFRLFAKHSRWRWVQSNARLIYRN--GRPDYIIATQRPLTDEEGREHLQK  391 (848)
HIF1A_MOUSE  PLDSKTFLSRHSLDMKFSYCDERITELMGYEPEELLGR-SIYEYYHALDSDHLTKTHHDMFTKG-----QVTTGQYRMLAKRGGYVWVETQATVIYNTKNSQPQCIVCVNYVVSGIIQHDLIFS  353 (836)
EPAS1_MOUSE  PLDSKTFLSRHSMDMKFTYCDDRILELIGYHPEELLGR-SAYEFYHALDSENMTKSHQNLCTKG-----QVVSGQYRMLAKHGGYVWLETQGTVIYNPRNLQPQCIMCVNYVLSEIEKNDVVFS  355 (874)
HIF3A_MOUSE  PLGRGAFLSRHSLDMKFTYCDERIAEVAGYSPDDLIGC-SAYEYIHALDSDAVSRSIHTLLSKG-----QAVTGQYRFLARTGGYLWTQTQATVVSGGRGPQSESIICVHFLISRVEETGVVLS  340 (662)
SIM1_MOUSE   KLHSNMFMFRASLDMKLIFLDSRVAELTGYEPQDLIEK-TLYHHVHGCDTFHLRCAHHLLVKG-----QVTTKYYRFLAKQGGWVWVQSYATIVHNSRSSRPHCIVSVNYVLTDTEYKGLQLS  343 (765)
SIM2_MOUSE   KLHSNMFMFRASLDLKLIFLDSRVTELTGYEPQDLIEK-TLYHHVHGCDTFHLRYAHHLLVKG-----QVTTKYYRLLSKLGGWVWVQSYATVVHNSRSSRPHCIVSVNYVLTDVEYKELQLS  343 (657)
SIM_DROME    KLHQNMFMFRAKLDMKLIFFDARVSQLTGYEPQDLIEK-TLYQYIHAADIMAMRCSHQILLYKG-----QVTTKYYRFLTKGGGWVWVQSYATLVHNSRSSREVFIVSVNYVLSEREVKDLVLN  391 (697)
BMAL1 Mouse  RVKSMEYVSRHAIDGKFVFVDQRATAILAYLPQELLGT-SCYEYFHQDDIGHLAECHRQVLQTRE----KITTNCYKFKIKDGSFITLRSRWFSFMNPWTKEVEYIVSTNTVVLANVLEGGDPT  453 (626)
BMAL2_MOUSE  NVRPAEFITRFAMNGKFVYVDQRATAILGYLPQELLGT-SCYEYFHQDDHSSLTDKHKAVLQSKE----KILTDSYKFRVKDGAFVTLKSEWFSFTNPWTKELEYIVSVNTLVLGRSETRLSLL  422 (579)
CYCL_DROME   NIRHVLFISRHSGEGKFLFIDQRATLVIGFLPQEILGT-SFYEYFHNEDIAALMESHKMVMQVPE----KVTTQVYRFRCKDNSYIQLQSEWRAFKNPWTSEIDYIIAKNSVFL----------  413 (413)
ARNT2_MOUSE  MSVPTEFLSRHNSDGIITFVDPRCISVIGYQPQDLLGK-DILEFCHPEDQSHLRESFQQVVKLKG----QVLSVMYRFRTKNREWLLIRTSSFTFQNPYSDEIEYVICTNTNVKQLQQQAELE  449 (712)
ARNT_MOUSE   ICQPTEFISRHNIEGIFTFVDHRCVATVGYQPQELLGK-NIVEFCHPEDQQLLRDSFQQVVKLKG----QVLSVMFRFRSKTREWLWMRTSSFTFQNPYSDEIEYIICTNTNVKNSSQEPRPTL  475 (791)
ARNT_DROME   SNNQSEFITRHAMDGKFTFVDQRVLNILGYTPTELLGK-ICYDFFHPEDQSHMKESFDQVLKQKG----QMFSLLYRARAKNSEYVWLRTQAYAFLNPYTDEVEYIVCTNSSGKTMHGAPLDAA  397 (644)
