Supplementary Information for
Casein kinase 1 and disordered clock proteins form functionally equivalent phospho-based circadian modules in fungi and mammals

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This PDF file includes:

Supplementary Figures and Figure Legends S1 to S5
Supplementary Methods
Supplementary References
**Supplementary Figure 1**

**A**

- CK1α
- CK1α-R181Q
- CK1αΔC

**B**

- M₁
- CK1α
- CK1αΔC
- CK1α-R181Q
- CIP
- CK1α

**C**

- Graph showing temperature (°C) vs. ratio and first derivative for CK1α, CK1α-R181Q, CK1αΔC, and Buffer.

| Protein          | Onset #1 for Ratio | Inflection Point #1 for Ratio |
|------------------|--------------------|-------------------------------|
| CK1α             | 35.5 °C            | 45.9 °C                       |
| CK1α-R181Q       | 34.2 °C            | 45.3 °C                       |
| CK1αΔC           | 35.1 °C            | 46.1 °C                       |

**D**

- CK1δ
- CK1δ-R178Q
- CK1δΔC

**E**

- Graph showing temperature (°C) vs. ratio and first derivative for CK1δ, CK1δ-R178Q, CK1δΔC, and Buffer.

| Protein          | Onset #1 for Ratio | Inflection Point #1 for Ratio |
|------------------|--------------------|-------------------------------|
| CK1δ             | 45.9 °C            | 56.3 °C                       |
| CK1δ*            | 45.7 °C            | 55.9 °C                       |
| CK1δ-R178Q*      | 46.8 °C            | 56.6 °C                       |
| CK1δΔC           | 44.2 °C            | 54.4 °C                       |

**F**

- Graph showing temperature (°C) vs. ratio and first derivative for CK1δ, CK1δ*, CK1δ-R178Q*, Buffer, and *+λPPase.

**G**

- CK1δ (FLAG) at 4 °C, 20 °C after 0, 0.3, 1 h.
- CK1δ-R178Q (FLAG) at 4 °C, 20 °C after 0, 0.3, 1 h.
**SI Figure S1.** Characterization of CK1α and CK1δ variants

A  Schematic of CK1α. Grey and white boxes represent the conserved kinase domains and the non-conserved C-terminal tails. Blue boxes represent His-tags.

B  SDS-PAGE analysis and Coomassie stain of recombinant N-terminally His-tagged Neurospora CK1α variants (left) and dephosphorylation of autophosphorylated CK1α with calf intestine phosphatase, CIP (right).

C  Nano differential scanning fluorimetry (nanoDSF) analysis of recombinant CK1α variants. Temperatures of onsets and inflection points of unfolding were obtained from the first derivative of the ratio of tryptophan fluorescence at 330 nm : 350 nm.

D  Schematic of CK1δ versions. Grey and white boxes represent the conserved kinase domains and the non-conserved C-terminal tails. Blue and red boxes represent His- and FLAG-tags, respectively.

E  SDS-PAGE analysis and Coomassie stain of recombinant N-terminally His- and C-terminally FLAG-tagged human CK1δ variants (arrows). *: Kinase versions were co-expressed with λ-phosphatase (λPPase) to antagonize autophosphorylation.

F  NanoDSF analysis of recombinant CK1δ variants.

G  CK1δ and CK1δ-R178Q autophosphorylate in a temperature-dependent fashion. Unphosphorylated kinases were incubated with ATP at 4 and 20 °C for the indicated time periods and then analyzed by Western blot with FLAG antibodies.
**SI Figure S2.** CK1a supports progressive hyperphosphorylation of sFRQ *in vitro*

A
Schematic of FRQ structure and phosphorylation sites. Domains required for dimerization (coiled coil, CC), for CK1a binding (FCD1 and 2), and for binding of FRH (FFD) are depicted. Phosphorylation sites found *in vivo* (1, 2) are indicated. Blue: priming independent phosphorylation sites, i.e. phosphorylated sites without phosphorylated residue in the -3 position. Red: The 19 potential phosphate-directed phosphorylation motifs, i.e. pS/T-X-X-pS/T, where both S/T sites were found to be phosphorylated (note: not necessarily in the same peptide).

