Supporting Information

Snijder et al. 10.1073/pnas.1314326111

SI Experimental Procedures

Expression and Purification of KaiC and KaiB. The recombinant *Synechococcus* GST (GST)-KaiB and GST-KaiC proteins were produced in *Escherichia coli* BL21 strains, kindly provided by T. Kondo, Nagoya University, Nagoya, Japan. Expression was carried out for 72 h (GST-KaiC) or 20 h (GST-KaiB) at 37 °C in Terrific Broth medium containing 100 $\mu g \cdot mL^{-1}$ ampicillin. GST-KaiB expression was induced by 1 mM isopropyl β-D-1-thio-galactopyranoside. Cell lysis was performed in ice-cold extraction buffer [50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, and 1 mM DTT (5 mM $MgCl_2$ and 1 mM ATP for purification of KaiC)]. GST-KaiB and GST-KaiC were bound to Protino Gluthatione Agarose 4B (Macherey-Nagel) and cleaved of their GST tag by incubation with PreScission protease (GE Healthcare) in cleavage buffer [50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT (5 mM $MgCl_2$ and 0.5 mM ATP for purification of KaiC)]. If homogeneity of the affinity-purified proteins was not sufficient, they were further purified by ion-exchange chromatography. For this, KaiBor KaiC were applied to a MonoQ 5/50 GL or ResourceQ column (GE Healthcare) and eluted with 20 column volumes of a 0–1 M NaCl gradient in elution buffer [50 mM Tris·HCl, pH 8.0, 1 mM EDTA, and 1 mM DTT (5 mM MgCl2 and 0.5 mM ATP for purification of KaiC)]. Purified proteins were dialyzed in reaction buffer (RB) (20 mM Tris·HCl, pH 8, 150 mM NaCl, 0.5 mM EDTA, 5 mM MgCl2, and 1 mM ATP) using Vivaspin ultrafiltration spin columns (Satorius Stedim). Protein concentrations were determined using the Lowry or Bradford method.

Native PAGE and SDS/PAGE Analyses. To analyze KaiC/KaiBC complexes, the recombinant Kai proteins were incubated in RB as described in *Results*, mixed with 2× sample buffer (100 mM NaCl, 100 mM imidazole, 4 mM 6-aminocaproic acid, 2 mM EDTA, 0.02% Ponceau S, and 20% glycerol) and applied to SERVAGelN 4–16 vertical native gels (SERVA Electrophoresis GmbH). Electrophoresis was performed overnight at 4 °C using 50 mM BisTris, pH 7.0, as anode buffer and 50 mM Tricine, 15 mM BisTris, and 1 mM ATP as cathode buffer. Separated protein complexes were stained with Coomassie brilliant blue. To determine KaiC's phosphorylation state under the conditions indicated in Fig. 2, aliquots of the reaction mixtures containing 0.75, 1, 1.5, and 2 μg KaiC were mixed with SDS sample buffer and heated for 5 min at 95 °C. Denatured proteins were electrophoretically separated in polyacrylamide gels (11% T and 0.67% C). Phosphoforms of KaiC were visualized by Coomassie staining and analyzed by Molecular Imager ChemiDoc XRS+ (Bio-Rad) and ImageLab software (Bio-Rad).

Native MS. KaiB, KaiC, and KaiCB mixtures were prepared and incubated in RB as indicated in *Results*. Before MS, samples were transferred to an MS-compatible buffer of 75 mM ammonium acetate, pH 6.8, using Vivaspin 500 5K (MWCO) centrifugal filter units. Buffer exchange was performed at 4 °C for six rounds of concentration/dilution over the course of ∼90 min. For the study of KaiB tetramerization, we determined the concentration of a stock solution in ammonium acetate (buffer exchanged directly from KaiB stock solution in RB without additional incubation steps) from the absorbance at a wavelength of 280 nm, using the KaiB sequence to estimate the extinction coefficient. This stock solution was diluted in 75 mM ammonium acetate to arrive at the concentrations as indicated in Fig. 1*B*. With the KaiC and KaiCB samples, a total volume of 50 μL for each sample was incubated for either 6 h or 7 d at 30 °C or 4 °C in RB, respectively. The KaiC concentration in RB was 3 μM in all samples, and the KaiB concentration was 3 μM in the 30 °C sample and 9 μM in the 4 °C sample. After the final round of concentration for buffer exchange, the total volumes had been reduced to 25 μL. We estimated a sample loss on the order of 50%, yielding similar final concentration in the buffer-exchanged samples. The Kai samples were kept on ice until analyzed. Samples were loaded into gold-coated boro-silicate capillaries prepared in-house. KaiB tetramerization was studied on an LCT1 (Micromass; Waters, U.K.) with elevated source pressure (7–9 mbar) for optimal transmission of larger complexes (1). Capillary voltage was set between 1,200–1,400 V, and the cone voltage at 160 V. KaiC/KaiCB complexes were analyzed on a modified QToF II (MS Vision; Waters, U.K.), operated at 10 mbar source pressure, 1,300–1,500 V capillary voltage, 160 V cone voltage, 50 V collision energy with $1–2 \times 10^{-2}$ mbar pressure in the collision cell using xenon as collision gas (2, 3). Tandem MS spectra were acquired at a collision energy of 80 V. KaiC phosphorylation was determined by dividing the mass deviations stated in Table S1 by the theoretical mass of 12 phosphorylations (960 Da). Ion mobility spectrometry was performed on a Synapt G1 (Waters, U.K.) operated at 9 mbar source pressure. Capillary voltage was set at 1,300 V, cone voltage at 160 V, and TRAP/TRANSFER at 10/30 V collision energy, with a pressure of $1–2 \times 10^{-2}$ mbar pressure using xenon as collision gas. Traveling wave ion mobility was performed using a wave height of 2–12 V ramped over 60% of the cycle, with nitrogen as buffer gas at a pressure of 5.55×10^{-1} mbar. The collisional cross-section was calibrated using denatured ubiquitin, cytochrome C, and myoglobin, as well as native GroEL (4, 5). The reported cross-sections and 95% confidence intervals are based on four replicate analyses. Theoretical cross-sections were calculated with the Driftscope Projection Approximation algorithm (Waters, U.K.).

Hydrogen–Deuterium Exchange MS. In hydrogen–deuterium exchange (HDX)-MS, proteins are transferred into a deuterated solution, resulting in the exchange of backbone amide protons for deuterons from the solution. After quenching the reaction before rapid proteolytic digestion, the levels of deuterium uptake can be determined for all of the resulting peptide products using MS. The observed deuterium uptake can then be mapped back onto the amino acid sequence. Because deuterium uptake is largely determined by solvent exposure and secondary structure, binding interfaces and (allosteric) structural transitions can be observed with HDX-MS.

