Title: *Synechocystis* KaiC3 displays temperature and KaiB dependent ATPase activity and is important for viability in darkness

Running title: Biochemical characterization of *Synechocystis* KaiC3

Authors: Anika Wiegard[1,*,#], Christin Köbler[2], Katsuaki Oyama[3,‡], Anja K. Dörrich[4], Chihiro Azai[3,5], Kazuki Terauchi[3,5,#], Annegret Wilde[2], Ilka M. Axmann[1]

Author affiliations:

[1] Institute for Synthetic Microbiology, Cluster of Excellence on Plant Sciences (CEPLAS), Heinrich Heine University Duesseldorf, Duesseldorf, Germany

[2] Institute of Biology III, Faculty of Biology, University of Freiburg, Freiburg, Germany

[3] Graduate School of Life Sciences, Ritsumeikan University, Kusatsu, Shiga, Japan

[4] Institute for Microbiology and Molecular Biology, Justus-Liebig University, Giessen, Germany

[5] College of Life Sciences, Ritsumeikan University, Kusatsu, Shiga, Japan

* present address: Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden

‡ present address: Graduate School of Medicine, Kobe University, Kobe, Hyougo, Japan

Correspondence and requests for materials should be addressed to A. Wiegard (email: anika.wiegard@ki.se) or K. Terauchi (email: terauchi@fc.ritsumei.ac.jp)

Abstract:

Cyanobacteria form a heterogeneous bacterial group with diverse lifestyles, acclimation strategies and differences in the presence of circadian clock proteins. In *Synechococcus elongatus* PCC 7942, a unique posttranslational KaiABC oscillator drives circadian rhythms. ATPase activity of KaiC correlates with the period of the clock and mediates temperature compensation. *Synechocystis* sp. PCC 6803 expresses additional Kai proteins, of which KaiB3 and KaiC3 proteins were suggested to fine-tune the standard KaiAB1C1 oscillator. In the present study, we therefore characterised the enzymatic activity of KaiC3 as a representative of non-standard KaiC homologs *in vitro*. KaiC3 displayed ATP synthase and ATPase activities, which were lower compared to the *Synechococcus* 7942 KaiC protein. ATP hydrolysis showed no temperature compensation. Hence, KaiC3 is missing a defining feature of the model cyanobacterial circadian oscillator. Yeast two-hybrid analysis showed that KaiC3 interacts with KaiB3, KaiC1 and KaiB1. Further, KaiB3 and KaiB1 reduced *in vitro* ATP hydrolysis by KaiC3. Spot assays showed that chemoheterotrophic growth in constant darkness is completely abolished after deletion of Δ*kaiAB1C1* and reduced in the absence of *kaiC3*. We therefore suggest a role for adaptation to darkness for KaiC3 as well as a crosstalk between the KaiC1 and KaiC3 based systems.

Importance: The circadian clock is known to influence the cyanobacterial metabolism globally and a deeper understanding of its regulation and fine-tuning will be important for metabolic optimizations in context of industrial applications. Due to the heterogeneity of cyanobacteria, the characterization of clock systems in organisms apart from the standard circadian model *Synechococcus elongatus* sp. PCC 7942 is required. *Synechocystis* sp. PCC 6803 is of special interest, because it represents a major cyanobacterial model organism and harbors diverged

43 homologs of the clock proteins, which are present in various other non-cyanobacterial
44 prokaryotes. By our *in vitro* studies we unravel the interplay of the multiple *Synechocystis* Kai
45 proteins and characterize the enzymatic activities of the non-standard clock homolog KaiC3. We
46 show that the deletion of *kaiC3* affects growth in constant darkness suggesting its involvement in
47 the regulation of non-photosynthetic metabolic pathways.

48 **Introduction:**

49 Cyanobacteria have evolved the circadian clock system to sense, anticipate and respond to
50 predictable environmental changes based on the rotation of Earth and the resulting day-night
51 cycle. Circadian rhythms are defined by three criteria: (i) oscillations with a period of about 24
52 hours without external stimuli, (ii) synchronization of the oscillator with the environment and (iii)
53 compensation of the usual temperature dependence of biochemical reactions, so that the period
54 of the endogenous oscillation does not depend on temperature in a physiological range (1). The
55 cyanobacterial circadian clock system has been studied in much detail in the unicellular model
56 cyanobacterium *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus* 7942). Its core
57 oscillator is composed of three proteins, which are unique to prokaryotes: KaiA, KaiB, and KaiC
58 (from now on $KaiA_{7942}$, $KaiB_{7942}$ and $KaiC_{7942}$; please note that some of the hereafter cited
59 information were gained studying proteins from *Thermosynechococcus elongatus* BP-1 though)
60 (2). The level of $KaiC_{7942}$ phosphorylation and $KaiC_{7942}$'s ATPase activity represent the key features
61 of the biochemical oscillator. $KaiA_{7942}$ stimulates autophosphorylation and ATPase activity of
62 $KaiC_{7942}$, whereas $KaiB_{7942}$ binding to the complex inhibits $KaiA_{7942}$ action, stimulates
63 autodephosphorylation activity and reduces ATPase activity of $KaiC_{7942}$ (3-5). As a consequence of

64 dynamic interactions with $KaiA_{7942}$ and $KaiB_{7942}$, $KaiC_{7942}$ rhythmically phosphorylates and
65 dephosphorylates with a 24-hour period (2).

66 $KaiC_{7942}$ consists of two replicated domains (CI and CII) which assemble into a hexamer forming
67 an N-terminal CI ring and a C-terminal CII ring (6-8). Phosphorylation takes place in the CII ring (9),
68 whereas ATP hydrolysis occurs in both rings (10). In the CII ring, ATP hydrolysis is part of the
69 dephosphorylation mechanism (11, 12). ATP hydrolysis in the CI ring correlates with the period of
70 the clock and temperature compensation and is further required for a conformational change of
71 $KaiC_{7942}$, which allows binding of $KaiB_{7942}$ (4, 13, 14). The levels of phosphorylation and ATP
72 hydrolysis of the $KaiC_{7942}$ serve as the read-out for regulatory proteins, which orchestrate the
73 circadian output (15, 16). For a recent review on the functioning of the $KaiABC_{7942}$ system, see
74 Swan *et al.* (17).

75 Cyanobacteria represent one of the most diverse prokaryotic phyla(18) and little is known about
76 timekeeping mechanisms in other cyanobacteria than *Synechococcus* 7942. A core diurnal
77 genome has been described (19), but temporal coordination varies and, based on genomic
78 analyses, large variations in the cyanobacterial clock systems can be expected (20-25).

79 The cyanobacterial model strain *Synechocystis* sp. PCC 6803 (from now on *Synechocystis* 6803)
80 contains a standard *kai* operon, encoding homologs of the *kaiA*, *kaiB* and *kaiC* genes (in the
81 following designated $kaiA_{6803}$, $kaiB1_{6803}$ and $kaiC1_{6803}$) as well as two additional *kaiB* and *kaiC*
82 genes (26). The $kaiC2_{6803}$ and $kaiB2_{6803}$ genes form an operon, whereas $kaiC3_{6803}$ and $kaiB3_{6803}$ are
83 orphan genes. Bioinformatic studies revealed that all cyanobacteria, which do contain at least one
84 *kaiB* gene, always encode orthologs of KaiB1 and KaiC1. Additional KaiB3 and KaiC3 homologs are
85 present in about one third of cyanobacterial genera, with a slightly higher occurrence of KaiC3.

86 KaiC3 is also present in bacteria and archaea, showing a higher distribution than KaiC1 and KaiC2
87 homologs (19). The reported number of oscillating genes in *Synechocystis* 6803 varies largely
88 between different studies, which is likely due to differences in growth conditions (27-30). These
89 results imply that *Synechocystis* 6803 might be able to fine-tune its circadian rhythm in response
90 to environmental conditions. In line with this, Aoki and Onai (21) suggested that $KaiC3_{6803}$ and
91 $KaiB3_{6803}$ modulate the amplitude and period of the $KaiAB1C1_{6803}$ oscillator, whereas, to our
92 knowledge, no function for $kaiC2B2_{6803}$ has been described so far.

93 The three KaiC proteins from *Synechocystis* 6803 show high conservation of the kinase motif in
94 the CII domain and all three proteins were shown to exhibit autophosphorylation activity (19, 23).
95 $KaiA_{6803}$ stimulates the autophosphorylation of $KaiC1_{6803}$, but does not affect the phosphorylation
96 of the two other KaiC homologs. Based on sequence analysis and experimental validation of
97 kinase activity, Schmelling *et al.* (19) concluded that autokinase and autophosphatase activities
98 are highly conserved features of all cyanobacterial KaiC homologs. Likewise, ATPase motifs in the
99 N-terminal CI domain of KaiC are highly conserved in all cyanobacterial KaiC homologs (19).
100 However, ATP hydrolysis was only characterised for several KaiC1 homologs and one non-
101 cyanobacterial KaiC2 protein (10, 31-33).

102 In a natural day and night cycle, *Synechococcus* 7942 orchestrates its metabolism in a precise
103 temporal schedule, with the metabolism being adjusted by the clock but also feeding input
104 information to the clock (34-36). Knockout of the *Synechococcus* 7942 *kai* genes leads to a growth
105 disadvantage. When grown in competition with the wild type under light-dark conditions, the
106 clock deficient mutant cells are eliminated from the culture (37). In *Synechocystis* 6803 deletion
107 of the *kaiAB1C1* gene cluster causes reduced growth in light-dark rhythms, even when grown as

108 single culture (38). A comparably strong impact on growth in a light-dark cycle was observed in
109 both strains after deletion of the regulator of phycobilisome association A (RpaA) (34, 39), the
110 master transcription factor of circadian gene expression. In addition, studies on single nucleotide
111 polymorphisms identified the *rpaA* gene as one of three genes responsible for the faster growth
112 of *Synechococcus elongatus* UTEX 2973 compared to *Synechococcus* 7942(40). Using a transposon
113 library, David *et al.* (41) showed that KaiA, despite being non-essential for growth in light-dark
114 cycles, strongly contributes to the fitness of *Synechococcus* 7942 under these conditions.
115 Decreased fitness most likely occurs due to reduced phosphorylation of RpaA in the *kaiA* knockout
116 strain (41). Overall, these data demonstrate the value of the cyanobacterial clock system for
117 metabolic orchestration under natural conditions, with the clock system considered especially
118 important for the transition from light to darkness (42).