B
Hyperphosphorylation kinetics of sFRQ is independent of priming. sFRQ (8.3 nM) was incubated with CK1a or CK1a-R181Q (95 nM) and an ATP regenerating system at 4 °C and 20 °C for up to 48 h. Phosphorylation kinetics was analyzed by Western blot with FLAG antibodies.

C
Substantial phosphorylation of sFRQΔFCD1 by CK1a requires high kinase concentration. sFRQΔFCD1 (8.3 nM) was incubated with the indicated concentrations of CK1a and phosphorylation was measured as above.

D
Deletion of the C-terminal tail of CK1a accelerates phosphorylation of sFRQ. Incubation of sFRQ (8.3 nM) with CK1aΔC (112 nM) at 4 and 20 °C.

E
Phosphorylation kinetics of sFRQ is dependent on CK1aΔC concentration. sFRQ (8.3 nM) was incubated with the indicated concentrations of CK1aΔC.
preincubation of CK1δ for:

- 0
- 3
- 6
- 12
- 24 h

mPER2 (V5)

+ mPER2 for 6 h after preincubation

CK1δΔC

mPER2 (V5)

4 °C

0, 6, 12, 24 h

mPER2 shift (a.u.)

4 °C

0, 2, 4, 6

0, 6, 12, 18, 24 h

mPER2 shift (a.u.)

40 nM CK1δΔC

20 °C

0, 2, 4, 6

0, 6, 12, 18, 24 h

mPER2 shift (a.u.)

40 nM CK1δΔC

42nM CK1δ

840nM CK1δ

42nM CK1δ

840nM CK1δ

Supplementary Figure 3
**SI Figure S3.** Hyperphosphorylation of mPER2 by CK1δ does not require priming and is attenuated by autophosphorylation and autoinhibition of the kinase

A  Preincubation of CK1δ decreases its activity towards mPER2. Unphosphorylated CK1δ (+λ,PP) was preincubated at 4°C with ATP for the indicated time periods. The pre-treated kinase samples were subsequently assayed for their activity to phosphorylate mPER2 for a fixed time period of 6 h at 4°C (left panel) and 20°C (right panel).

B  CK1δΔC rapidly hyperphosphorylates mPER2. Hyperphosphorylation kinetics of mPER2 by CK1δΔC (39 nM) at 4 and 20°C. Upper panels: Western blot. Lower panels: Quantification of the phosphorylation-dependent electrophoretic shift. The electrophoretic position of the 130 kDa and 170 kDa markers was arbitrarily set to 0 and 0.5, respectively.

C  Hyperphosphorylation of mPER2ΔCKBD requires high concentration of CK1δ. mPER2ΔCKBD was incubated at 4 and 20°C with 42 nM (white circles) and 840 nM (black circles) CK1δ. The phosphorylation-dependent electrophoretic shift of mPER2 after the indicated reaction periods was determined (see B).
Supplementary Figure 4
SI Figure S4. CK1α variants can substitute for CK1δ.

A Sequence alignment of the conserved kinase domains of CK1δ (H. sapiens) and CK1α (N. crassa). Red: identical amino acid residues; black: nonidentical residues; orange: similar residues (R,K – E,D – I,L,M,V – S,T – F,Y – D,N – E,Q).

B CK1α hyperphosphorylates mPER2 in a priming-independent fashion. In vitro phosphorylation kinetics for 48 h at 4 and 20 °C of mPER2 (WCL of HEK293 cells) by 290 nM CK1α and CK1α-R181Q, respectively. Quantification of phosphorylation-dependent electrophoretic mobility shift is shown.

C Deletion of the C-terminal tail of CK1α accelerates hyperphosphorylation of mPER2. CK1αΔC (337 nM) was incubated with mPER2 for the indicated time periods at 4 and 20 °C.