HDX-MS was performed on free KaiC, free KaiB, and two distinct KaiCB mixtures. Free KaiC and free KaiB were incubated for 1 wk at 4 °C in RB at a concentration of 30 μM to match the incubations of the KaiCB complexes. Bound KaiB was prepared by incubating 30 μM KaiB in the presence of 90 μM KaiC for 1 wk at 4 °C in RB. Similarly, for bound KaiC, 30 μM KaiC was incubated in the presence of 90 μM KaiB for 1 wk at 4 °C in RB. The HDX reaction was started by diluting the above stock solutions 20-fold to a final volume of 40 μL in D_2O. The reaction was carried out on ice for 1, 10, and 60 min before quenching by 2:1 dilution into ice-cold 6 M guanidine·HCl, 300 mM Tris (2-carboxyethyl) phosphine, with pH adjusted to give a final pH of 2.5.

Immediately after quenching, the sample was injected into a Waters HDX/ nanoAcquity system for digestion on an online

pepsin column (25 °C, at a flow rate of 50 μL·min^{-1}) followed by separation on a 10-min RP-UPLC gradient at 0 °C and MS on a Waters Xevo QToF G2. For peptide identification, samples of KaiC and KaiB were prepared under identical conditions in H_2O and analyzed using MS^e data acquisition. Data for peptide identification was processed with ProteinLynx Global Server 2.5 software. Deuterium uptake was calculated compared with the control samples in H_2O using Waters DynamX 1.0.0 software.

The experiments were performed in triplicate. HDX-MS was performed in time-course experiments with 0-, 1-, 10-, and 60-min exposure to D_2O. For KaiB, 29 peptides could be identified and quantified after digestion with pepsin, covering 96% of the full sequence (Fig. S3). For KaiC, 119 peptides could be identified and quantified, covering 92% of the full sequence (Fig. S4). The experiments were performed in triplicate. The kinetics of deuterium uptake were similar between bound and unbound states, but there were substantial differences in total deuterium uptake, which is most clearly seen in the 60-min time points. Observed changes in deuterium uptake between two samples were filtered to $P < 0.01$, as calculated from pairwise two-tailed Student t testing between the 60-min time points. Overviews of the HDX-MS data, including P values for KaiB and KaiC, are provided in Tables S2 and S3, respectively.

Structural Modeling and Analysis. For KaiB we used the four monomers from the crystal structure (PDB ID code 4kso) to create an ensemble of four starting structures that was used as input for the docking, taking advantage of the ensemble docking ability of HADDOCK. Only one monomer (chain A) required homology modeling to rebuild the missing 7 Nter residues, based on the three other monomers. This was done using a multiple template approach with the MODELER package version 9.11 (6). For KaiC, we extracted a dimer from the available structure of *Synechococcus elongatus* KaiC (PDB ID code 3dvl). The docking simulations were performed using the HADDOCK web server (7) (http://haddock.science.uu.nl/services/HADDOCK). We generated 10,000/400/400 structures for the different it0/it1/water stages of HADDOCK. The HDX-MS information was mapped onto KaiB and KaiC solvent-accessible surfaces, resulting in two lists of active residues used to derive ambiguous interaction restraints (8). ATP and Mg^{2+} were restrained using specific geometrical distances derived from the crystal structure of KaiC. After clustering, all of the representative solutions were inspected and the top four clusters based on the HADDOCK score were considered for further analyzes and discussion (*Results*).

1. Tahallah N, Pinkse M, Maier CS, Heck AJR (2001) The effect of the source pressure on the abundance of ions of noncovalent protein assemblies in an electrospray ionization orthogonal time-of-flight instrument. *Rapid Commun Mass Spectrom* 15(8):596–601.
2. van den Heuvel RHH, et al. (2006) Improving the performance of a quadrupole time-of-flight instrument for macromolecular mass spectrometry. *Anal Chem* 78(21):7473–7483.
3. Lorenzen K, Versluis C, van Duijn E, van den Heuvel RHH, Heck AJR (2007) Optimizing macromolecular tandem mass spectrometry of large non-covalent complexes using heavy collision gases. *Int J Mass Spectrom* 268(2–3):198–206.
4. Ruotolo BT, Benesch JLP, Sandercock AM, Hyung SJ, Robinson CV (2008) Ion mobility-mass spectrometry analysis of large protein complexes. *Nat Protoc* 3(7):1139–1152.
5. Bush MF, et al. (2010) Collision cross sections of proteins and their complexes: A calibration framework and database for gas-phase structural biology. *Anal Chem* 82(22):9557–9565.
6. Eswar N, et al. (2006) Comparative protein structure modeling using modeller. *Curr Protoc Bioinformatics* Chap 5, Unit 5.6.
7. de Vries SJ, van Dijk M, Bonvin AM (2010) The HADDOCK web server for data-driven biomolecular docking. *Nat Protoc* 5(5):883–897.
8. de Vries SJ, et al. (2007) HADDOCK versus HADDOCK: New features and performance of HADDOCK2.0 on the CAPRI targets. *Proteins* 69(4):726–733.