119 Here, we aim at a detailed biochemical characterization of KaiC3$_{6803}$ and the role of *Synechocystis*
120 *6803* KaiB$_{6803}$ homologs in modulation of KaiC3$_{6803}$ function. Further, we demonstrate that the
121 Δ*kaiC3* mutant strain has a growth defect under chemoheterotrophic growth conditions, which is
122 similar, but less pronounced compared to Δ*kaiAB1C1* and Δ*rpaA* strains (38, 39). Our data support
123 the idea of a function of KaiC3$_{6803}$ and KaiB3$_{6803}$ in fine-tuning the central oscillator composed of
124 KaiA$_{6803}$, KaiB1$_{6803}$ and KaiC1$_{6803}$ in *Synechocystis* 6803.

125 **Results:**

126 **Recombinant KaiC3$_{6803}$ displays ATP synthase activity**

127 Previous bioinformatic analysis predicted that kinase, dephosphorylation and ATPase activities
128 are conserved in KaiC3$_{6803}$ (19). So far, only the kinase activity of KaiC3$_{6803}$ was experimentally
129 confirmed which differed from the activity of KaiC$_{7942}$ by lacking stimulation by KaiA$_{7942}$ and

130 $KaiA_{6803}$ (23). Therefore, we aimed at the characterization of further predicted activities of
131 $KaiC3_{6803}$.

132 The first step in $KaiC_{7942}$ dephosphorylation is regarded as a reversal of phosphorylation: $KaiC_{7942}$
133 transfers its bound phosphoryl group to ADP and thereby synthesizes ATP (11, 12). To investigate
134 reversibility of intrinsic phosphorylation, we tested whether $KaiC3_{6803}$ can synthesize [α-^{32}P]ATP
135 from [α-^{32}P]ADP (Fig. 1). As control, we used phosphorylated and dephosphorylated $KaiC_{7942}$ (Fig.
136 S1.). Immediately after adding [α-^{32}P]ADP, all KaiC proteins started to synthesize [α-^{32}P]ATP.
137 Phosphorylated as well as non-phosphorylated $KaiC_{7942}$ both showed higher initial ATP synthesis
138 than $KaiC3_{6803}$ (Fig. 1). After incubating phosphorylated and dephosphorylated $KaiC_{7942}$ for 2 hours
139 at 30°C with [α-^{32}P]ADP, a relative [α-^{32}P]ATP level of 26-28 % was detected. $KaiC3_{6803}$ was also
140 capable of intrinsic ATP synthesis, but compared to $KaiC_{7942}$ less than one third of relative [α-
141 ^{32}P]ATP was formed. This can be explained by two hypotheses: (i) $KaiC3_{6803}$ showed lower
142 phosphotransferase activity or (ii) higher consumption of the produced ATP. The latter would
143 require that $KaiC3_{6803}$ exhibits higher ATPase activity than $KaiC_{7942}$. Therefore, we next examined
144 ATP hydrolysis activity of $KaiC3_{6803}$.

145 **$KaiC3_{6803}$ displays ATPase activity**

146 ATP hydrolysis can be measured by quantifying ADP production in the presence of KaiC over time
147 (4). Conservation of WalkerA and WalkerB motifs in the CI domain of $KaiC3_{6803}$ proteins suggested
148 their capability to hydrolyze ATP (19). We confirmed the predicted ATPase activity *in vitro* using
149 Strep-$KaiC3_{6803}$, which produced 8.5 ± 1.0 ADP molecules per monomer and day (mean ± s.d., Fig.
150 2). This activity was about 45 % lower than the one measured for $KaiC_{7942}$ (19.1 ± 3.3 ADP per
151 day (4)). Hence, the above-observed lower level of net ATP production by $KaiC3_{6803}$ was not based

152 on a higher ATP consumption, but due to lower dephosphorylation activity *per se*. In KaiC$_{7942}$, the
153 ATPase activity changes after substitution of the phosphorylation sites (4). We therefore
154 generated variants of Strep-KaiC3$_{6803}$, in which residues S423 and T424 are either replaced with
155 aspartate and glutamate (Strep-KaiC3$_{6803}$-DE) or replaced with two alanine residues (Strep-
156 KaiC3$_{6803}$-AA). Strep-KaiC3$_{6803}$-AA, showed more than two fold increased ATPase activity, whereas
157 ATP hydrolysis by Strep-KaiC3$_{6803}$-DE was only slightly different from the wild-type protein (Fig. 2)
158 and not reduced as reported for KaiC$_{7942}$ (4).

159 **KaiC3$_{6803}$ interacts with KaiB3$_{6803}$ and components of the standard KaiAB1C1$_{6803}$ oscillator**

160 *In vitro* and *in silico* studies suggested that KaiA$_{6803}$, KaiB1$_{6803}$ and KaiC1$_{6803}$ form the standard
161 clock system of *Synechocystis* 6803 (21, 23). Since Aoki and Onai (21) suggested that KaiC3$_{6803}$
162 might modulate the main oscillator function, we performed protein-protein interaction studies in
163 order to reveal a possible crosstalk between the multiple Kai proteins. First, interaction was
164 determined by yeast-two hybrid analysis using KaiC1$_{6803}$, KaiC3$_{6803}$, KaiB1$_{6803}$ and KaiB3$_{6803}$ fused
165 to AD and BD domains, respectively. The color change of the colonies indicates β-galactosidase
166 activity and is an estimate for the interaction of the respective two proteins. As expected, these
167 experiments showed self-interaction of KaiC3$_{6803}$ (Fig. 3A), since KaiC homologs are known to form
168 hexamers (6, 8). In addition, an interaction of KaiC3$_{6803}$ with KaiB3$_{6803}$ was detected (Fig. 3A) which
169 is in line with bioinformatic analysis showing frequent co-occurrence of *kaiC3* and *kaiB3* genes in
170 genomes (19). We could not detect an interaction between KaiC3$_{6803}$ and KaiA$_{6803}$ by yeast-two
171 hybrid analysis (Fig. 3B), but detected a heteromeric interaction between KaiC1$_{6803}$ and KaiC3$_{6803}$
172 using different protein fusion variants (Fig. 3C). These results confirm previous *in vivo* data, which
173 showed co-purification of KaiA$_{6803}$ with KaiC1$_{6803}$ but not with KaiC3$_{6803}$, and a weak interaction

174 between the two KaiC homologs KaiC1$_{6803}$ and KaiC3$_{6803}$ (23). Besides KaiC1$_{6803}$, also its respective
175 KaiB protein, KaiB1$_{6803}$, showed an interaction with KaiC3$_{6803}$ in our analysis (Fig. 3C),
176 corroborating the hypothesis that there is a cross talk between the putative KaiC3$_{6803}$-B3$_{6803}$
177 system and the core oscillator KaiAB1C1$_{6803}$. Such a possible cross talk via the KaiB proteins was
178 further supported by our *in vitro* pull-down assays, in which KaiC3$_{6803}$ interacted with KaiB3$_{6803}$
179 (Fig. S3B,D) and further with KaiB1$_{6803}$ (Fig. S3B,D). Also KaiC1$_{6803}$ interacted with both, KaiB1$_{6803}$
180 and KaiB3$_{6803}$ homologs (Fig. S3,A,C). One must take into account that based on the *in vitro* pull-
181 down assays using *Synechocystis* 6803 whole cell extracts we cannot exclude indirect interactions.
182 As we have shown that KaiC1$_{6803}$ and KaiC3$_{6803}$ interact with each other, both proteins could bind
183 as a hetero-hexamer to the GST-tagged KaiB$_{6803}$ proteins and therefore co-elute from the affinity
184 matrix.

185 **ATPase activity of KaiC3$_{6803}$ is reduced in the presence of KaiB1$_{6803}$ and KaiB3$_{6803}$**

186 The interaction of KaiC3$_{6803}$ with KaiB1$_{6803}$ and KaiB3$_{6803}$ (Fig. 3 and Fig. S3) suggested a regulation
187 of KaiC3$_{6803}$ activity by these two KaiB proteins. We therefore measured ATP hydrolysis by Strep-
188 KaiC3$_{6803}$ in the presence of KaiB1$_{6803}$ and KaiB3$_{6803}$ proteins, respectively (Fig. 4A). After size
189 exclusion chromatography KaiB1$_{6803}$ was mainly eluted as tetramer (44 and 72 kDa, respectively,
190 depending on the column), whereas KaiB3$_{6803}$ was eluted as monomer (13/23 kDa) and tetramer
191 (41/70 kDa) (Fig. S4). Therefore, the monomeric and tetrameric KaiB3$_{6803}$ fractions were tested
192 separately. In all measurements, the ATPase activity was linear and showed no oscillations. ATP
193 hydrolysis was reduced by 55 % after the addition of the KaiB3$_{6803}$ monomer, but not affected by
194 the KaiB3$_{6803}$ tetramer. The KaiB1$_{6803}$ tetramer also inhibited the ATPase activity of Strep-
195 KaiC3$_{6803}$, though to a lesser extend (35 % reduction compared to Strep-KaiC3$_{6803}$ alone, Fig. 4).

196 Because ATPase activity of $KaiC_{7942}$ is influenced by $KaiA_{7942}$ (4), we also investigated the effect of
197 $KaiA_{6803}$ on $KaiC3_{6803}$, but in line with the lack of interaction in our yeast two-hybrid analysis data
198 (Fig. 3B), ATPase activity of Strep-$KaiC3_{6803}$ was not clearly affected by $KaiA_{6803}$ (Fig. 4A).