D Neurospora CK1α does not support hyperphosphorylation of mPER2ΔCKBD. Quantification of the mPER2ΔCKBD phosphorylation kinetics by 290 nM CK1α at 4 and 20 °C.

E Expression of Neurospora CK1α and CK1α-R181Q shortens circadian period length of T-REx-U2OS cells. Luciferase reporter assay: T-REx-U2OS cells harboring DOX-inducible stably integrated ck1α (left panel) or ck1α-R181Q (right panel) genes were transiently transfected with a pBmal1-luc reporter plasmid (3). Expression of the CK1α versions were either induced with DOX or left uninduced (PBS) and bioluminescence was recorded for 96 h. Data were detrended and plotted. The dotted lines represent the standard deviation (± S.D.) of 4 technical replicates. Circadian period length is indicated.
**PER** (1218 aa)

N+C disorder: 1034 aa
Ser: 150 14.5%
Thr: 50 4.8%

**mPER1** (1291 aa)

N+C disorder: 949 aa
Ser: 128 13.9%
Thr: 91 9.8%

Supplementary Figure 5
**SI Figure S5.** Domain architecture of mPER1 and *Drosophila* PER

Schematic and disorder plot of mPER1 (upper panel) and *Drosophila* PER (lower panel). PAS-PAS domains, indicated by red boxes, correspond to T209-P465 of mPER1 and R231-P499 of PER.

Binding of CK1δ/ε and DBT and of CRY1/2 and TIM to mPER1 and PER, respectively, is shown. Disorder plots were generated by IUPred2A. Red traces: IUPred2A (long); blue traces: ANCHOR2. The total lengths of the regions N- and C-terminal of the dimerization domains (referred to as N + C disorder), and their seryl and threonyl content are indicated.
SI Methods

Mg$^{2+}$ chelation-enhanced fluorescence-based assay (ChEF assay)

ChEF assay from AssayQuant Technologies, Inc. (formerly available as Omnia kinase assay kit from Invitrogen) was performed with a plate reader (EnSpire, PerkinElmer) placed in an incubator (CLF Plant Climatics E41L1C8). Sulfonamido-oxine (SOX)-labelled, primed peptide, prephosphorylated in the -3 position (P24: KRRRALSpSVASL-SOX), and unprimed peptide (P24SA: KRRRALSAVASL-SOX) were purchased from AssayQuant Technologies, Inc. Phosphorylation of the seryl in the +1 position of the peptide induces together with the sulfonamido-oxine chelation of Mg$^{2+}$ and thereby enhances fluorescence of the chromophore (4).

The assay was performed in a 384-well plate from Corning (# 3574) in a final volume of 30 µL per well. 15 µL master mix was suspended per well and the kinase reaction was initiated by addition of 15 µL enzyme mix and incubation at 20 °C. The final composition of the reaction was 1x kinase buffer, 200 µM DTT, 1 mM ATP, and 10 µM peptide. Final kinase concentration was 135 nM CK1 in case of P24, and a 20-fold higher concentration for P24SA. The plate reader was set to $\lambda_{em}$ 360 nm, $\lambda_{ex}$ 485 nm. Measurement was performed at 81 s intervals. For each kinetics 2 independent experiments were performed with 3 technical replicates (n = 6). For each kinetics 2 independent experiments were performed with 3 technical replicates. Rates were calculated from the initial slopes (20 to 40% product formation, see Supplementary Information Excel files for individual kinetics).

The activity of CK1α and CK1β at 4 °C was measured by a modification of the ChEF assay, since the EnSpire plate reader cannot be cooled to 4 °C. Hence, separate phosphorylation reactions were carried out for each individual time point in a 384-well plate at 4 °C with a delayed start protocol, such that at the end of the reaction periods all samples could be measured at the same time in the EnSpire at 20 °C. For CK1β one and for CK1α two independent experiments with two technical replicates were performed.