PER2_MOUSE   PPEKRIFTTTHTPNCLFQAVDERAVPLLGYLPQDLIET-PVLVQLHPSDRPLMLAIHKKILQAGGQ---PFDYSPIRFRTRNGEYITLDTSWSSFINPWSRKISFIIGRHKVRVGPLNEDVFAA  444 (1257)
PER1_MOUSE   PPDKRIFTTRHTPSCLFQDVDERAAPLLGYLPQDLLGA-PVLLFLHPEDRPLMLAIHKKILQLAGQ---PFDHSPIRFCARNGEYVTMDTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVF-T  473 (1291)
PER_DROME    SQKSPKFAIRHTATGIISHVDSAAVSALGYLPQDLIGR-SIMDFYHHEDLSVMKETYETVMKKGQTAGASFCSKPYRFLIQNGCYVLLETEWTSFVNPWSRKLEFVVGHHRVFQGPKQCNVFEA  507 (1224)
```

Fig. S4. Multiple sequence alignments of the PAS domains among selected bHLH-PAS family proteins. Secondary structure elements are shown at the top of the alignments with "h" denoting α helices and "e" denoting β strands. Residue number of each sequence is indicated at the end of each block and the total number of residues in each protein is shown in bracket at the end of the alignment. Conserved hydrophobic or neutral residues are highlighted in yellow, and polar/small residues in gray. Residues that were mutated in our mutagenesis analyses are highlighted in magenta.

Figure S5

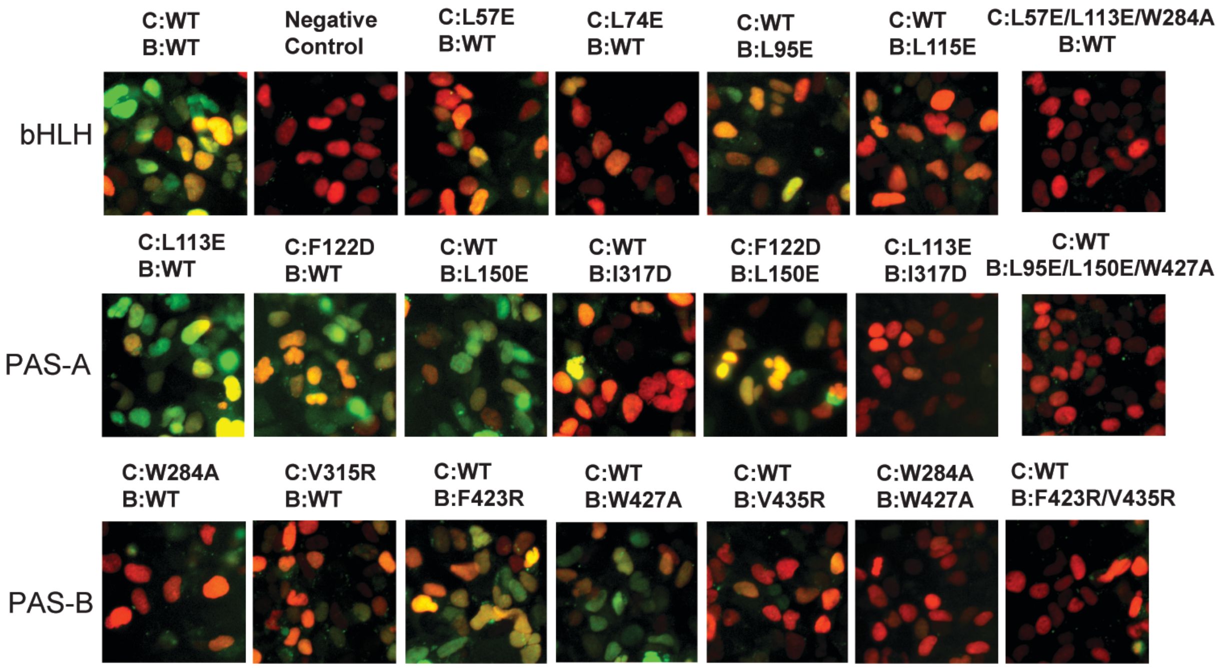

Figure S5. Representative cell images from Bimolecular fluorescence complementation (BiFC) experiments of WT and mutant CLOCK:BMAL1 truncated constructs (for details see Material and Methods). Complementation of the bHLH-PAS-AB construct of CLOCK:BMAL1 generates green fluorescence of Venus. Red fluorescence is generated by RFP-tagged H2B for nuclear staining. While the red fluorescence signal from RFP is present in all images, a strong green Venus signal can obscure the red signal in some images. Absence of green fluorescence from Venus indicates weak or no association between CLOCK and BMAL1 subunits.