199 **ATPase activity of $KaiC3_{6803}$ is temperature dependent**

200 True circadian clocks are characterized by temperature compensated oscillations, which ensure
201 robust time measurements under temperature fluctuations. In the *Synechococcus* 7942 KaiABC
202 clock, overall temperature compensation is derived from $KaiC_{7942}$'s ATPase activity, which is stable
203 between 25 and 35 °C (Q_{10}=1.2, (4)). We therefore asked whether N-Strep-$KaiC3_{6803}$, as a
204 representative of non-standard KaiC homologs, shows temperature compensation as well.
205 Measurements at 25, 30 and 35 °C, however, revealed a temperature-dependent ADP production
206 by $KaiC3_{6803}$ (Q_{10}=2.4, Fig. 4B; activation energy = 66.5 kJ mol^{-1}, Fig. S5A). Hence, $KaiC3_{6803}$ is
207 lacking a characteristic feature of circadian oscillators. In accordance, dephosphorylation of Strep-
208 $KaiC3_{6803}$ was higher at 25 °C than at 30 and 35 °C (Fig. S5B)

209 **The non-standard $KaiC3_{6803}$ protein supports growth of *Synechocystis* 6803 cells in the dark**

210 As *in vitro* analyses suggested an interaction of $KaiC3_{6803}$ with $KaiB1_{6803}$ and $KaiC1_{6803}$, we aimed
211 at elucidating the role of $KaiC3_{6803}$ and its crosstalk with the putative main oscillator in the cell.
212 Clock factors are reported to be essential for cell viability in light-dark cycles (34, 39, 41).
213 Accordingly, deletion of the *kaiAB1C1* cluster of *Synechocystis* 6803 resulted in growth defects in
214 light-dark cycles (38). Lower viability of the Δ*kaiAB1C1* strain was more pronounced under
215 photomixotrophic (0.2 % glucose) compared to photoautotrophic conditions, whereas deletion
216 of *kaiC3* had no effect on cell viability in light-dark cycles (38). As KaiC3 homologs are also present
217 in non-photosynthetic bacteria (19), we were interested in the growth of the Δ*kaiC3* strain in the

218 dark. The *Synechocystis* 6803 WT strain used here, in contrast to previous studies (43), is able to
219 grow in complete darkness when supplemented with glucose (39). We therefore analyzed the
220 viability of Δ*kaiC3* cells via spot assays (44) in constant light and in complete darkness on agar
221 plates containing 0.2 % glucose (Fig. 5). Under photomixotrophic growth conditions with
222 continuous illumination, wild type and Δ*kaiC3* showed similar viability, whereas the viability of
223 the Δ*kaiAB1C1* strain was reduced. Further, in complete darkness, wild type and Δ*kaiC3* displayed
224 detectable growth, while growth of Δ*kaiAB1C1* was abolished completely. Growth inhibition of
225 the Δ*kaiAB1C1* mutant under mixotrophic conditions in the light and in the dark highlights the
226 importance of the KaiAB1C1$_{6803}$ oscillator for the switch between these two different metabolic
227 modes of *Synechocystis* 6803. Comparing wild-type and Δ*kaiC3* strains, the latter showed reduced
228 growth in complete darkness. Thus, KaiC3$_{6803}$ seems to be linked to dark adaption of *Synechocystis*
229 6803 cells, yet not as essential as the core oscillator KaiAB1C1$_{6803}$.

230 **Discussion:**

231 *Characterization of enzymatic activities of KaiC3$_{6803}$*

232 Previous sequence analysis suggested that the three enzymatic activities of KaiC$_{7942}$, which are
233 autophosphorylation, autodephosphorylation and ATPase activity, are conserved in all
234 cyanobacterial KaiC proteins (19). This hypothesis is supported by experiments demonstrating
235 autophosphorylation of all KaiC$_{6803}$ proteins (23), different cyanobacterial KaiC1 and KaiC3
236 homologs (19, 31, 33), and even non-cyanobacterial KaiC homologs from *Rhodopseudomonas*
237 *palustris* (32), *Legionella pneumophila* (45) as well as two thermophilic Archaea (19). In the
238 current paper, we provide experimental evidence that dephosphorylation and ATPase activities
239 are conserved in KaiC3$_{6803}$ as well. However, the level of the ATPase activity seems to vary

240 between KaiC proteins. While ATPase activity of the KaiC from *Prochlorococcus marinus* MED4
241 was reported to have the same activity as $KaiC_{7942}$ (31), we show here that the ATPase activity of
242 $KaiC3_{6803}$ is lower. In contrast, ATP hydrolysis activity of the KaiC2 homolog from *Legionella*
243 *pneumophila* and KaiC1 homolog from *Gloeocapsa* sp. PCC 7428 were reported to be elevated
244 when compared to the standard $KaiC_{7942}$ protein (32, 33).

245 ATPase activity of $KaiC3_{6803}$ further differed from that of $KaiC_{7942}$, by lacking temperature
246 compensation (Fig. 4). Therefore, $KaiC3_{6803}$ cannot be the core component of a true circadian
247 oscillator. This is further supported by differences in the ATP hydrolysis rate between the
248 phosphomimetic variants of $KaiC_{7942}$ and $KaiC3_{6803}$. ATPase activity of the true clock protein
249 $KaiC_{7942}$ depends on its phosphorylation status. In the $KaiC_{7942}$-AA variant, which mimics the
250 dephosphorylated state of $KaiC_{7942}$, the ATPase activity is higher than average, whereas, in
251 $KaiC_{7942}$-DE, mimicking the phosphorylation state, it is diminished (4). In our analysis, ATP
252 hydrolysis by Strep-$KaiC3_{6803}$-AA was similarly increased in comparison to Strep-$KaiC3_{6803}$.
253 However, Strep-$KaiC3_{6803}$-DE did not show reduced activity as was shown for $KaiC_{7942}$-DE, implying
254 that the phosphorylation state of $KaiC3_{6803}$ does not influence $KaiC3_{6803}$'s ATPase activity.

255 However, the ATPase activity of Strep-$KaiC3_{6803}$ was affected decisively by the addition of
256 $KaiB3_{6803}$ and $KaiB1_{6803}$. Both KaiB homologs led to decreased ATPase activity of Strep-$KaiC3_{6803}$,
257 with $KaiB3_{6803}$ having a stronger impact (Fig. 4A). $KaiB_{7942}$ exists as a monomer or a tetramer in
258 solution (46). As a tetramer, $KaiB_{7942}$ subunits adopt a unique fold, which was also observed in
259 crystals of $KaiB1_{6803}$ (47). Notably, $KaiB_{7942}$ binds as a monomer to $KaiC_{7942}$, in which it adopts a
260 different, thioredoxin-like fold (48-50). The formation of $KaiB_{7942}$ tetramers is concentration-
261 dependent and thereby ensures that the effective monomer concentration is stable over a broad

262 range of total $KaiB_{7942}$ concentrations (46). This points at a putative mechanism how $KaiC3_{6803}$
263 might fine-tune the standard oscillator: Binding of $KaiB1_{6803}$ to $KaiC3_{6803}$ will reduce the free
264 $KaiB1_{6803}$ concentration in the cell and thereby influence the $KaiAB1C1_{6803}$ oscillator. In contrast,
265 only the monomeric form of $KaiB3_{6803}$ had a regulatory effect on ATP hydrolysis of $KaiC3_{6803}$
266 suggesting that $KaiB3_{6803}$ bound as a monomer to $KaiC3_{6803}$, but no transition from tetramer to
267 monomer occurred. This finding is surprising, because residues K57, G88 and D90, which are
268 important for fold switching of $KaiB_{7942}$ (50), are conserved in $KaiB3_{6803}$. However, we show only
269 *in vitro* data here. In the cell, protein concentrations as well as spatial and temporal separation of
270 the different proteins might have an influence on these interactions. In addition, we do not take
271 possible heteromeric interactions among the KaiB proteins into account.

272 *Function of* $KaiC3_{6803}$ *in an extended network*

273 Our interaction studies imply a crosstalk between $KaiC1_{6803}$ and $KaiC3_{6803}$ (Fig. 6). KaiC1 is believed
274 to form a standard oscillator together with $KaiA_{6803}$ and $KaiB1_{6803}$ (21, 23), which is supported by
275 the interaction with $KaiB1_{6803}$ observed in this paper (Fig. 3C, Fig. S3). We further hypothesize that
276 $KaiC3_{6803}$ acts in a separate non-circadian regulatory system together with $KaiB3_{6803}$. In
277 accordance with bioinformatic analysis, which showed a significant co-occurrence of KaiB3 and
278 KaiC3 in Cyanobacteria (19), $KaiB3_{6803}$ had a stronger effect on the ATPase activity of $KaiC3_{6803}$
279 than $KaiB1_{6803}$ (Fig. 4A). Since $KaiC3_{6803}$ is able to form hetero-oligomers with $KaiC1_{6803}$ and was
280 shown to interact with $KaiB1_{6803}$ as well, interference between the $KaiC3_{6803}$-$KaiB3_{6803}$ system and
281 the standard oscillator is very likely. However, we exclude that $KaiA_{6803}$ is involved in the putative
282 crosstalk by four reasons: (i) $KaiA_{6803}$ did neither stimulate ATP hydrolysis nor kinase activity of
283 $KaiC3_{6803}$ (this paper and Wiegard *et al.* (23)); (ii) We were not able to show an interaction of

284 KaiC3$_{6803}$ and KaiA$_{6803}$ using different approaches (this paper and Wiegard *et al.* (23)); (iii) KaiA
285 interacting residues are not conserved in cyanobacterial KaiC3 homologs, and (iv) KaiC3 homologs
286 are present in organisms which do not harbor KaiA (19).

287 The growth defect of the mutant strains, Δ*kaiAB1C1* and Δ*kaiC3,* in complete darkness suggests
288 that the here proposed putative KaiC3-KaiB3 system and the KaiAB1C1$_{6803}$ oscillator may target
289 similar cellular functions. The metabolic network of cyanobacteria is described as temporally
290 partitioned with extensive effects of day-night transitions, involving shifts in ATP and reductant
291 levels and alterations of the carbon flux (41). In *Synechococcus* 7942, environmental signals can
292 be fed into the main clock output system via the transcriptional regulator RpaB (51). In contrast
293 to *Synechococcus* 7942, *Synechocystis* 6803 is able to grow in complete darkness, which adds
294 another layer of complexity to day-night transitions and demand for further regulatory elements.
295 The here observed impaired mixotrophic growth of the *kaiC3$_{6803}$* mutant in darkness supports the
296 idea of KaiC3$_{6803}$ and KaiB3$_{6803}$ functioning as such additional elements to adjust the state of the
297 main *Synechocystis* 6803 oscillator. Conversely, it is also possible that KaiC3$_{6803}$ function is
298 controlled by the KaiAB1C1$_{6803}$ clock system. Köbler *et al.* (39) demonstrated that solely KaiC1$_{6803}$,
299 but not KaiC3$_{6803}$, interacts with the main output histidine kinase SasA in the *Synechocystis* 6803
300 timing system. Thus, in *Synechocystis* 6803, only the main oscillator feeds timing information into
301 the SasA-RpaA output system to control the expression of many genes involved in dark growth
302 (39). The output pathway for KaiC3$_{6803}$ is unknown so far and it might be possible that the only
303 function of KaiC3$_{6803}$ is to modulate the function of the main oscillator in response to a yet
304 unknown input factor.