In-vitro phosphorylation assay and quantification

Creatine phosphokinase (CPK, Roche) was prepared essentially as described (5): 100 mg CPK in 1 mL activation buffer (40 mM HEPES pH 7.5, 40 mM DTT) were incubated for 30 min at RT. CPK was then diluted to 5 mg/mL (= 1750 u/mL) by the addition of 19 mL dilution buffer (40 mM HEPES pH 7.5, 50% glycerol). The enzyme stock (40 mM HEPES pH 7.5, 47.5% glycerol, 2 mM DTT) was aliquoted, frozen in liquid nitrogen, and stored at -80 °C until further use.

The phosphorylation assay was carried out below 20 °C because the quantitative recovery of FRQ or PER2 was not reproducible at higher temperatures; the large disordered proteins are presumably clipped in the course of a 24 h incubation. The phosphorylation assay was prepared on ice in 1.5 mL low-binding tubes (Sarstedt). To start the reaction, 60 µL master mix containing kinase and target protein (in 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol) was combined with 60 µL 2x RRXN mix (70 mM HEPES pH 7.5, 160 mM NaCl, 20 mM MgCl$$_2$$, 4 mM DTT, 2x PhosSTOP phosphatase inhibitor cocktail (Roche), 10 mM ATP, 10 ng/µL leupeptin, 10 ng/µL pepstatin A, 34 ng/µL PMSF, 0.5 µg/µL CPK, 20 mM creatine phosphate (Calbiochem)) and then incubated at the indicated temperature. Reactions were stopped by the addition of 40 µL 4x SDS-sample buffer and boiling at 95 °C for 5 min. Samples were analyzed by Western blot. Blots were quantified by densitometry using the Gel Analysis tool of ImageJ. The diagrams show the positions of the center of mass for each band. Protein marker bands were set as reference points. In FRQ blots the 95 kDa marker band corresponds to 0 and the 170 kDa band to 1. In PER2 blots the 130 kDa band corresponds to 0 and the 170 kDa to 0.5, and accordingly, an equidistant position above the 170 kDa band was defined as 1.

Transfection and lysis of HEK293T cells

HEK293T cells were seeded and grown to confluency in 10 cm culture dishes overnight. Using polyethanolamine (PEI), cells were then transfected with pcDNA4/TO vector containing different alleles of V5 epitope-tagged mPer2. 40 h post-transfection, total protein lysate was extracted using 0.4 mL ice-cold lysis buffer (25 mM TrisHCl, pH 8.0, 150 mM NaCl, 0.5% Triton X 100, 2 mM EDTA, 1 mM NaF supplemented with 1x Complete protease inhibitor cocktail mix (Roche)). Lysates were then sonicated on ice water in an ultrasonic bath (Merck) for 5 min followed by centrifugation at 20,000 g for 15 min at 4 °C to remove cell debris. The resulting
supernatant was transferred to a fresh cooled 1.5 mL tube. Total protein concentration was determined by UV Spectrophotometry using a nanophotometer (Implen).

**Time-course bioluminescence measurements**

Bioluminescence of luciferase reporter genes was measured with a plate reader (EnSpire from PerkinElmer) placed in a temperature-controlled incubator (CLF Plant Climatics E41L1C8).

T-REx-U2OS cells expressing pBmal1-luc:

T-REx-U2OS cells stably expressing DOX-inducible versions of CK1δ and CK1α, respectively, were seeded in a white 96-well plate (Costar), grown to confluence overnight and then transfected using Xfect with pBmal1-luc vector (3)). 12 h post-transfection, the cells were synchronized by media change to luminescence medium (DMEM without phenol red supplemented with 10% FBS, 1x PenStrep, 0.125 µM luciferin, 0.1 mg/mL normocin) containing either PBS or DOX. After a 20 min incubation at 37 °C and 5% CO₂, the plate was sealed and bioluminescence was recorded at 30 min intervals at 37 °C. Three independent experiments with four wells per cell line were carried out. Resulting bioluminescence traces were detrended using a sliding 24h-time window for baseline subtraction and a sliding 3h-time window for smoothening. Circadian period was determined using cosinor analysis in MATLAB (https://de.mathworks.com/products/matlab.html).