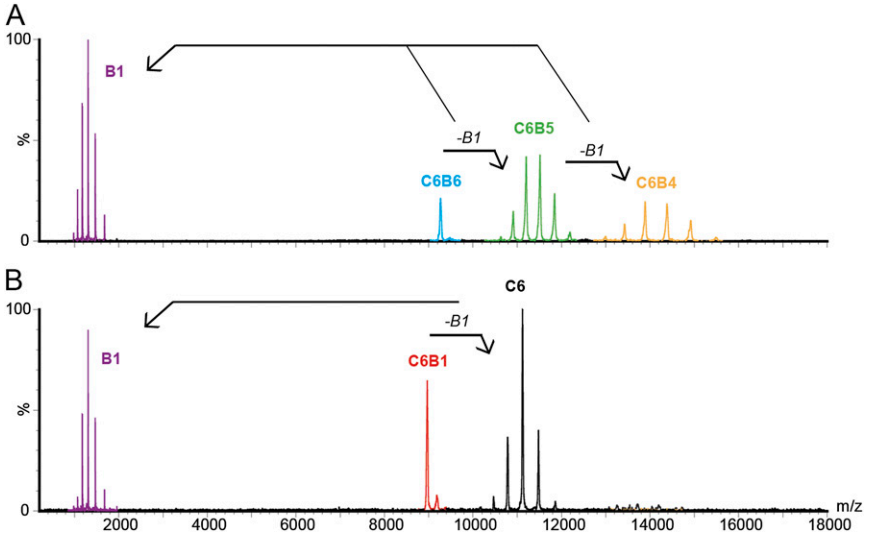

Fig. S1. Tandem MS analysis of KaiCB complexes. Single charge states of KaiCB complexes are selected from the MS1 spectra shown in *Results* (Fig. 2). Applying higher collision energies (80 V) results in the dissociation of highly charged KaiB subunits. The masses of all detected components are listed in Table S1. (*A*) Tandem MS of KaiC6B6 complexes; the mass of the first high *m/z* product ion corresponds to KaiC6B5 and the second high *m/z* product ion to KaiC6B4, thus confirming that the precursor is KaiC6B6. (*B*) Tandem MS of KaiC6B1. The dominant product ions corresponds to KaiC6 and B1, thus confirming that the precursor is KaiC6B1.

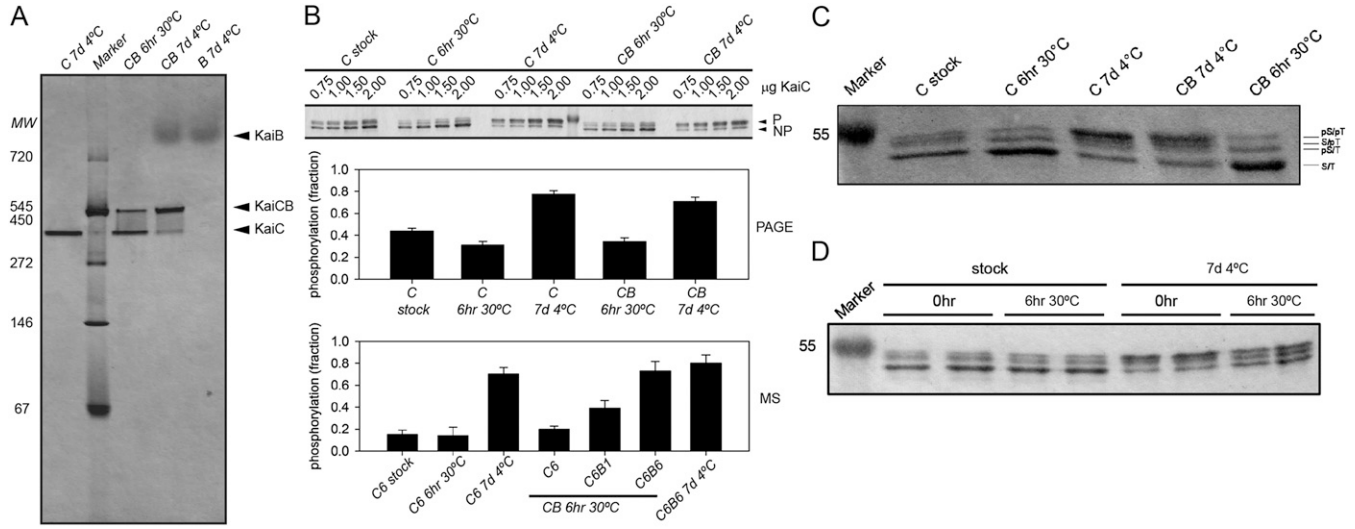

Fig. S2. Native PAGE and phosphorylation analysis of KaiCB complexes. (*A*) Native PAGE of KaiCB complexes under standard conditions and at reduced temperature. (*B*) SDS/PAGE and native MS analysis of KaiC phosphorylation. In the MS analyses of KaiC and KaiCB, substantial mass deviations were observed compared with theoretical masses calculated from the protein sequence (Table S1). These residual masses were interpreted as phosphorylation of KaiC. In SDS/PAGE, the phosphorylated forms of KaiC have a reduced mobility, allowing the phospholevels to be determined by densitometry. Low levels of phosphorylation were determined in KaiC input material. Similar levels were detected after incubating KaiC alone or in the presence of KaiB at 30 °C. Whereas SDS/PAGE yields one bulk value of KaiC phosphorylation in the KaiCB preparation under standard conditions, native MS can determine the individual phosphostates of all three detected components. Low phospholevels of free KaiC but high phospholevels in $KaiC_6B_6$ were observed, confirming that KaiB binds preferentially to phosphorylated KaiC. Intermediate phospholevels were determined for the $KaiC_6B_1$ complex, indicating again that phosphorylation is limiting to the formation of full $KaiC_6B_6$ complexes. As reported previously, higher levels were detected when KaiC was incubated alone at 4 °C and similarly high levels were detected when KaiC was incubated in the presence of KaiB at 4 °C, thus explaining the higher propensity for KaiB binding. (*C*) Individual phosphostates can be distinguished, as indicated. After incubation at 4 °C, the pS/pT form of KaiC is most enriched in both KaiC and KaiCB preparations. (*D*) Aliquots from stock solution or after incubation at 4 °C were frozen at −20 °C while an additional 6-h incubation at 30 °C was carried out. Samples were loaded on gel to monitor phosphorylation. There is a decrease in the overall phosphorylation level of KaiC when incubated at 30 °C after 1 wk of incubation at 4 °C, indicating that the dephosphorylation activity was preserved.

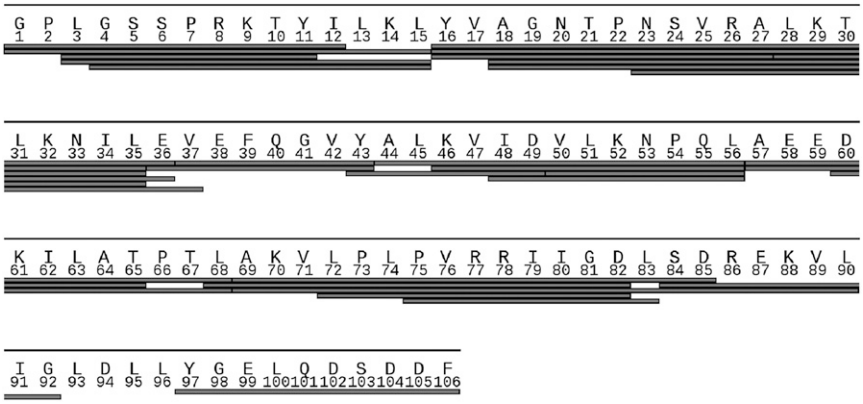

Fig. S3. Peptide analysis of pepsin-digested KaiB. A total of 29 peptides are identified with sufficient intensity for HDX analysis, covering 96% of the sequence. The 29 peptides used for HDX analysis are mapped onto the full sequence.