Figure S6

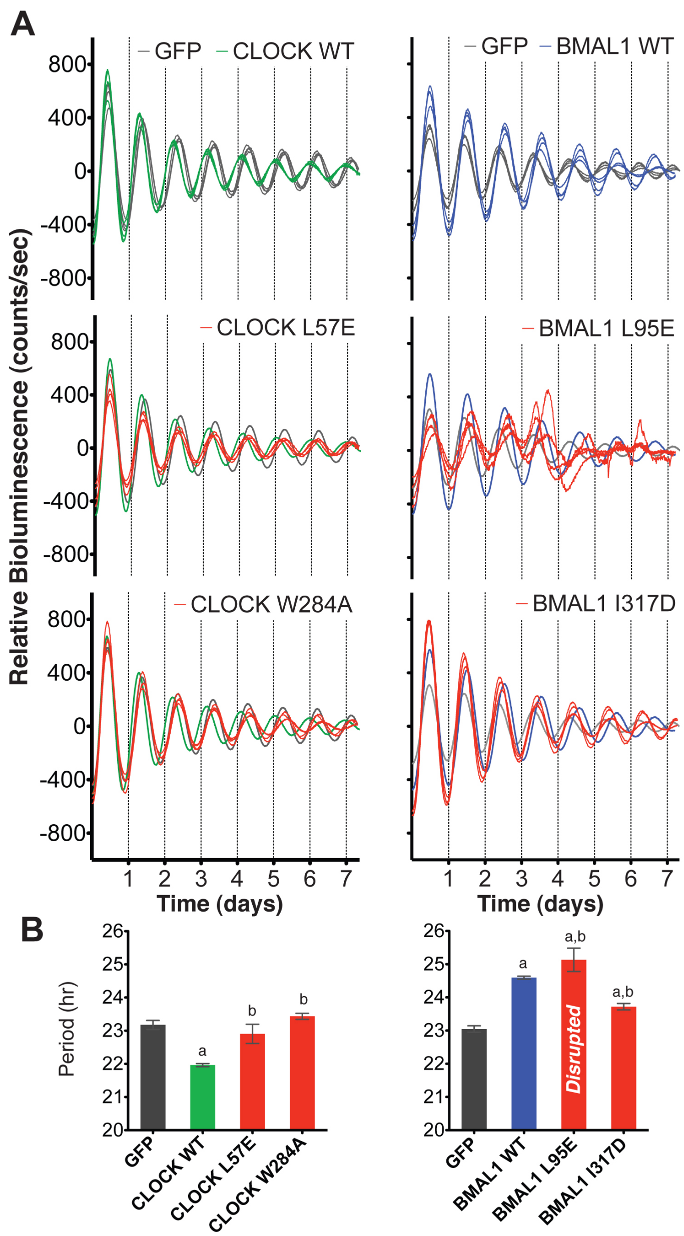

Fig. S6. CLOCK:BMAL1 mutants alter circadian PER2::luciferase rhythms in fibroblasts. (**A**) Representative bioluminescence records of *Per2*^{Luc} fibroblasts overexpressing GFP (gray), WT CLOCK (green) and WT BMAL1 (blue) are shown (*top two panels*, n=4). Lumicycle traces for CLOCK and BMAL1 mutants (red) are shown overlapping with the mean traces of GFP (gray) and WT CLOCK or BMAL1 (as above) overexpressing cells for comparison (*lower four panels*, n=4). (**B**) Average period of the circadian rhythms in cells overexpressing GFP, WT and mutant CLOCK (*left panel*), one-way ANOVA p< 0.0001; and GFP, WT and mutant BMAL1 (*right panel*), one-way ANOVA p< 0.0001. Tukey's multiple comparison posthoc test: "a" = different from GFP control at p<0.01; "b" = different from WT control at p<0.01. BMAL1 L95E rhythms were disrupted as indicated in the bar graph so period values were based on the first 3 days.