**Neurospora strains expressing frq-lucPEST (6):**

White 96-well plates (Costar) were filled with 150 µL/well luciferase medium (1% agarose, 50 ng/µL biotin, 1x Vogel's solution, 1% sorbose, 0.05% glucose, 0.05% fructose, 50 µM luciferin). Wells were inoculated with 5 µL conidial suspension (OD420 2.2 = 6.3 x10⁶ conidia/mL) and the plate was sealed. Plates were incubated at RT for several days, synchronized by exposure to high light for 12 h and then placed in the EnSpire (incubator at 25 °C) in the dark. Bioluminescence was measured at 30 min intervals for several days. Experiments were carried out twice with one clone for wt and two different clones for the overexpressing strain. Results were plotted using Microsoft Excel.

**Generation of stable U2OStx cell lines**

pBmal1-luc vector was kindly provided by Dr. Steven A. Brown (3), pcDNA4/TO-derived vectors were constructed by inserting the coding regions of human casein kinase 1 isoform δ (CK1δ), Neurospora crassa casein kinase 1 a (CK1α), and V5-tagged murine Period circadian protein homologue 2 (mPER2), respectively, downstream of the CMV-TetO promoter which allows for doxycycline-induced protein expression in cells containing the tetracycline-repressor cassette (T-REx). T-REx-U2OS cells were seeded in a 24-well plate and grown to confluence overnight. Cells were transfected with pcDNA4/TO vectors containing the desired alleles of FLAG epitope-tagged CK1δ and CK1α, respectively. Stable transfectants were selected by growing cells to sub-confluence in complete media supplemented with 50 µg/mL hygromycin and 100 µg/mL zeocin (Invitrogen) over the course of two weeks. Resulting single cell colonies after selection were isolated for further experimentation. Inducible protein expression was assayed by the addition of 10 ng/mL doxycycline (DOX).

**Construction of genomically modified Neurospora crassa**

The ck1a strain was generated by replacing in a Δmus52 ras-rfd his-3 strain via homologous recombination the region downstream of the first intron of endogenous ck1a by the corresponding cDNA coding for the short, major splice-isofrom of CK1α (363 aa) (7) followed by the trpC terminator and a hygromycin B cassette (8). The strain was then crossed with ras-rfd pfrq-lucPEST (his+) resulting in a homokaryotic strain.

Cloning and crossing of Neurospora was performed as recommended by the Fungal Genetics Stock Center (http://www.fgsc.net, (9)), resulting in N. crassa ras-rfd his+ pfrq-lucPEST ck1a:ck1a short-hph here referred to as ck1a-OX. N. crassa ras-rfd his+ pfrq-lucPEST (6) was used as a ck1a wild type control strain.

**Race tube assay**

The circadian conidiation rhythms of Neurospora strains were analyzed using race tubes (10-12).
SDS-PAGE
SDS polyacrylamide gels (7.5 cm x 15.0 cm x 1 mm) were run at 5 to 7 mA for 18 h with SDS running buffer (48.7 mM Tris, 3.84 mM glycine, 0.1% (w/v) SDS). The stacking gel (60 mM TrisHCl pH 6.8, 0.1% (w/v) SDS) contained 5% acrylamide and the resolving gel (376 mM TrisHCl pH 8.8, 0.1% (w/v) SDS) contained 10% acrylamide (acrylamide:bisacrylamid 29.5:0.5) for the analysis of mPER2, 14% (29.5:0.5) for CK1, and 12% (29.8:0.2) for FRQ.

Protein samples were diluted in SDS sample buffer and boiled at 95 °C for 5 min before loading onto the SDS gel.

SDS-PAGE for Coomassie staining was carried out with mini gels (cast with equipment from Bio-Rad) at 80 V for 30 min, followed by 150 V for 60 min. Gels were stained with Coomassie (13) and destained as described by (14).