305

306 **Material and Methods:**

307 **Cloning, expression and purification of recombinant Kai proteins**

308 In this paper, we name the *Synechocystis kai* genes according to the definition introduced by
309 Schmelling *et al.* (19) (Please note that naming of KaiB3/KaiB2 and KaiC3/KaiC2 is not consistent
310 in the literature). Genes encoding $KaiB1_{6803}$ (ORF *slr0757*) and $KaiB3_{6803}$ (ORF *sll0486*) were
311 amplified from genomic *Synechocystis* 6803 wild type DNA using specific primers (Table S1) and
312 Phusion Polymerase (New England Biolabs). After restriction digest with BamHI and NotI,
313 amplified fragments were inserted into pGEX-6P1 (GE Healthcare) and the resulting plasmids
314 were used for heterologous expression. For production of recombinant $KaiC_{7942}$ as well as
315 $KaiA_{6803}$, $KaiB1_{6803}$, $KaiB3_{6803}$ and $KaiC3_{6803}$, we used pGEX-based plasmids described in Wiegard
316 *et al.* (23) (see also Table S2 for a list of all plasmids used in this study). A detailed protocol of
317 expression and purification can be found on protocols.io
318 (https://dx.doi.org/10.17504/protocols.io.48ggztw). Briefly, proteins were expressed as GST-
319 fusion proteins in *E. coli* BL21 [DE3] or NEB Express (New England Biolabs) and lysed in 50 mM
320 Tris/HCl (pH8), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT (+ 5 mM $MgCl_2$, 1 mM ATP for KaiC
321 proteins). Purification was performed via *batch* affinity chromatography using glutathione
322 agarose 4B (Macherey and Nagel) or glutathione sepharose 4B (GE Healthcare) in the same buffer.
323 Finally, the GST-tag was cleaved off using PreScission protease (GE Healthcare) in 50 mM Tris/HCl
324 (pH8), 150 mM NaCl, 1 mM EDTA, 1 mM DTT (+ 5 mM $MgCl_2$, 1 mM ATP for KaiC proteins). If
325 homogeneity of the proteins was not sufficient, they were further purified via anion exchange
326 chromatography using a MonoQ or ResourceQ column (GE Healthcare Life Sciences) using 50 mM

327 Tris/HCl (pH8), 1 mM EDTA, 1 mM DTT (+ 5 mM $MgCl_2$, 1 mM ATP for KaiC proteins) and a 0-1M
328 NaCl gradient.

329 To produce Strep-KaiC3$_{6803}$ variants with amino acid substitutions, the *kaiC3$_{6803}$* gene in the pGEX-
330 kaiC3$_{6803}$ vector was modified by site directed mutagenesis using the Quick-Change Site-Directed
331 Mutagenesis Kit (Stratagene) or Q5 Site-Directed Mutagenesis Kit (New England Biolabs). Base
332 triplets encoding S423 and T424 were changed to code for alanine or for aspartate and glutamate
333 resulting in *kaiC3$_{6803}$-AA* and *kaiC3$_{6803}$-DE* genes, respectively. To generate *kaiC3$_{6803}$-catE1$^-$catE2*,
334 two subsequent site-directed mutagenesis reactions were performed to exchange nucleotides
335 encoding E67 and E68 as well as nucleotides encoding E310 and E311 all with bases encoding
336 glutamine (all primers used for mutagenesis are listed in Table S1). Afterwards, *kaiC3$_{6803}$* WT and
337 modified *kaiC3$_{6803}$* genes were amplified with KOD-Plus-Neo polymerase (Toyobo) using pASK-
338 kaiC3 primers (Table S1). Amplicons were digested with SacII and HindIII and ligated into the
339 respective restriction sites of pASK-IBA5plus (IBA Life sciences). For purification of recombinant
340 Strep-KaiC$_{7942}$, the pASK-IBA-5plus based vector described in Oyama *et al.* (14) was used. Strep-
341 KaiC3$_{6803}$ proteins were expressed in *E. coli* Rosetta gamiB (DE3) or Rosetta gami2 (DE3) cells
342 (Novagen). Expression of Strep-KaiC$_{7942}$ was carried out in *E. coli* DH5α. Cells were cultured in LB
343 medium containing 100 µg ml^{-1} ampicillin with vigorous agitation at 37 °C. At OD_{600nm} 0.33-0.68
344 protein expression was induced with 200 ng ml^{-1} anhydrotetracycline and the strains were further
345 incubated as following: Strep-KaiC$_{7942}$: 7h at 37°C, Strep-KaiC3$_{6803}$-WT: 5h at 35°C or 3.5h at 37°C,
346 Strep-KaiC3$_{6803}$-AA and Strep-KaiC3$_{6803}$-catE1$^-$catE2$^-$: 18°C overnight, Strep-KaiC3$_{6803}$-DE: 25°C
347 overnight. Cells were harvested and lysed by sonication in ice-cold buffer W [20mM Tris/HCl
348 (pH8), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ATP (+ 2 mM DTT for Strep-KaiC3$_{6803}$ proteins)] including

protease inhibitors (protease inhibitor cocktail, Roche or Nacalai). Soluble proteins were loaded on self-prepared columns packed with Strep-Tactin XT superflow or Strep-Tactin Sepharose (IBA lifesciences) and purified under gravity flow. After washing with buffer W, Strep-KaiC proteins were eluted with ice cold buffer W + 50 mM D(+)biotin (for Strep-Tactin XT Superflow) or ice cold buffer W + 2.5 mM desthiobiotin (for Strep tactin Superflow). See https://dx.doi.org/10.17504/protocols.io.meac3ae for a detailed protocol.

All proteins used for ATPase activity measurements were further purified via size exclusion chromatography. Strep-KaiC3$_{6803}$ and Strep-KaiC$_{7942}$ proteins were applied on a Sephacryl S300 HR HiPrep 16/60 Sephacryl column (GE Healthcare) and separated in 20 or 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 2 mM DTT, 1 mM ATP and 5 mM MgCl2. For separation of KaiB1$_{6803}$ and KaiB3$_{6803}$, a Sephacryl S200 HR HiPrep 16/60 column (GE Healthcare) or Superdex 200 Increase 10/30 GL column (GE Healthcare) and 20 or 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 2 mM DTT as running buffer were used. KaiA$_{6803}$ was purified on a Superdex 200 Increase 10/30 GL column (GE Healthcare) in 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 2 mM DTT. See https://dx.doi.org/10.17504/protocols.io.mdtc26n for further details.

ATPase activity

For ATPase measurements, KaiC proteins fused to an N-terminal Strep-tag were used and all Kai proteins were purified via size exclusion chromatography (see above). 3.45 µM of Strep-KaiC3$_{6803}$ variants were incubated in ATPase buffer (20 mM Tris/HCl (pH8), 150 mM NaCl, 1 mM ATP, 5 mM $MgCl_2$) at 25 °C, 30 °C or 35 °C for 24 hours. To analyze the influence of KaiA$_{6803}$ and KaiB$_{6803}$ proteins on KaiC3$_{6803}$ ATPase activity, we mixed 0.2 mg ml^{-1} KaiC3$_{6803}$ with 0.04 mg ml^{-1} KaiA$_{6803}$ or 0.04 mg ml^{-1} KaiB$_{6803}$ and incubated the mixtures for 24 hours at 30 °C. Monomeric and

371 oligomeric $KaiB3_{6803}$ were analyzed separately. To monitor ADP production, every 3, 4 or 6 hours
372 2 µl of the reaction mixture were applied on a Shim-Pack-VP-ODS column (Shimadzu) and
373 separated using 100 mM phosphoric acid, 150 mM triethylamine, 1 % acetonitrile as running
374 buffer. ADP production per monomer KaiC and 24 hours was calculated using a calibration curve.
375 A detailed protocol can be found on protocols.io
376 (https://dx.doi.org/10.17504/protocols.io.mebc3an). The Q_{10} value was calculated from ATPase
377 measurements at 25 °C and 35 °C, using the formula $Q_{10} = \left(\frac{R_2}{R_1}\right)^{10°C/(T_2-T_1)}$

378 **ATP synthase activity**

379 To investigate dephosphorylation via ATP synthesis, we used KaiC proteins which were expressed
380 as GST-fusion proteins and subsequently cleaved off their GST-tag. 3 µM KaiC in ATP synthesis
381 buffer (20 mM Tris/HCl(pH8), 150 mM NaCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mM ATP) was mixed
382 with 0.8 µCi ml^{-1} [α^{32}P]ADP and stored at -20 °C or incubated for 2 hours at 30 °C. As a control,
383 the same experiment was performed in the presence of 0.5 mM ADP. After 20-fold dilution,
384 nucleotides in a 0.5 µl reaction mixture were separated via thin layer chromatography using TLC
385 PEI Cellulose F plates (Merck Millipore) and 1 M LiCl as solvent. [α-^{32}P]ADP and [γ-^{32}P]ATP were
386 separated in parallel to identify the signals corresponding to ADP and ATP, respectively. Dried
387 plates were subjected to autoradiography and signals were analysed using a Personal Molecular
388 Imager FX system (Bio-Rad) and ImageLab software (Bio-Rad). For each reaction mixture, the
389 relative intensity of [α-^{32}P]ATP was calculated as percentage of all signals in the corresponding
390 lane. Because [α-^{32}P]ATP was already synthesized during mixing of the samples, the relative ATP
391 intensity measured in the -20 °C sample containing 0.5 mM ADP was subtracted for normalization.

392 The principle of this method is based on Egli *et al.* (12). A detailed protocol is available on
393 protocols.io (https://dx.doi.org/10.17504/protocols.io.48qgzvw).