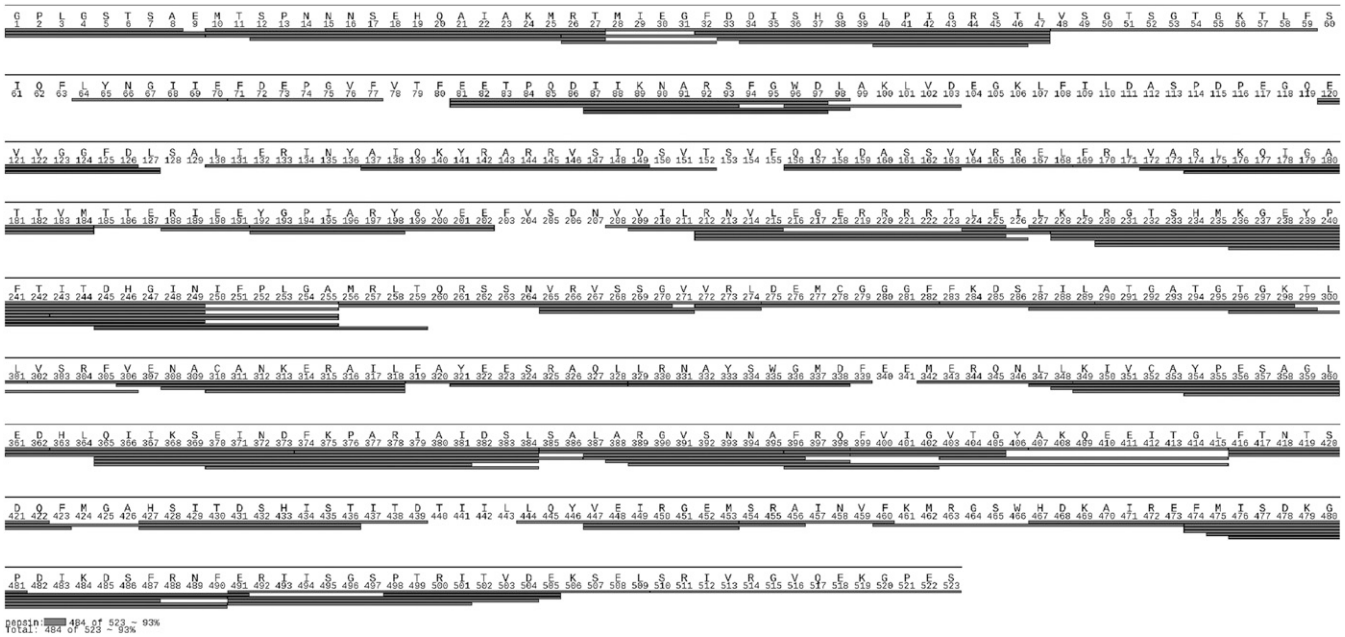

Fig. S4. Peptide analysis of pepsin-digested KaiC. A total of 119 peptides are identified with sufficient intensity for HDX analysis, covering 92% of the sequence. The 119 peptides used for HDX analysis are mapped onto the full sequence.

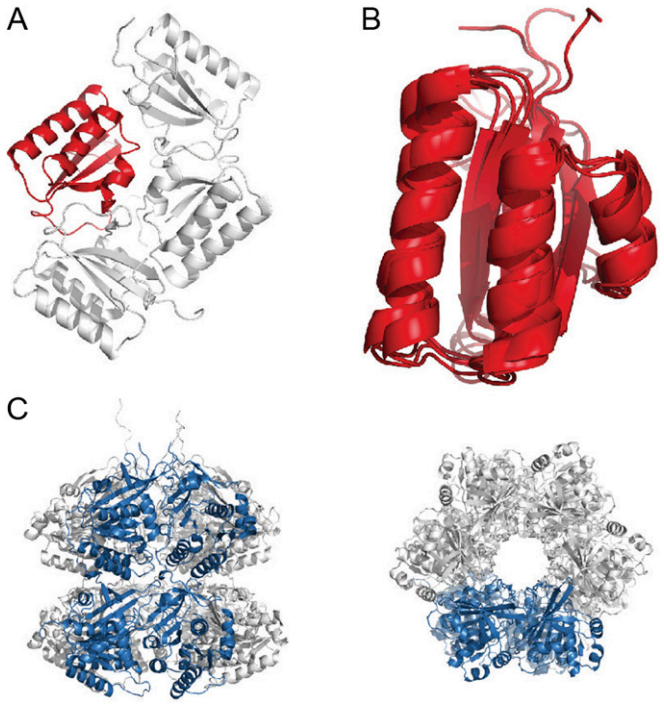

Fig. S5. Kai structures used for HADDOCK. (*A*) Crystal structure of KaiB (PDB ID code 4kso). (*B*) Ensemble of four monomer models of lowest energy for *S. elongatus* KaiB. The entire ensemble of four structures was used to dock on KaiC. (*C*) Side and top views of the KaiC structure used for HADDOCK. The crystal structure of KaiC (PDB ID code 3dvl) was used as a starting model. Two adjacent subunits were isolated from the full hexamer structure and the 13 first residues of the flexible C terminus were truncated.