Western blotting
Proteins were transferred from gel to nitrocellulose membrane (Protran supported from GE) via semi-dry Western blotting (15) at 250 mA per gel (7.5 cm x 15 cm) for 2 h 20 min. Blotting buffer contained 20 mM Tris, 150 mM glycine and 20% methanol. The membrane was subsequently incubated in Ponceau S staining solution (0.2% trichloroacetic acid, 3.0% (w/v) Ponceau S) for 2 min and rinsed with plenty of water.

Membranes were blocked with 5% skim milk in TBS (10 mM Tris, 150 mM NaCl, HCl ad pH 7.4) for 30 min. Antibodies were diluted in 5% skim milk in TBS. Primary antibody was incubated for either 2 h at RT or overnight at 4 °C. The membrane was washed three times for 10 min with TBS and incubated with secondary antibody overnight at 4 °C or for 2 h at RT and then washed three times for 10 min with TBS.

Membranes were incubated with primary and secondary antibodies according to the table below.

| Antibody | Origin | Dilution | Incubation conditions | Secondary antibody |
|----------|--------|----------|-----------------------|--------------------|
| αCK1a    | Pineda Antikörper-Service* | 1:500    | overnight at 4 °C or 2 h at RT | αrabbit           |
| αFLAG    | Monoclonal ANTI-FLAG M2, Sigma-Aldrich (F3165) | 1:5000   | overnight at 4 °C or 2 h at RT | αmouse            |
| αV5      | Anti-V5 Epitope antibody, antibodies-online.com (ABIN933572) | 1:2500   | overnight at 4 °C or 2 h at RT | αmouse            |
| αmouse   | goat anti-mouse IgG (H+L)-HRP conjugate, Bio-Rad(170-6516) | 1:10 000 | 2 h at RT or overnight at 4 °C |                   |
| αrabbit  | goat anti-rabbit IgG (H+L)-HRP conjugate, Bio-Rad(172-1019) | 1:10 000 | 2 h at RT or overnight at 4 °C |                   |

secondary antibodies were conjugated with horseradish peroxidase (HRP)

* antibody from rabbit sera (Pineda Antikörper-Service) was affinity purified in-house

Membranes were treated with freshly prepared luminol mix (100 mM TrisHCl pH 8.5, 2.2 x 10⁻² % (w/v) luminol, 3.3 x 10⁻³ % (w/v) coumaric acid, 9.0 x 10⁻³ % (v/v) H₂O₂) for about 10 s and put in a plastic bag into a film cassette. Membranes were exposed for a time series to several X-ray films (Fujifilm). Films were then developed (Medical film processor from Konica Minolta).

Expression and purification of recombinant proteins
The following E. coli expression strains and plasmids were used:
Rosetta 2 [pLysS] with pET-Ascl-6His-2FLAG-shortFRQ (and variants of sFRQ) as well as pQE30-6His-CK1α-FLAG (WT and ∆C);
Rosetta 2 [pLysS, pREP4, pET-2α-phosphatase] with pQE30-6His-CK1α-FLAG (WT, R178Q); M15 [pREP4] with pQE30-6His-TEV-CK1a short (and variants).

The respective E. coli strain was freshly inoculated with an overnight culture and incubated for 2 to 4 h at 37 °C. The culture was cooled down and overexpression was induced with 0.5 mM
IPTG. Cells were grown for 12 to 16 h at 18 to 20 °C and then lysed with a microfluidizer (M1-10L from Microfluidics).

Proteins were purified by affinity chromatography with a HisTrap HP columns (GE). CK1 protein preparations were rebuffered after affinity purification with a PD-10 desalting column (GE) in Storage Buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 10% glycerol, 10 mM DTT). FRQ variants were further purified and concentrated by anion exchange chromatography (HiTrap Q HP, GE) in elution buffer (20 mM HEPES pH 7.5, 371 mM NaCl, 10% glycerol, 2 mM DTT).

Proteins were aliquoted at the desired concentration, frozen in liquid nitrogen and stored at -80 °C.