394 **Yeast two-hybrid assays**

395 For yeast two-hybrid assays, vectors containing the GAL4 activation domain (AD) and the GAL4
396 DNA-binding domain (BD) were used. Genes of interest were amplified from *Synechocystis* 6803
397 wild type genomic DNA with the Phusion Polymerase (NEB) according to manufacturer's
398 guidelines. Indicated restriction sites were introduced via the oligonucleotides listed in Table S1.
399 Vectors and PCR fragments were cut with the respective restriction enzymes (Thermo Fisher
400 Scientific) and the gene of interest was ligated into the vector, leading to a fusion protein with an
401 AD- or BD-tag either at the N- or C-terminus. All constructed plasmids are listed in Table S2.
402 Transformation of yeast cells was performed according to manufacturer's guidelines using the
403 Frozen-EZ Yeast Transformation II Kit (Zymo Research) and cells were selected on complete
404 supplement mixture (CSM) lacking leucine and tryptophan (-Leu -Trp) dropout medium (MP
405 Biochemicals) at 30 °C for 3–4 days. Y190 (Clontech) cells were used for measuring
406 β-galactosidase activity. Formed colonies were spotted on a second plate (CSM -Leu -Trp) and
407 incubated for 2 days. Afterwards a colony-lift filter assay was performed as described by Breeden
408 *et al*. (52). A detailed protocol can be found on protocols.io
409 (https://dx.doi.org/10.17504/protocols.io.v7ve9n6).

410 **Bacterial strains and growth conditions**

411 Wild type *Synechocystis* 6803 (PCC-M, re-sequenced, (53)) and the *kaiC3* deletion mutant (38)
412 were cultured photoautotrophically in BG11 medium (54) supplemented with 10 mM TES buffer
413 (pH 8) under constant illumination with 50 µmol photons $m^{-2}s^{-1}$ of white light (Philips TLD Super

414 80/840) at 30 °C. Cells were grown either in Erlenmeyer flasks with constant shaking (140 rpm) or
415 on plates (0.75 % Bacto-Agar, Difco) supplemented with 0.3 % thiosulfate. Detailed recipes can
416 be found on protocols.io (https://dx.doi.org/10.17504/protocols.io.wj5fcq6).

417 **Spot assays**

418 Experiments were performed as previously described (44). Strains were propagated
419 mixotrophically on BG11 agar plates with the addition of 0.2 % (w/v) glucose. Dilution series of
420 cell cultures started with OD_{750nm} 0.2 and OD_{750nm} 0.4, followed by incubation of the plates for 6
421 or 28 days under constant light conditions and in complete darkness, respectively.

422

423 **Acknowledgement:**

424 We thank Junko Moriwaki, Nancy Sauer, Jennifer Andres, Lukas Pohlig, Thomas Volkmer and
425 Megumi Fujimoto for technical assistance. This work was funded by the Deutsche
426 Forschungsgemeinschaft (DFG, German Research Foundation) under Germany´s Excellence
427 Strategy – EXC 2048/1 – Project ID: 390686111 to A. Wiegard and I.M. Axmann. The work was
428 financially supported by Heine Research Academies - Travel Grants, and EMBO ASTF 145 to A.
429 Wiegard, by grants AX 84/1-3 and EXC 1028 from the German Research Foundation to I.M.
430 Axmann, by JSPS Grants-in-Aid 16H00784, 17K19247 and 19K05833 to K. Terauchi, and by grant
431 WI2014/5-3 from the German Research Foundation to A. Wilde.

432 **Author contributions:**

433 I.M. Axmann, A. Wilde, A. Wiegard, C. Köbler and K. Terauchi conceived the project. A. Wiegard,
434 C. Köbler A.K. Dörrich and K. Oyama designed, performed and analyzed experiments. I.M Axmann,

435 A. Wilde and K. Terauchi supervised the study. All authors interpreted and discussed the data. A.
436 Wiegard, C. Köbler and A. Wilde wrote the manuscript. I.M. Axmann, C. Azai, K. Terauchi, A.K.
437 Dörrich, and K. Oyama commented essentially on the manuscript. All authors approved the
438 manuscript.

439 **This manuscript contains supplementary information:**

440 **Supplementary methods**

441 **Table S1.** Oligonucleotides used in this study.

442 **Table S2.** Plasmids used in this study.

443 **Figure S1.** Initial phosphorylation level of KaiC proteins.

444 **Figure S2.** Original scans of the KaiC3 yeast two-hybrid interactions.

445 **Figure S3.** Interaction of $KaiB_{6803}$ and $KaiC_{6803}$ proteins in pull down analysis.

446 **Figure S4.** Purification of KaiB1 and KaiB3 proteins used for ATPase analysis

447 **Figure S5.** ATPase activity and dephosphorylation of $KaiC3_{6803}$ are temperature dependent

448 **References:**

449 1. Ditty JL, Williams SB, Golden SS. 2003. A cyanobacterial circadian timing mechanism. Annu Rev
450 Genet 37:513-43.
451 2. Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T. 2005.
452 Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation *in vitro*. Science
453 308:414-5.
454 3. Iwasaki H, Nishiwaki T, Kitayama Y, Nakajima M, Kondo T. 2002. KaiA-stimulated KaiC
455 phosphorylation in circadian timing loops in cyanobacteria. Proc Natl Acad Sci U S A 99:15788-93.
456 4. Terauchi K, Kitayama Y, Nishiwaki T, Miwa K, Murayama Y, Oyama T, Kondo T. 2007. ATPase activity
457 of KaiC determines the basic timing for circadian clock of cyanobacteria. Proc Natl Acad Sci U S A
458 104:16377-81.
459 5. Kitayama Y, Iwasaki H, Nishiwaki T, Kondo T. 2003. KaiB functions as an attenuator of KaiC
460 phosphorylation in the cyanobacterial circadian clock system. EMBO J 22:2127-34.
461 6. Hayashi F, Suzuki H, Iwase R, Uzumaki T, Miyake A, Shen JR, Imada K, Furukawa Y, Yonekura K,
462 Namba K, Ishiura M. 2003. ATP-induced hexameric ring structure of the cyanobacterial circadian
463 clock protein KaiC. Genes Cells 8:287-96.
464 7. Mori T, Saveliev SV, Xu Y, Stafford WF, Cox MM, Inman RB, Johnson CH. 2002. Circadian clock
465 protein KaiC forms ATP-dependent hexameric rings and binds DNA. Proc Natl Acad Sci U S A
466 99:17203-8.
467 8. Pattanayek R, Wang J, Mori T, Xu Y, Johnson CH, Egli M. 2004. Visualizing a circadian clock protein:
468 crystal structure of KaiC and functional insights. Mol Cell 15:375-88.
469 9. Nishiwaki T, Iwasaki H, Ishiura M, Kondo T. 2000. Nucleotide binding and autophosphorylation of
470 the clock protein KaiC as a circadian timing process of cyanobacteria. Proc Natl Acad Sci U S A
471 97:495-9.

472 10. Murakami R, Miyake A, Iwase R, Hayashi F, Uzumaki T, Ishiura M. 2008. ATPase activity and its
473 temperature compensation of the cyanobacterial clock protein KaiC. Genes Cells 13:387-95.
474 11. Nishiwaki T, Kondo T. 2012. Circadian autodephosphorylation of cyanobacterial clock protein KaiC
475 occurs via formation of ATP as intermediate. J Biol Chem 287:18030-5.
476 12. Egli M, Mori T, Pattanayek R, Xu Y, Qin X, Johnson CH. 2012. Dephosphorylation of the core clock
477 protein KaiC in the cyanobacterial KaiABC circadian oscillator proceeds via an ATP synthase
478 mechanism. Biochemistry 51:1547-58.
479 13. Phong C, Markson JS, Wilhoite CM, Rust MJ. 2013. Robust and tunable circadian rhythms from
480 differentially sensitive catalytic domains. Proc Natl Acad Sci U S A 110:1124-9.
481 14. Oyama K, Azai C, Nakamura K, Tanaka S, Terauchi K. 2016. Conversion between two
482 conformational states of KaiC is induced by ATP hydrolysis as a trigger for cyanobacterial circadian
483 oscillation. Sci Rep 6:32443.
484 15. Shultzaberger RK, Boyd JS, Diamond S, Greenspan RJ, Golden SS. 2015. Giving Time Purpose: The
485 Synechococcus elongatus Clock in a Broader Network Context. Annu Rev Genet 49:485-505.
486 16. Dong G, Yang Q, Wang Q, Kim YI, Wood TL, Osteryoung KW, van Oudenaarden A, Golden SS. 2010.
487 Elevated ATPase activity of KaiC applies a circadian checkpoint on cell division in *Synechococcus*
488 *elongatus*. Cell 140:529-39.
489 17. Swan JA, Golden SS, LiWang A, Partch CL. 2018. Structure, function, and mechanism of the core
490 circadian clock in cyanobacteria. J Biol Chem 293:5026-5034.
491 18. Shih PM, Wu D, Latifi A, Axen SD, Fewer DP, Talla E, Calteau A, Cai F, Tandeau de Marsac N, Rippka
492 R, Herdman M, Sivonen K, Coursin T, Laurent T, Goodwin L, Nolan M, Davenport KW, Han CS, Rubin
493 EM, Eisen JA, Woyke T, Gugger M, Kerfeld CA. 2013. Improving the coverage of the cyanobacterial
494 phylum using diversity-driven genome sequencing. Proc Natl Acad Sci U S A 110:1053-8.
495 19. Schmelling NM, Lehmann R, Chaudhury P, Beck C, Albers S-VV, Axmann IMM, Wiegard A. 2017.
496 Minimal Tool Set for a Prokaryotic Circadian Clock. bioRxiv doi:10.1101/075291.