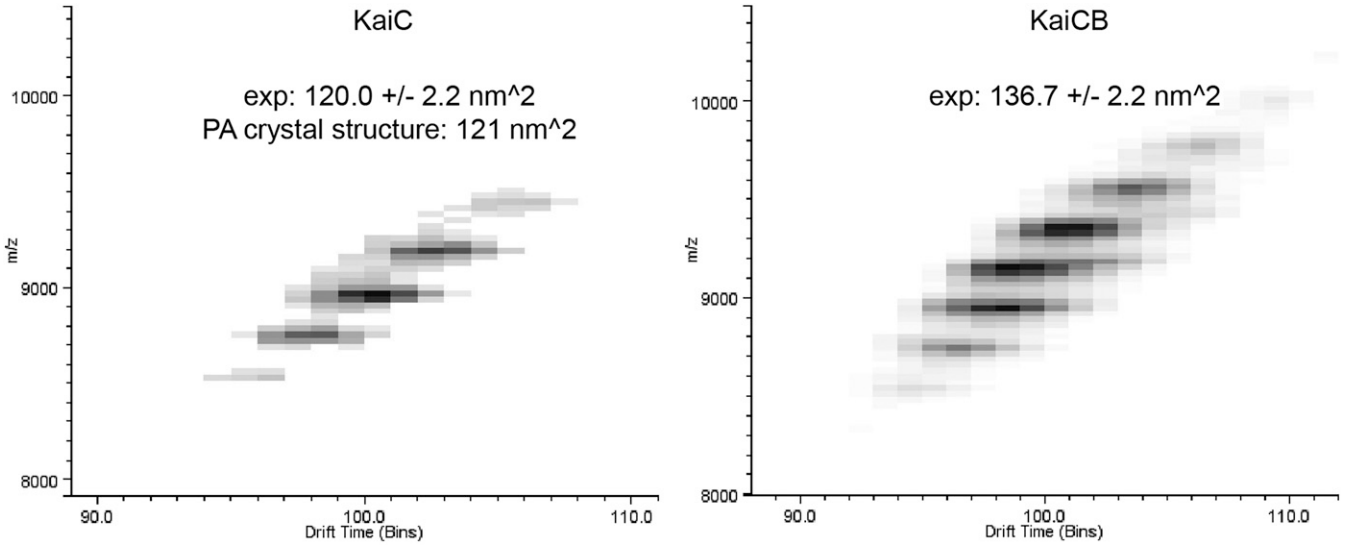

Fig. S6. Ion mobility MS of KaiC and KaiCB complexes. For both KaiC and KaiCB complexes, the drift-time vs. *m/z* contour plots are shown. The average collisional cross-sections for the complexes are indicated on the plots. For the KaiC hexamer, we calculated the theoretical collisional cross-section based on the crystal structure of KaiC (PDB ID code 3dvl).

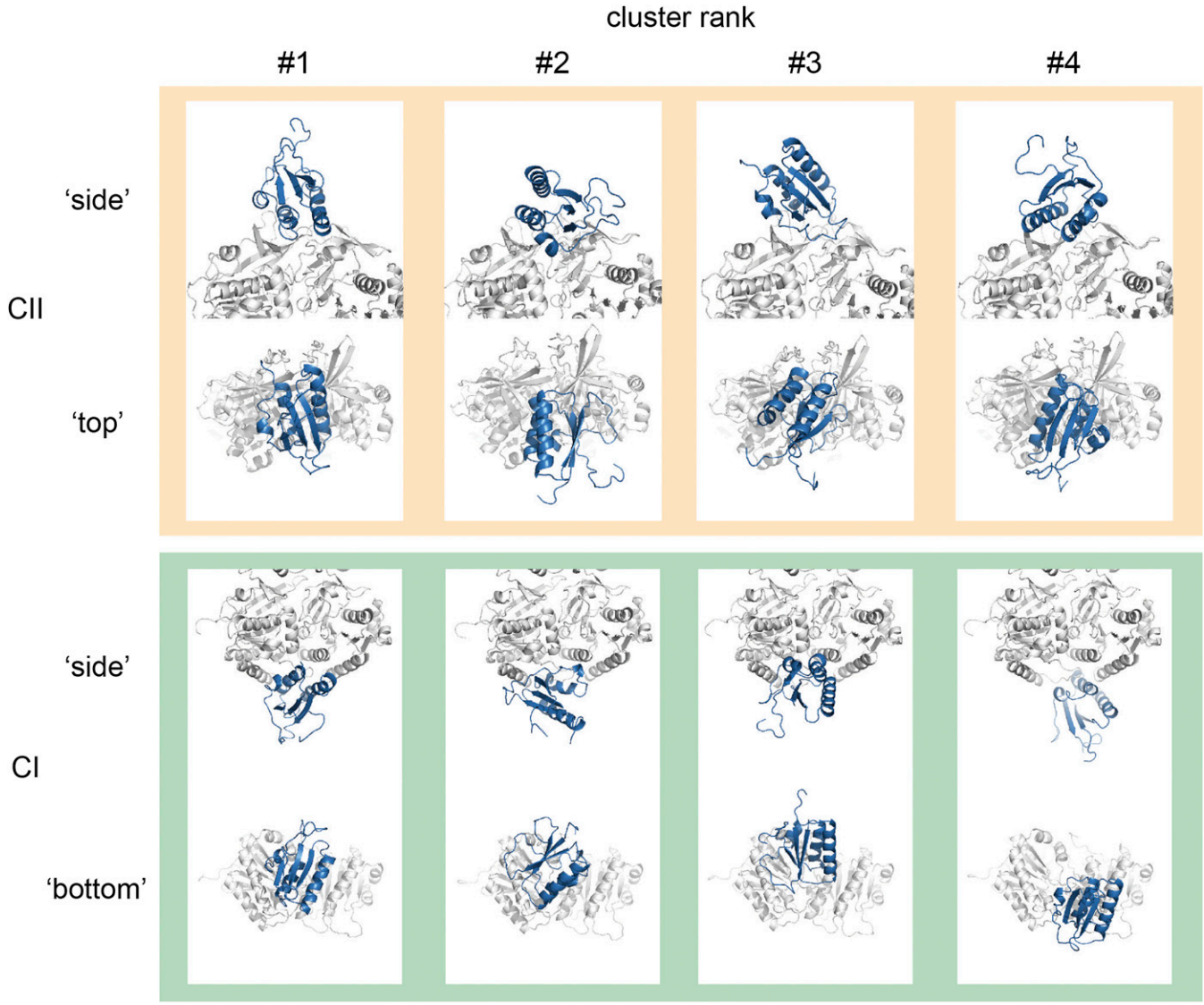

Fig. S7. Detailed views the four best-scoring HADDOCK cluster of KaiB–KaiC binding on either the protected region of the CII or CI domain.

Table S1. Masses of Kai proteins and protein complexes

Complex	Origin	Mass/Da		
		Average	SD	Mass deviation*
KaiB				
B1	Stock	11,740	1	23
B2		23,481	1	47
B4		46,961	2	93
KaiC				
C6	Stock	355,922	38	140
	30 °C 6 h	355,972	27	190
KaiCB				
C6B1	30 °C 6 h	367,870	70	371
C6B6	30 °C 6 h	426,782	86	698
	4 °C 1 wk	426,851	74	767
CID products	Precursor			
C6	C6B1	355,827	42	45
B1		11,719	1	2
C6B5	C6B6	414,489	43	122
C6B4		402,729	70	79

*The mass deviation is calculated from theoretical masses of 11,717 Da for KaiB and 58,283 Da for KaiC. All KaiC assemblies are assumed to have 12 ATP (507 Da) bound. CID, collision-induced dissociation.