Figure S7

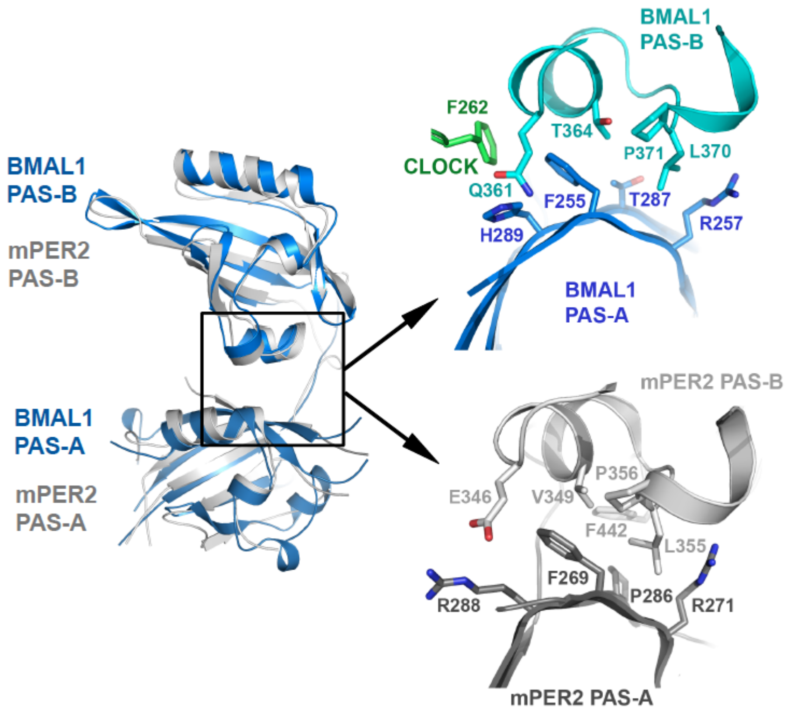

Fig. S7. Spatial arrangement of the two PAS domains in BMAL1 is similar to that in PER. Superposition of mouse PERIOD2 protein (mPER2, colored gray) with BMAL1 (blue). The detailed interface between the two PAS domains in the two proteins is shown in enlarged insets (*right panels*). The two PAS domains in BMAL1 have a similar spatial arrangement to that observed in mouse PER2 and *Drosophila* PER (*35*) (*left*). While the Cα rmsd between individual PAS-A or PAS-B domains of BMAL1 and mPER range from 0.9-1.3Å, when the tandem PAS-A and PAS-B domains in the two proteins are superimposed, the Cα rmsd increased only moderately to 1.9Å.