497 20. Axmann IM, Hertel S, Wiegard A, Dorrich AK, Wilde A. 2014. Diversity of KaiC-based timing systems
498 in marine Cyanobacteria. Mar Genomics 14C:3-16.
499 21. Aoki S, Onai K. 2009. Circadian clocks of *Synechocystis* sp. strain PCC 6803, *Thermosynechococcus*
500 *elongatus*, *Prochlorococcus* spp., *Trichodesmium* spp. and other species, p 259-282. *In* Ditty JL,
501 Mackey SR, Johnson CH (ed), Bacterial circadian programs. Springer Berlin Heidelberg.
502 22. Dvornyk V, Vinogradova O, Nevo E. 2003. Origin and evolution of circadian clock genes in
503 prokaryotes. Proc Natl Acad Sci U S A 100:2495-500.
504 23. Wiegard A, Dörrich AK, Deinzer HT, Beck C, Wilde A, Holtzendorff J, Axmann IM. 2013. Biochemical
505 analysis of three putative KaiC clock proteins from *Synechocystis* sp. PCC 6803 suggests their
506 functional divergence. Microbiology 159:948-958.
507 24. Johnson CH, Egli M. 2014. Metabolic compensation and circadian resilience in prokaryotic
508 cyanobacteria. Annu Rev Biochem 83:221-47.
509 25. Holtzendorff J, Partensky F, Mella D, Lennon JF, Hess WR, Garczarek L. 2008. Genome streamlining
510 results in loss of robustness of the circadian clock in the marine cyanobacterium *Prochlorococcus*
511 *marinus* PCC 9511. J Biol Rhythms 23:187-99.
512 26. Kanesaki Y, Shiwa Y, Tajima N, Suzuki M, Watanabe S, Sato N, Ikeuchi M, Yoshikawa H. 2012.
513 Identification of substrain-specific mutations by massively parallel whole-genome resequencing of
514 Synechocystis sp. PCC 6803. DNA Res 19:67-79.
515 27. Kucho K-i, Okamoto K, Tsuchiya Y, Nomura S, Nango M, Kanehisa M, Ishiura M. 2005. Global
516 analysis of circadian expression in the cyanobacterium *Synechocystis* sp. strain PCC 6803. J
517 Bacteriol 187:2190-9.
518 28. Beck C, Hertel S, Rediger A, Lehmann R, Wiegard A, Kolsch A, Heilmann B, Georg J, Hess WR,
519 Axmann IM. 2014. A daily expression pattern of protein-coding genes and small non-coding RNAs
520 in Synechocystis sp. PCC 6803. Appl Environ Microbiol doi:10.1128/AEM.01086-14.

521 29. van Alphen P, Hellingwerf KJ. 2015. Sustained Circadian Rhythms in Continuous Light in
522 Synechocystis sp. PCC6803 Growing in a Well-Controlled Photobioreactor. PLoS One 10:e0127715.
523 30. Saha R, Liu D, Hoynes-O'Connor A, Liberton M, Yu J, Bhattacharyya-Pakrasi M, Balassy A, Zhang F,
524 Moon TS, Maranas CD, Pakrasi HB. 2016. Diurnal Regulation of Cellular Processes in the
525 Cyanobacterium Synechocystis sp. Strain PCC 6803: Insights from Transcriptomic, Fluxomic, and
526 Physiological Analyses. MBio 7.
527 31. Axmann IM, Dühring U, Seeliger L, Arnold A, Vanselow JT, Kramer A, Wilde A. 2009. Biochemical
528 evidence for a timing mechanism in *Prochlorococcus*. J Bacteriol 191:5342-7.
529 32. Ma P, Mori T, Zhao C, Thiel T, Johnson CH. 2016. Evolution of KaiC-Dependent Timekeepers: A
530 Proto-circadian Timing Mechanism Confers Adaptive Fitness in the Purple Bacterium
531 Rhodopseudomonas palustris. PLoS Genet 12:e1005922.
532 33. Mukaiyama A, Ouyang D, Furuike Y, Akiyama S. 2019. KaiC from a cyanobacterium Gloeocapsa sp.
533 PCC 7428 retains functional and structural properties required as the core of circadian clock
534 system. International Journal of Biological Macromolecules 131:67-73.
535 34. Diamond S, Jun D, Rubin BE, Golden SS. 2015. The circadian oscillator in Synechococcus elongatus
536 controls metabolite partitioning during diurnal growth. Proc Natl Acad Sci U S A 112:E1916-25.
537 35. Welkie DG, Rubin BE, Diamond S, Hood RD, Savage DF, Golden SS. 2019. A Hard Day’s
538 Night: Cyanobacteria in Diel Cycles. Trends Microbiol 27:231-242.
539 36. Pattanayak G, Rust MJ. 2014. The cyanobacterial clock and metabolism. Curr Opin Microbiol 18:90-
540 5.
541 37. Woelfle MA, Ouyang Y, Phanvijhitsiri K, Johnson CH. 2004. The adaptive value of circadian clocks:
542 an experimental assessment in cyanobacteria. Curr Biol 14:1481-6.
543 38. Dorrich AK, Mitschke J, Siadat O, Wilde A. 2014. Deletion of the Synechocystis sp. PCC 6803
544 kaiAB1C1 gene cluster causes impaired cell growth under light-dark conditions. Microbiology
545 doi:10.1099/mic.0.081695-0.

546 39. Kobler C, Schultz SJ, Kopp D, Voigt K, Wilde A. 2018. The role of the Synechocystis sp. PCC 6803
547 homolog of the circadian clock output regulator RpaA in day-night transitions. Mol Microbiol
548 110:847-861.
549 40. Ungerer J, Wendt KE, Hendry JI, Maranas CD, Pakrasi HB. 2018. Comparative genomics reveals the
550 molecular determinants of rapid growth of the cyanobacterium Synechococcus elongatus UTEX
551 2973. Proc Natl Acad Sci U S A doi:10.1073/pnas.1814912115.
552 41. Welkie DG, Rubin BE, Chang YG, Diamond S, Rifkin SA, LiWang A, Golden SS. 2018. Genome-wide
553 fitness assessment during diurnal growth reveals an expanded role of the cyanobacterial circadian
554 clock protein KaiA. Proc Natl Acad Sci U S A 115:E7174-e7183.
555 42. Diamond S, Rubin BE, Shultzaberger RK, Chen Y, Barber CD, Golden SS. 2017. Redox crisis underlies
556 conditional light-dark lethality in cyanobacterial mutants that lack the circadian regulator, RpaA.
557 Proc Natl Acad Sci U S A 114:E580-e589.
558 43. Anderson SL, McIntosh L. 1991. Light-activated heterotrophic growth of the cyanobacterium
559 Synechocystis sp. strain PCC 6803: a blue-light-requiring process. J Bacteriol 173:2761-7.
560 44. Dörrich AK, Wilde A. 2015. Spot Assays for Viability Analysis of Cyanobacteria. Bio-protocol
561 5:e1574.
562 45. Loza-Correa M, Sahr T, Rolando M, Daniels C, Petit P, Skarina T, Gomez Valero L, Dervins-Ravault
563 D, Honore N, Savchenko A, Buchrieser C. 2014. The Legionella pneumophila kai operon is
564 implicated in stress response and confers fitness in competitive environments. Environ Microbiol
565 16:359-81.
566 46. Snijder J, Burnley RJ, Wiegard A, Melquiond AS, Bonvin AM, Axmann IM, Heck AJ. 2014. Insight
567 into cyanobacterial circadian timing from structural details of the KaiB-KaiC interaction. Proc Natl
568 Acad Sci U S A 111:1379-84.

569 47. Hitomi K, Oyama T, Han S, Arvai AS, Getzoff ED. 2005. Tetrameric architecture of the circadian
570 clock protein KaiB. A novel interface for intermolecular interactions and its impact on the circadian
571 rhythm. J Biol Chem 280:19127-35.
572 48. Snijder J, Schuller JM, Wiegard A, Lossl P, Schmelling N, Axmann IM, Plitzko JM, Forster F, Heck AJ.
573 2017. Structures of the cyanobacterial circadian oscillator frozen in a fully assembled state. Science
574 355:1181-1184.
575 49. Tseng R, Goularte NF, Chavan A, Luu J, Cohen SE, Chang YG, Heisler J, Li S, Michael AK, Tripathi S,
576 Golden SS, LiWang A, Partch CL. 2017. Structural basis of the day-night transition in a bacterial
577 circadian clock. Science 355:1174-1180.
578 50. Chang YG, Cohen SE, Phong C, Myers WK, Kim YI, Tseng R, Lin J, Zhang L, Boyd JS, Lee Y, Kang S,
579 Lee D, Li S, Britt RD, Rust MJ, Golden SS, LiWang A. 2015. Circadian rhythms. A protein fold switch
580 joins the circadian oscillator to clock output in cyanobacteria. Science 349:324-8.
581 51. Espinosa J, Boyd JS, Cantos R, Salinas P, Golden SS, Contreras A. 2015. Cross-talk and regulatory
582 interactions between the essential response regulator RpaB and cyanobacterial circadian clock
583 output. Proc Natl Acad Sci U S A 112:2198-203.
584 52. Breeden L, Nasmyth K. 1985. Regulation of the yeast HO gene. Cold Spring Harb Symp Quant Biol
585 50:643-50.
586 53. Trautmann D, Voss B, Wilde A, Al-Babili S, Hess WR. 2012. Microevolution in cyanobacteria: re-
587 sequencing a motile substrain of Synechocystis sp. PCC 6803. DNA Res 19:435-48.
588 54. Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Generic assignments, strain
589 histories and properties of pure cultures of cyanobacteria. J Gen Microbiol 111:1-61.

590

591 **Figure legends:**

592 **Figure 1: KaiC3$_{6803}$ showed decreased ATP synthase activity compared to KaiC$_{7942}$.** Prior to the
593 experiment, KaiC$_{7942}$ was incubated for 2 weeks at 4 °C or overnight at 30 °C to generate fully
594 phosphorylated (P-KaiC$_{7942}$) and dephosphorylated (NP-KaiC$_{7942}$) protein, respectively (Fig. S1). **A:**
595 Representative autoradiograph of separation of [γ-^{32}P]ATP (product) and [α-^{32}P]ADP (substrate)
596 via thin layer chromatography after incubation with indicated KaiC proteins for 2h. Controls show
597 the ATP signal in the presence of an excess of cold ADP. For size control [γ-^{32}P]ATP and [α-^{32}P]ADP
598 were separated on the same cellulose F plate. **B:** Relative ATP signals after 2 hours incubation
599 displayed as percentage of all radioactive signals in the corresponding lane (mean with standard
600 deviation from two experiments, each analysed in duplicates). All values are normalised to the
601 relative ATP signal at 0h incubation time in the presence of an excess of cold ADP.