Table S2. HDX-MS analysis of KaiB: free vs. KaiC-bound

Peptide Residue†		Relative deuterium uptake*							
		Free			Bound			Difference	
Start	End	1 min	10 min	60 min	1 min	10 min	60 min	$Bound_{60min} - Free_{60min}$	*P* value‡
−3	8	0.46	0.51	0.58	0.60	0.65	0.68	−0.10	0.0003
−1	7	0.53	0.59	0.67	0.67	0.74	0.74	−0.08	0.0267
−3	11	0.27	0.31	0.38	0.38	0.42	0.44	−0.07	0.0256
−1	11	0.27	0.31	0.38	0.36	0.40	0.45	−0.06	0.0012
0	11	0.28	0.33	0.40	0.40	0.45	0.47	−0.06	0.0054
12	23	0.31	0.51	0.58	0.25	0.38	0.48	0.11	0.0029
12	31	0.15	0.30	0.42	0.12	0.23	0.31	0.10	0.0023
12	32	0.15	0.31	0.43	0.12	0.24	0.33	0.10	0.0057
14	31	0.15	0.33	0.43	0.12	0.22	0.31	0.12	0.0003
14	32	0.16	0.33	0.49	0.12	0.23	0.34	0.15	0.0001
19	33	0.20	0.31	0.43	0.12	0.23	0.32	0.11	0.0024
24	31	0.07	0.20	0.30	0.09	0.22	0.28	0.02	0.2929
32	39	0.37	0.47	0.54	0.44	0.57	0.64	−0.11	<0.0001
33	39	0.34	0.44	0.51	0.39	0.52	0.56	−0.05	<0.0001
39	45	0.28	0.37	0.43	0.34	0.38	0.39	0.04	0.5728
40	52	0.18	0.31	0.40	0.20	0.30	0.36	0.04	0.0976
42	52	0.18	0.31	0.40	0.19	0.29	0.35	0.05	0.0019
44	52	0.16	0.31	0.39	0.19	0.30	0.36	0.02	0.2624
46	52	0.26	0.47	0.56	0.26	0.45	0.56	0.00	0.8591
53	61	0.15	0.22	0.29	0.20	0.25	0.30	−0.01	0.6631
53	64	0.14	0.23	0.33	0.12	0.16	0.22	0.12	0.0009
56	64	0.14	0.27	0.38	0.10	0.17	0.25	0.13	0.0049
64	78	0.23	0.36	0.48	0.16	0.29	0.41	0.08	0.0360
65	81	0.24	0.38	0.51	0.21	0.32	0.43	0.08	0.0134
68	78	0.28	0.45	0.58	0.17	0.27	0.45	0.12	0.0038
71	79	0.27	0.43	0.55	0.18	0.29	0.43	0.12	0.0079
65	86	0.23	0.40	0.50	0.23	0.37	0.47	0.03	0.0030
80	88	0.24	0.33	0.44	0.25	0.47	0.59	−0.15	0.0017
93	102	0.38	0.39	0.40	0.34	0.36	0.38	0.02	0.2981

*Relative deuterium uptake was determined as a fraction of maximum uptake (number of residues in the peptide minus one, minus prolines) and is reported as the average of triplicate experiments.
†Residues are numbered according to wild-type *S. elongatus* sequence. Numbering starts at −4 to account for the missing N-terminal methionine and the extra residual GST-tag amino acids (five residues).
‡*P* values from pairwise two-tailed Student *t* testing between the 60-min time points.

Table S3. HDX-MS analysis of KaiC: free vs. KaiB-bound

Peptide Residue[†]		Relative deuterium uptake*							
		Free			Bound			Difference	
Start	End	1 min	10 min	60 min	1 min	10 min	60 min	$Bound_{60min} - Free_{60min}$	*P* value[‡]
−3	4	0.50	0.62	0.64	0.56	0.59	0.63	0.02	0.4933
−3	5	0.52	0.61	0.62	0.55	0.59	0.62	0.00	0.8150
−3	23	0.39	0.47	0.54	0.41	0.46	0.48	0.06	0.1208
6	21	0.44	0.54	0.55	0.47	0.51	0.55	0.00	0.8645
6	23	0.42	0.53	0.55	0.44	0.49	0.54	0.01	0.4720
8	23	0.42	0.55	0.57	0.41	0.51	0.52	0.05	0.2304
22	28	0.21	0.26	0.26	0.20	0.22	0.26	0.00	0.9500
22	43	0.10	0.13	0.18	0.11	0.10	0.13	0.05	0.0305
24	43	0.09	0.13	0.18	0.08	0.10	0.13	0.05	0.0091
28	43	0.09	0.13	0.19	0.08	0.10	0.15	0.04	0.0163
29	43	0.11	0.15	0.21	0.09	0.12	0.17	0.05	0.0139
30	43	0.11	0.15	0.19	0.09	0.13	0.16	0.03	0.0192
36	42	0.12	0.18	0.25	0.07	0.14	0.19	0.06	0.0486
44	55	0.12	0.15	0.17	0.11	0.12	0.14	0.04	0.0290
60	66	0.07	0.08	0.08	0.06	0.05	0.06	0.02	0.0600
67	73	0.09	0.09	0.08	0.06	0.06	0.06	0.02	0.0876
77	89	0.18	0.25	0.30	0.16	0.23	0.27	0.02	0.4189
77	93	0.15	0.21	0.26	0.14	0.18	0.23	0.03	0.0576
77	94	0.14	0.20	0.24	0.12	0.16	0.20	0.04	0.0370
83	93	0.13	0.17	0.20	0.12	0.15	0.18	0.02	0.0969
83	94	0.10	0.13	0.17	0.09	0.12	0.15	0.02	0.1449
92	99	0.20	0.38	0.48	0.15	0.18	0.29	0.19	0.0084
116	122	0.33	0.39	0.45	0.21	0.31	0.35	0.09	0.0063
116	123	0.34	0.42	0.43	0.18	0.27	0.35	0.08	0.0313
117	123	0.33	0.40	0.39	0.42	0.50	0.50	−0.11	0.0046
126	132	0.11	0.22	0.35	0.08	0.11	0.15	0.19	0.0051
133	145	0.10	0.18	0.28	0.08	0.13	0.17	0.11	0.0068
133	148	0.11	0.20	0.30	0.09	0.15	0.20	0.10	0.0062
152	159	0.47	0.59	0.57	0.48	0.53	0.59	−0.02	0.2959
152	164	0.31	0.44	0.48	0.27	0.34	0.41	0.08	0.0133
165	171	0.05	0.08	0.12	0.05	0.05	0.07	0.05	0.0437
168	180	0.14	0.25	0.33	0.12	0.19	0.26	0.07	0.0157
170	180	0.13	0.27	0.34	0.11	0.22	0.27	0.07	0.0122
172	180	0.16	0.27	0.34	0.15	0.23	0.27	0.07	0.0032
181	187	0.32	0.44	0.48	0.34	0.42	0.49	−0.01	0.5964
184	198	0.33	0.46	0.54	0.36	0.45	0.53	0.01	0.4986
188	194	0.38	0.54	0.61	0.40	0.52	0.63	−0.02	0.5675
188	198	0.33	0.46	0.53	0.34	0.45	0.52	0.01	0.5594
205	211	0.12	0.18	0.24	0.07	0.14	0.19	0.04	0.0451
204	221	0.11	0.15	0.21	0.08	0.12	0.15	0.05	0.0025
208	221	0.21	0.29	0.34	0.17	0.24	0.30	0.04	0.0274
208	222	0.18	0.25	0.28	0.15	0.20	0.25	0.03	0.0340
208	223	0.17	0.23	0.27	0.14	0.18	0.23	0.04	0.0448
224	238	0.12	0.15	0.16	0.11	0.13	0.14	0.03	0.0015
220	245	0.17	0.24	0.26	0.15	0.18	0.20	0.06	0.0787
224	245	0.10	0.14	0.17	0.10	0.13	0.13	0.03	0.0501
226	245	0.13	0.17	0.18	0.10	0.13	0.15	0.03	0.0940
223	251	0.17	0.21	0.25	0.15	0.18	0.22	0.04	0.0075
224	251	0.18	0.24	0.26	0.15	0.19	0.23	0.03	0.0761
226	251	0.18	0.24	0.26	0.16	0.20	0.23	0.03	0.0311
232	251	0.23	0.32	0.31	0.18	0.26	0.29	0.02	0.2232
239	251	0.22	0.29	0.31	0.21	0.27	0.31	0.00	0.6008
241	255	0.52	0.62	0.63	0.50	0.60	0.64	−0.01	0.4704
252	266	0.42	0.53	0.55	0.43	0.49	0.55	0.00	0.9492
252	270	0.37	0.45	0.49	0.37	0.42	0.46	0.03	0.1865
261	267	0.16	0.22	0.23	0.16	0.21	0.20	0.03	0.2161
261	270	0.27	0.34	0.37	0.28	0.32	0.35	0.02	0.1017
271	278	0.15	0.18	0.22	0.12	0.14	0.18	0.04	0.0019
268	294	0.16	0.20	0.22	0.12	0.17	0.19	0.03	0.0642
279	285	0.08	0.09	0.10	0.07	0.07	0.09	0.02	0.0155
283	295	0.16	0.30	0.34	0.13	0.17	0.23	0.11	0.0152
286	297	0.18	0.31	0.38	0.14	0.18	0.25	0.13	0.0001