Protein extraction from S. cerevisiae

135 mL YPD medium (1% yeast extract, 2% tryptone, 2% glucose) was inoculated with S. cerevisiae W303 (from a glycerol stock) and incubated at 30 °C shaking overnight. Cells were pelleted at 3,500 g for 10 min at 4 °C in 50 mL tubes, washed with 50 mL ddH2O and resuspended in 1 mL ddH2O. Cell suspension was frozen in liquid nitrogen in small drops and stored at -80 °C.

The frozen pellet was continuously cooled in liquid nitrogen while ground to very fine powder with mortar and pestle. The powder was transferred into a pre-cooled 1.5 mL tube and an equivalent volume protein buffer was added (PB, 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol) supplemented with protease inhibitors (5 ng/µL leupeptin, 5 ng/µL pepstatin A, and 17 ng/µL (≈ 1 mM) PMSF) and 1x PhosSTOP (Roche). The mixture was vortexed vigorously 4 times at 5 min intervals and cell debris was pelleted by centrifugation at 10,000 g at 4 °C for 10 min.

Cloning with restriction enzymes

Enzymes used for cloning were obtained from NEB (New England Biolabs). Restriction digestion (KpnI-HF, XhoI, XmaI), dephosphorylation of vector backbone (calf intestinal alkaline phosphatase (CIP)) and ligation (T4 DNA ligase) was performed as recommended by NEB. Linear DNA was purified using NucleoSpin Gel and PCR Clean-up kit from Macherey-Nagel. Plasmids were extracted with GeneJET Plasmid Miniprep kit from Thermo Fisher Scientific.

Cloning with overlapping regions (restriction enzyme-free cloning)

Ligation-free overlap cloning was used for site-directed mutagenesis (at single or multiple sites), insertions and deletions (16). Enzymes (Q5 DNA polymerase, restriction enzyme DpnI and T4 DNA polymerase) were obtained from NEB. DpnI digestion and exonuclease reaction with T4 DNA polymerase was performed in NEBuffer 2.1. E. coli was transformed with exonuclease treated PCR product(s) to facilitate in vivo plasmid assembly via overlapping DNA ends.
### Table of primers used for cloning

| Primer name | Primer sequence | Template plasmid Plasmid created |
|-------------|----------------|----------------------------------|
| AscI-6His-2FLAG-FRQshort fw | TTTTTTTTTTTGCGCGCCATGCATCACC ATCACACCAAGATGCATCTCCTCCTTATCAAAAGGAG | SF.5-HisTag-TEV-FRQ-FL (17) |
| FRQ-STOP-Not1 rev | TTTTTTTTTTTGCGCGCCATGCATCACC ATCACACCAAGATGCATCTCCTCCTTATCAAAAGGAG | pET-6His-2FLAG-shortFRQ |
| AscI-6His-2FLAG-FRQshort fw | see above | pBM60-Cla-4FRQ ΔFCD1 (18) |
| FRQ-STOP-Not1 rev | see above | pET-Ascl-6His-2FLAG-FRQshortΔFCD1 |
| CK1a R181Q fw | GGCACATGCCGATATGCTCC [ATCAACAAGCATCGGATCGAG AGGAGCATCTGGGATCG GCCAGGATACGGTGGGCTCGATTCA] | pQE30 CK1a short TEV (17) |
| CK1a R181Q rev | see above | pQE30-6His-TEV-CK1a short R181Q |
| hCK1 delta R178Q fw | CACCGGGGGCCGCGCTAGTACGGCTCCATC AACAGGC | pcDNA4TO-CSNK1D1FLAG/pQE30-6His-TEV-hCK1delta-FLAG |
| hCK1 delta R178Q rev | see above | pcDNA4TO-hCK1delta R178Q-FLAG/pQE30-6His-TEV-hCK1delta R178Q-FLAG |
| CKI delta ΔC FLAG fw | GAAATGCTGCAAAAATT GACTCAAAGACGATGACGACAAGTGAAA GC CGAATGGAGATCAAGGTCATCTCCTCCTTATCATGC | pQE30-6His-TEV-hCK1delta-FLAG |
| CKI delta ΔC FLAG rev | see above | pQE30-6His-TEV-hCK1deltaΔC-FLAG |
| V5:mPER2incDNA4X X hol_F | TTTTTTTTTCTCGAGACCATGGGTAAGCCTATCCCTAACCCTCCTCCGATCGAATGGGATACGGTGGGCTCGATTCA | pcDNA4TO-FLAG-mPER2 |
| V5:mPER2incDNA4X X hol_R | see above | pcDNA4TO-V5-mPER2 |
| mPER2 CK1BD-B fw | CCTATAAGAAGCAACGCTCTC AAGGACATTGACGAGCTTCTTATAGAGGAGGCTGGTGGCTTATTCCAAACATCCCATCC | pcDNA4TO-V5-mPER2 |
| mPER2 CK1BD-A rev | see above | pcDNA4TO-V5-mPER2 |
| mPER2 CK1BD-B fw | see above | pcDNA4TO-V5-mPER2 |
| mPER2 CK1BD-B rev | see above | pcDNA4TO-V5-mPER2 |
| mPER2 dCKBD mut F | see above | pcDNA4TO-V5-mPER2 |
| mPER2 dCKBD mut Rc | see above | pcDNA4TO-V5-mPER2 |
| CK1a in pGEM4 KpnI fw | see above | pQE30 CK1a short TEV (17) (cloning into pGEM4) |
| CK1a in pGEM4 XmaI rev | see above | pQE30 CK1a short TEV (17) (cloning into Neurospora) |
| Split 1 ck1a ORF fw | see above | pGEM4 variant |
| Trafo hyg +3'UTR rev | see above | ck1a short (cloning into Neurospora) |