602 **Figure 2: ATPase activity of Strep-KaiC3$_{6803}$ and variants Strep-KaiC3$_{6803}$-DE and Strep-KaiC3$_{6803}$-**
603 **AA.** Strep-KaiC3$_{6803}$, Strep-KaiC3$_{6803}$-DE and Strep-KaiC3$_{6803}$-AA were incubated for 24 hours at 30
604 °C and ADP production per day and per Strep-KaiC3$_{6803}$ monomer was calculated. As a control we
605 used Strep-KaiC3$_{6803}$-catE1^{-}catE2^{-}, which lacks both ATPase motifs due to replacement of catalytic
606 glutamates 67,68, 310 and 311 with glutamine residues. For comparison ADP production by
607 KaiC$_{7942}$ was monitored for 24 or 48 hours. Strep-KaiC$_{7942}$ was dephosphorylated prior to the
608 experiment by incubation at 30°C overnight. Shown are mean values with standard deviation of
609 at least five replicates. Please note that we averaged all measurements for Strep-KaiC3$_{6803}$ WT at
610 30 °C and therefore repeatedly display them in Fig. 2, Fig. 4A and Fig. 4B.

611 **Figure 3. KaiC3$_{6803}$ interacts with KaiB3$_{6803}$ and the proteins of the main oscillator KaiC1$_{6803}$,**
612 **KaiB1$_{6803}$. A, B, C:** Yeast two-hybrid reporter strains carrying the respective bait and prey plasmids

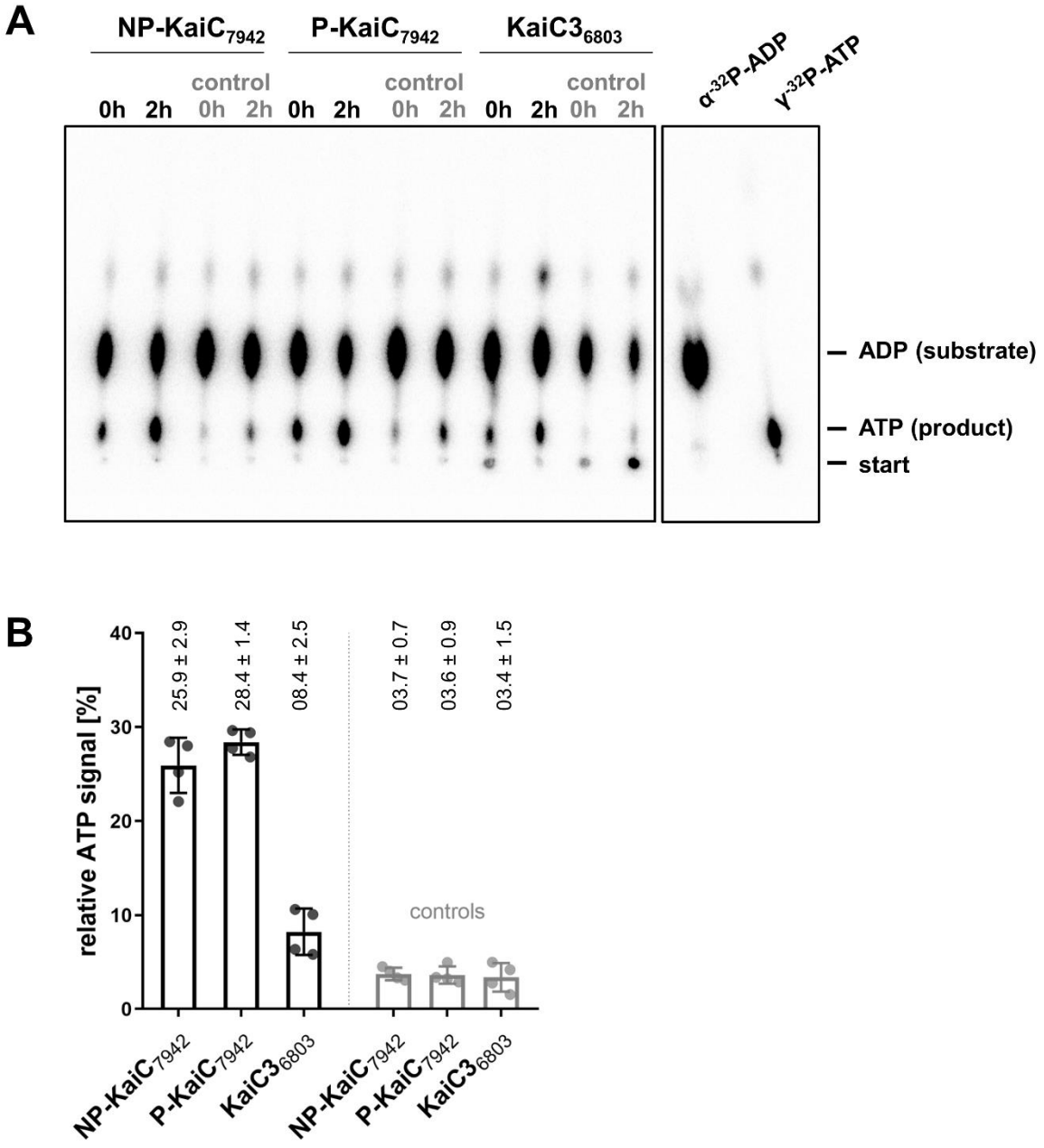
613 were selected by plating on complete supplement medium (CSM) lacking leucine and tryptophan
614 (-Leu -Trp). Physical interaction between bait and prey fusion proteins is indicated by a colour
615 change in the assays using 5-brom-4-chlor-3-indoxyl-β-D-galactopyranosid. **AD:** GAL4 activation
616 domain; BD, GAL4 DNA-binding domain. Shown are representative results of two replicates. For
617 clear presentation, spots were assembled from several assays (original scans are shown in Fig.
618 S2).

619 **Figure 4: ATPase activity of Strep-KaiC3$_{6803}$ is regulated by KaiB proteins and is temperature**
620 **dependent. A:** ATPase activity of Strep-KaiC3$_{6803}$ was decreased in the presence of KaiB1$_{6803}$ and
621 monomeric KaiB3$_{6803}$, but was not influenced by oligomeric KaiB3$_{6803}$ and by KaiA$_{6803}$. Shown are
622 mean values with standard deviation of at least three replicates. Controls show that KaiA$_{6803}$ and
623 KaiB$_{6803}$ proteins alone did not display ATPase activity (referring to Strep-KaiC3$_{6803}$ monomer
624 activity) **B:** ATPase activity of Strep-KaiC3$_{6803}$ is not temperature-compensated. Strep-KaiC3$_{6803}$
625 was incubated for 24 hours at the indicated temperatures and ADP production per day and
626 monomer Strep-KaiC3$_{6803}$ was calculated. Shown are mean values with standard deviation of at
627 least three replicates. Please note that we averaged all measurements for Strep-KaiC3$_{6803}$ WT at
628 30 °C and therefore repeatedly display them in Fig. 2, Fig. 4A and Fig. 4B.

629 **Figure 5. Reduced viability of the Δ*kaiC3* strain in complete darkness.** Viability of the
630 *Synechocystis* 6803 wild type, the *kaiAB1C1* deletion mutant (Δ*kaiAB1C1*) and the *kaiC3* deletion
631 mutant (Δ*kaiC3*) was tested under mixotrophic conditions in continuous light and dark conditions.
632 Strains were grown in liquid culture under constant light and different dilutions were spotted on
633 agar plates containing 0.2 % glucose. Plates were analysed after further incubation for 6 or 28

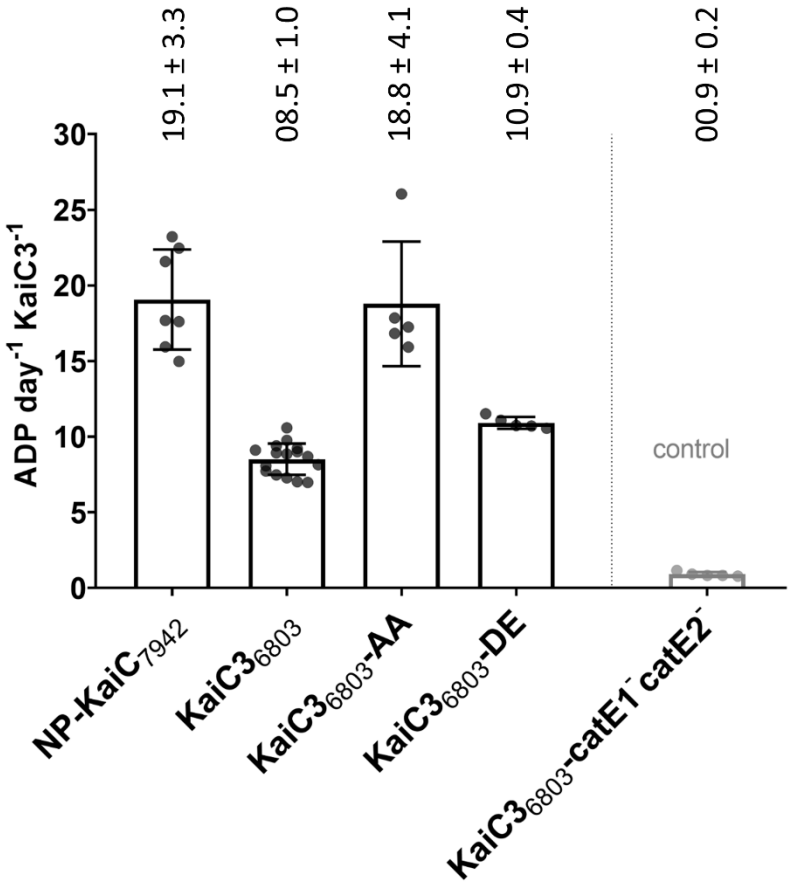
634 days of continuous light and darkness, respectively. A representative result of three independent
635 experiments is shown.