Table S3. Cont.

Peptide Residue†		Relative deuterium uptake*							
		Free			Bound			Difference	
Start	End	1 min	10 min	60 min	1 min	10 min	60 min	$Bound_{60min} - Free_{60min}$	*P* value‡
292	302	0.12	0.25	0.34	0.09	0.19	0.25	0.09	0.0228
298	314	0.07	0.09	0.16	0.06	0.09	0.13	0.03	0.6099
302	314	0.10	0.13	0.22	0.09	0.14	0.21	0.01	0.5741
304	314	0.09	0.13	0.22	0.08	0.13	0.20	0.02	0.4009
306	314	0.08	0.11	0.16	0.07	0.08	0.19	−0.03	0.3148
315	324	0.26	0.40	0.42	0.19	0.28	0.34	0.08	0.0264
317	324	0.28	0.41	0.45	0.21	0.30	0.38	0.06	0.0528
325	334	0.19	0.28	0.34	0.14	0.18	0.23	0.11	0.0544
325	335	0.15	0.23	0.30	0.09	0.11	0.13	0.16	0.0197
338	344	0.09	0.10	0.16	0.06	0.08	0.13	0.03	0.1464
344	356	0.31	0.42	0.45	0.25	0.31	0.38	0.07	0.0677
345	356	0.32	0.44	0.47	0.24	0.32	0.40	0.07	0.0238
345	358	0.32	0.42	0.43	0.25	0.32	0.40	0.03	0.0076
350	356	0.38	0.51	0.54	0.30	0.38	0.46	0.09	0.1591
343	369	0.16	0.23	0.25	0.14	0.18	0.22	0.03	0.0113
357	380	0.07	0.09	0.12	0.06	0.09	0.12	0.00	0.8445
361	377	0.08	0.11	0.13	0.07	0.10	0.13	0.00	0.0683
359	380	0.09	0.11	0.13	0.11	0.11	0.11	0.02	0.7616
361	380	0.09	0.12	0.14	0.08	0.10	0.13	0.02	0.0231
361	382	0.10	0.14	0.17	0.08	0.11	0.14	0.03	0.0214
366	380	0.06	0.08	0.10	0.05	0.08	0.08	0.02	0.0489
370	380	0.07	0.08	0.09	0.06	0.07	0.08	0.01	0.1628
381	391	0.36	0.49	0.51	0.36	0.42	0.48	0.03	0.1444
381	394	0.30	0.47	0.52	0.31	0.38	0.46	0.07	0.0571
383	392	0.38	0.51	0.55	0.34	0.43	0.52	0.03	0.3390
383	394	0.33	0.51	0.57	0.30	0.43	0.49	0.08	0.0085
384	394	0.35	0.49	0.57	0.34	0.42	0.50	0.07	0.0199
392	398	0.07	0.10	0.12	0.06	0.07	0.09	0.03	0.0073
392	401	0.05	0.07	0.09	0.05	0.06	0.07	0.02	0.0274
385	411	0.15	0.21	0.23	0.13	0.17	0.20	0.03	0.1142
395	401	0.07	0.07	0.08	0.06	0.06	0.07	0.01	0.3904
395	402	0.06	0.08	0.11	0.02	0.06	0.09	0.02	0.1894
399	411	0.11	0.16	0.24	0.11	0.15	0.22	0.02	0.3334
403	411	0.10	0.14	0.19	0.10	0.14	0.17	0.01	0.2903
412	418	0.35	0.44	0.45	0.27	0.36	0.44	0.01	0.7931
412	419	0.34	0.39	0.39	0.28	0.33	0.37	0.02	0.6262
412	432	0.32	0.41	0.46	0.34	0.37	0.42	0.04	0.1972
423	432	0.38	0.46	0.47	0.40	0.44	0.48	0.00	0.8484
423	435	0.32	0.39	0.43	0.27	0.34	0.39	0.04	0.0867
440	449	0.33	0.47	0.48	0.24	0.29	0.36	0.13	0.0050
443	449	0.41	0.54	0.55	0.31	0.38	0.44	0.11	0.0107
443	452	0.39	0.49	0.53	0.26	0.35	0.42	0.11	0.0041
450	456	0.07	0.12	0.16	0.04	0.07	0.10	0.06	0.0749
456	469	0.23	0.33	0.35	0.15	0.20	0.24	0.11	0.0086
463	477	0.02	0.11	0.12	0.01	0.04	0.09	0.04	0.1065
470	483	0.35	0.44	0.45	0.32	0.37	0.41	0.03	0.0286
470	486	0.39	0.48	0.49	0.35	0.41	0.46	0.03	0.0712
470	487	0.39	0.49	0.50	0.37	0.42	0.48	0.02	0.2411
471	486	0.34	0.44	0.45	0.33	0.39	0.44	0.01	0.4043
472	486	0.41	0.50	0.52	0.38	0.44	0.49	0.04	0.1146
487	497	0.47	0.61	0.61	0.39	0.56	0.63	−0.02	0.3929
487	500	0.50	0.65	0.66	0.48	0.60	0.69	−0.03	0.2637
487	501	0.50	0.63	0.63	0.48	0.60	0.64	−0.01	0.6437
487	505	0.47	0.59	0.61	0.45	0.54	0.58	0.03	0.2964
494	501	0.43	0.57	0.55	0.46	0.54	0.58	−0.03	0.2037
506	519	0.53	0.69	0.66	0.58	0.63	0.67	−0.01	0.5507