Figure S8

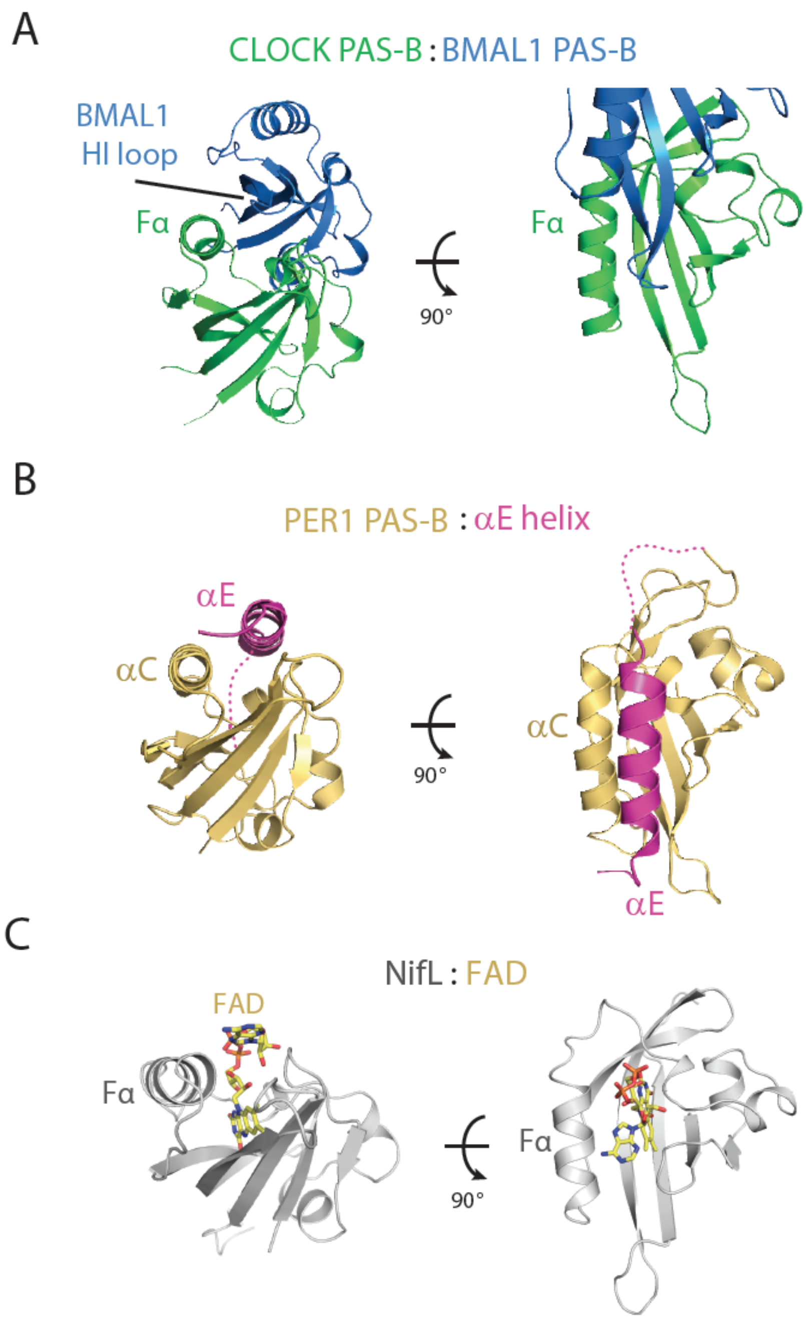

Fig. S8. A common protein-protein and protein-ligand binding site on the helical face of PAS domains. (A) CLOCK (green) and BMAL1 (blue) PAS-B interaction. (B) Helical interface of PER1 PAS-B (yellow) with its αE helix (magenta) (pdb: 4DJ2). Note: helix αC is equivalent to Fα helix, and αE is equivalent to Jα in canonical PAS nomenclature. (C) NifL oxygen sensing PAS domain (gray) in complex with FAD (yellow) (pdb: 2GJ3).

Table S1. Data collection and refinement statistics

	Native1	SeMet1	SeMet2
Data collection			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions			
a, *b*, *c* (Å)	67.19, 71.89, 174.17	67.10, 71.73, 172.76	67.24, 71.96, 173.31
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	50-2.56 (2.65-2.56)[1]	50-2.44 (2.48-2.44)[1]	50.0-2.28 (3.05-2.28)[1]
R_{sym} or R_{merge}	0.055 (0.770)	0.079 (0.780)	0.060 (0.864)
$I / \sigma I$	24.1 (1.7)	27.7 (1.9)	30.9 (1.8)
Completeness (%)	98.3 (99.8)	99.2 (94.5)	97.5 (100.0)
Redundancy	3.6 (3.5)	6.7 (5.6)	5.2 (5.3)
Unique reflections	27473	33106	39307
Refinement			
Resolution (Å)			37.0-2.28
No. reflections			36738
R_{work} / R_{free}			0.186/0.219
No. atoms			
Protein			5105
Water			172
B-factors			
Protein			71.7
Water			60.4
R.m.s. deviations			
Bond lengths (Å)			0.009
Bond angles (°)			0.809
Ramachandran Plot [2]			
Favored Regions (%)			98.4
Outliers (%)			0

[1]Values in parentheses are for highest-resolution shells.