636 **Figure 6: Model of the crosstalk between the KaiAB1C1$_{6803}$ standard oscillator and KaiC3$_{6803}$ in**
637 ***Synechocystis* 6803.** KaiC1$_{6803}$ forms a standard oscillator together with KaiA$_{6803}$ and KaiB1$_{6803}$.
638 The KaiAB1C1$_{6803}$ oscillator allows temporal metabolic orchestration. KaiC3$_{6803}$ forms an
639 additional regulatory mechanism together with KaiB3$_{6803}$. The two systems intertwine by
640 interactions of the KaiC$_{6803}$ proteins with each other and the non-corresponding KaiB$_{6803}$
641 homologs. Solid lines indicate experimentally verified physical interactions (this paper and
642 Wiegard *et al*.(23)), dotted arrows indicate stimulation of phosphorylation (23) and dotted lines
643 with bars show inhibition of ATPase activity.



644

645 Figure 1



646

647 Figure 2

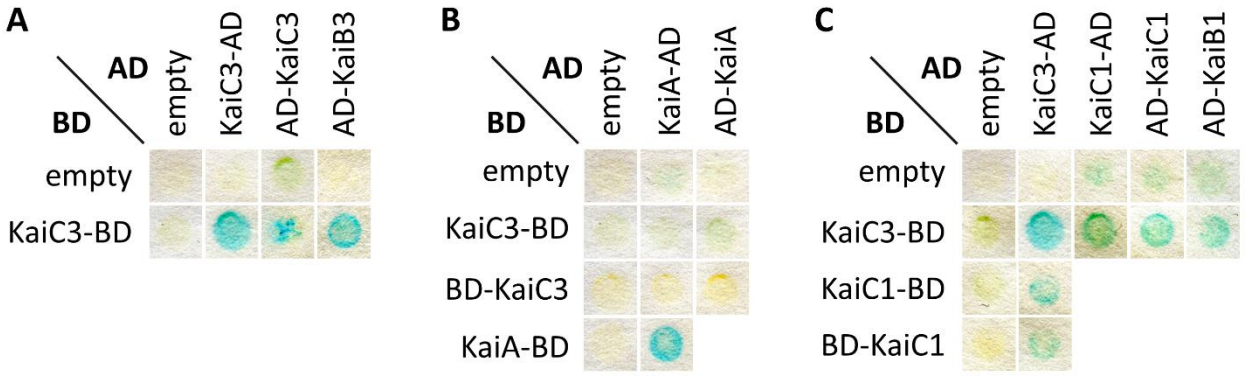
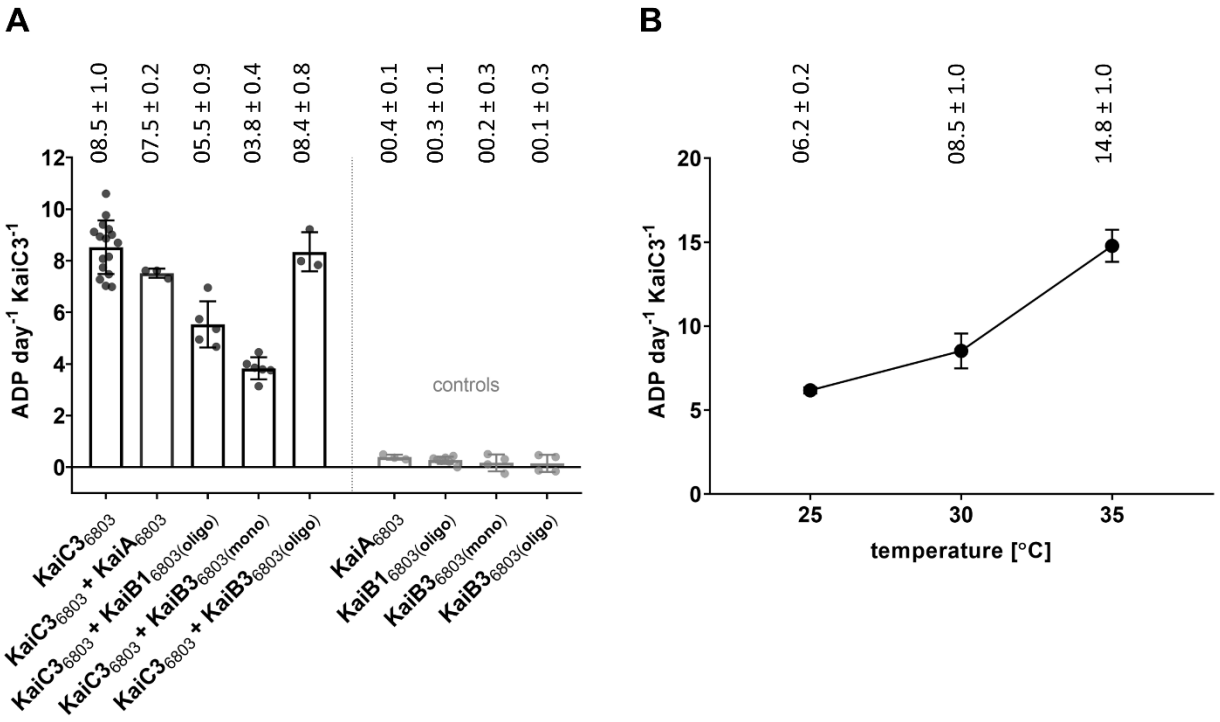
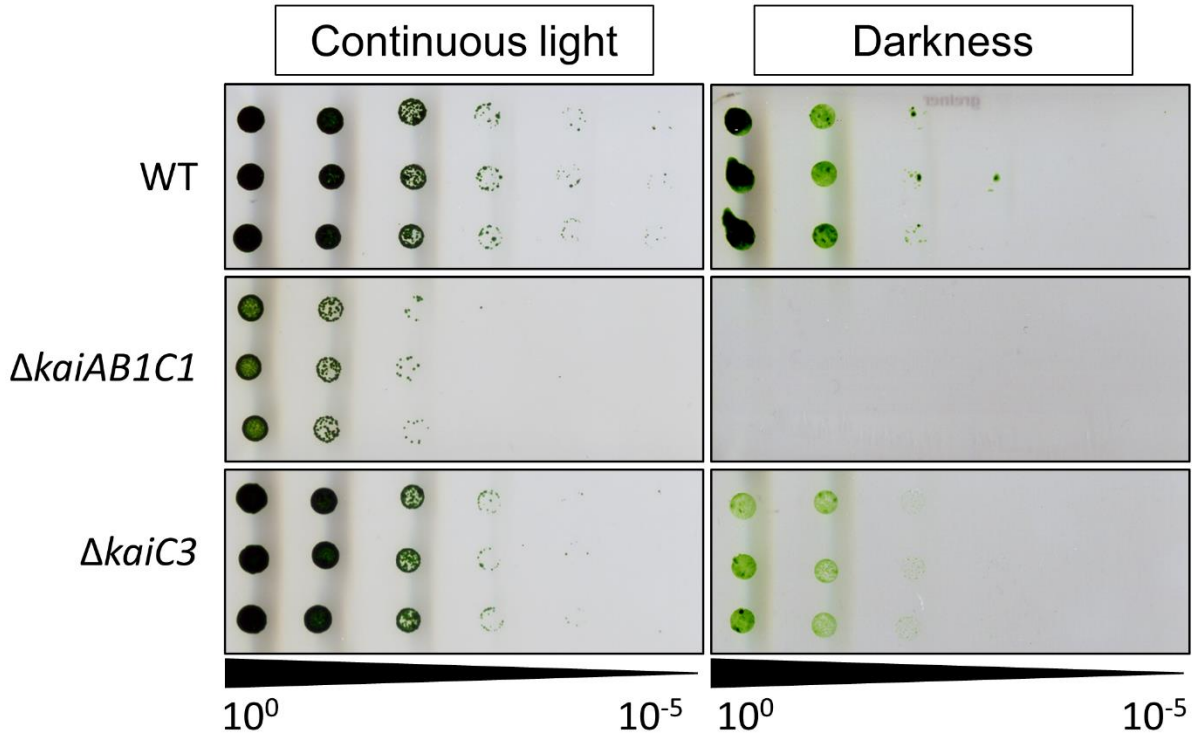

A
AD
BD
empty
KaiC3-AD
AD-KaiC3
AD-KaiB3
empty
KaiC3-BD
B
AD
BD
empty
KaiA-AD
AD-KaiA
empty
KaiC3-BD
BD-KaiC3
KaiA-BD
C
AD
BD
empty
KaiC3-AD
KaiC1-AD
AD-KaiC1
AD-KaiB1
empty
KaiC3-BD
KaiC1-BD
BD-KaiC1

Figure 3



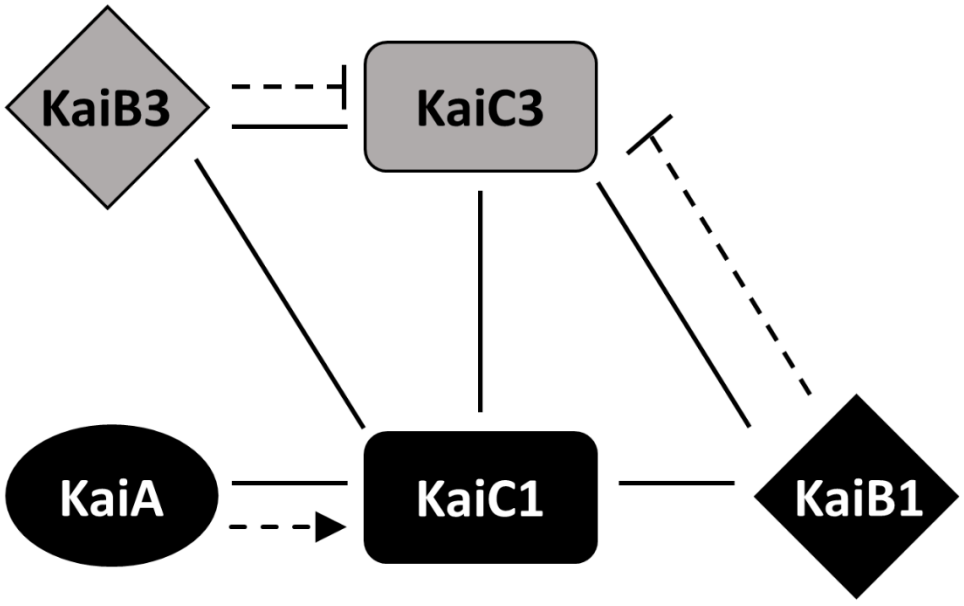
650

651 Figure 4



652

653 Figure 5



654

655 Figure 6