*Relative deuterium uptake was determined as a fraction of maximum uptake (number of residues in the peptide minus prolines) and is reported as the average of triplicate experiments.

†Residues are numbered according to wild-type *S. elongatus* sequence. Numbering starts at −4 to account for the missing N-terminal methionine and the extra residual GST-tag amino acids (five residues).

‡*P* values from pairwise two-tailed Student *t* testing between the 60-min time points.

Table S4. HADDOCK scores

Cluster rank	Haddock score	Nstruc	Eelec	Evdw	BSA	Edesolv
CII						
1	−76.0 ± 0.3	76	−759.1 ± 76.0	−56.1 ± 9.8	2533.9 ± 58.1	60.7 ± 10.5
2	−45.4 ± 4.7	14	−490.0 ± 37.0	−68.7 ± 10.1	2336.1 ± 166.2	47.7 ± 15.9
3	−42.6 ± 10.1	15	−357.1 ± 60.1	−77.2 ± 7.2	2328.8 ± 60.8	44.0 ± 10.8
4	−42.0 ± 9.7	19	−675.2 ± 106.1	−43.8 ± 11.2	2222.2 ± 159.9	61.0 ± 10.5
CI						
1	−48.1 ± 9.1	61	−575.8 ± 50.3	−73.4 ± 9.5	2676.1 ± 150.5	52.1 ± 5.7
2	−47.9 ± 6.1	35	−472.2 ± 98.0	−82.2 ± 6.0	2843.6 ± 164.5	53.5 ± 21.0
3	−45.6 ± 5.5	20	−488.3 ± 69.2	−90.2 ± 7.5	2936.8 ± 238.1	59.3 ± 6.7
4	−42.9 ± 15.4	15	−543.4 ± 125.2	−61.8 ± 4.3	2189.3 ± 183.0	37.5 ± 17.1

Scoring statistics of the models presented and evaluated in Fig. 5. Here we report the averaged HADDOCK scores for the four top-ranked models of each cluster (standard in HADDOCK analysis), the size of the clusters (Nstruc) and the contribution of different energy terms to the final HADDOCK score: Eelec (electrostatic energy), Evdw (van der Waals), BSA (buried surface area), and Edesolv (desolvation energy).

Table S5. Intermolecular contacts observed in cluster 1 (CII binding)

	Interacting residues		Hydrogen bonds			
Frequency	KaiB	KaiC	M–M	S–S	M–S	Nonbonded
0.5	P12	E276′	—	—	—	✗
0.3	N19	E276′	—	—	✗	—
0.9	N19	W466′	—	—	—	✗
0.7	R22	R273′	—	—	—	✗
0.8	R22	E276′	—	✗	—	✗
0.8	K25	D485′	—	✗	—	—
0.9	T26	R472′	—	✗	—	—
0.6	T26	D485′	—	✗	—	—
0.6	T26	R488′	—	—	—	✗
0.6	N29	D485′	—	✗	—	—
0.4	N29	R488′	—	—	✗	✗
0.3	I29	R488′	—	—	—	✗
0.3	E34	R488′	—	✗	—	—
0.5	E34	R450	—	✗	—	✗
0.5	E34	R500	—	✗	—	—
0.3	A65	K484	—	—	—	✗
0.4	K66	D485	—	✗	—	—
0.6	L70	E473	—	—	—	✗
0.4	L70	D485	—	—	—	✗
0.8	L70	F487	—	—	—	✗
0.4	P71	R450	—	—	—	✗
0.4	R73	K484	—	—	✗	✗
0.4	R73	D485	—	✗	—	✗
0.6	R74	E452	—	✗	—	✗
0.6	R74	S454	—	—	—	✗
0.7	R74	E473	—	✗	—	✗
0.3	R74	M475	—	—	—	✗
0.3	I75	E452	—	—	—	✗
0.6	G77	M475	—	—	—	✗
0.8	D78	W466	—	✗	—	—
0.3	L79	W466	—	—	—	✗
0.4	D81	S477	—	✗	—	✗
0.6	D81	K479	—	✗	—	✗
1	R82	W466	—	—	—	✗
0.3	R82	M475	—	—	—	✗
0.3	R82	I476	—	—	✗	—
0.5	R82	S477	—	—	—	✗
0.3	E83	W466	—	—	—	✗
0.9	K84	D478	—	✗	—	—

List of intermolecular contacts between KaiC and KaiB based on an analysis of the 10 best models of the top-ranking HADDOCK cluster (#1_CII). All contacts were analyzed using DIMPLOT. The frequency of a pairwise contact corresponds to its occurrence observed among the top 10 models of cluster #1_CII. For the hydrogen bonds, M refers to the main chain and S refers to the side chain. KaiC residues labeled with a prime indicate residues from the second chain in the model.