[2]Evaluated by MolProbity (*67*)

Table S2. Primers for the site-directed mutagenesis of CLOCK:BMAL1

Mutant	Primer	Sequence
CLOCK mutants:		
L57E:	F-	CAG TTC AAT GTC CTC ATT AAG GAG GAG GGG TCT ATG
	R-	AAT GAG GAC ATT GAA CTG ATC TCT ACG TTT CTT TTC TGA TTT
L74E:	F-	AGA AAG ATG GAC AAG TCT ACT GTT GAG CAG AAG AGC ATT
	R-	AGA CTT GTC CAT CTT TCT CGC GTT ACC AGG AAG CAT AGA CCC
L113E:	F-	CTT AGT AAT GAA GAG TTT ACA CAG GAG ATG TTA GAG GCT CTT
	R-	TGT AAA CTC TTC ATT ACT AAG GAA TGT GGG TTT CCA GTC CTG
F122D:	F-	TTA ATG TTA GAG GCT CTT GAT GGT TTT GAC TTA GCG ATC ATG ACA
	R-	ATC AAG AGC CTC TAA CAT TAA CTG TGT AAA CTC TTC ATT ACT AAG
W284A:	F-	TTT ACA TCT AGA CAC AGT TTA GAA GCG AAG TTT CTA TTT TTA GAT
	R-	TAA ACT GTG TCT AGA TGT AAA CTC TTC ATT TGG TTC TTC AAC AGT
V315R:	F-	GGA ACA TCA GGC TAT GAT TAC TAT CAT CGC GAT GAC CTA GAA
	R-	ATC ATA GCC TGA TGT TCC CAA GAC TTC AAA TGG CAA ATA
BMAL1 mutants:		
L95E:	F-	AAA ATG AAC AGT TTC ATT GAT GAA GAG GCT TCT TTG GTA CCA
	R-	ATC AAT GAA ACT GTT CAT TTT GTC CCG ACG CCT CTT TTC
L115E:	F-	AGG AAG TTA GAT AAA CTC ACC GTG GAG AGG ATG GCT GTT CAG
	R-	GGT GAG TTT ATC TAA CTT CCT GGA CAT TGC ATT GCA TGT TGG
L150E:	F-	TTT CTA TCA GAT GAC GAA CTG AAA CAC GAG ATT CTC AGG GCA
	R-	CAG TTC GTC ATC TGA TAG AAA TGT TGG CTT GTA GTT TGC TTC TGT
I317D:	F-	TGC AAC CTC AGC TGC CTC GTT GCA GAC GGG CGC CTG
	R-	GAG GCA GCT GAG GTT GCA GCC CTC GTT GTC TGG CTC
F423R:	F-	CTA CGA AGT CGA TGG TTC AGT CGC ATG AAC CCG CAC
	R-	GAA CCA TCG ACT TCG TAG CGT GAT AAA AGA ACC ATC TTT GAT CTT
W427A:	F-	CGA TGG TTC AGT TTC ATG AAC CCG GCG ACC AAG GAA GTT
	R-	CAT GAA ACT GAA CCA TCG ACT TCG TAG CGT GAT AAA AGA ACC
V435R:	F-	CCG TGG ACC AAG GAA GTT GAA TAC ATT CGC TCA ACC AAC
	R-	TTC CTT GGT CCA CGG GTT CAT GAA ACT GAA CCA TCG ACT TCG

The mutated codons are indicated in red.

Supplementary movie: CB.avi