Cultures of clonal cell lines

T-REX-U2OS (Life Technologies) and HEK293T (ATCC) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1x...
Penicillin-Streptomycin. Cell culture reagents were obtained from Life Technologies. Cells were grown at 37 °C in a humidified incubator containing 5% CO₂.

**Neurospora crassa strains and culture conditions**

*Neurospora crassa* strains carried the ras-1*<sup>td</sup>*, mutation (11) and the *frq-luc*PEST gene (6) in the his-3 locus (19), here referred to as wild-type (*wt*). *Neurospora* was grown on solid medium (2% sucrose, 2% agar, 1x Vogel’s) at RT and conidia were harvested with 1 M sorbitol. Flasks with liquid N medium (2% glucose, 0.5% L-arginine, 1x Vogel’s; (20)) were inoculated with conidia and incubated in a shaker at 25 °C.

**Dephosphorylation of CK1a**

5.6 µg purified CK1a in a volume of 10 µL was supplemented with 10 µL 2x CIP buffer (100 mM TrisHCl pH 8.0, 500 mM NaCl, 40 mM MgAc₂) and 5 u (= 0.5 µL 10 u/µL CIP (calf intestinal alkaline phosphatase, NEB) and incubated for 30 min at 30 °C. 7 µL 4x SDS sample buffer was added to stop the reaction. The sample was boiled at 95 °C for 5 min and 5 µL (= 1 µg CK1a) sample was loaded on an SDS-gel. After SDS-PAGE the gel was stained with Coomassie.

**Nano differential scanning fluorimetry**

The thermal stability of CK1 variants was determined by nano differential scanning fluorimetry (NanoDSF) using a Prometheus NT.48 (NanoTemper Technologies) and analyzed with the PR.ThermControl software (NanoTemper Technologies). The protein was diluted in storage buffer (StB, 20 mM HEPES pH 7.5, 500 mM NaCl, 10% glycerol 10 mM DTT) to a concentration of 3 mg/mL and loaded into standard capillaries (NanoTemper Technologies). Melting curves were generated by applying 30% excitation power and a constant temperature increase of 1.0 °C/min ranging from 15 to 90 °C. The ratio of tryptophan fluorescence at 330 and 350 nm was measured. The inflection point, determined by the maximum of the first derivative, corresponds to the melting temperature of the protein.
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