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Roles for ClpXP in regulating the circadian clock in *Synechococcus elongatus*

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In cyanobacteria, the KaiABC posttranslational oscillator drives circadian rhythms of gene expression and controls the timing of cell division. The Kai-based oscillator can be reconstituted in vitro, demonstrating that the clock can run without protein synthesis and degradation; however, protein degradation is known to be important for clock function in vivo. Here, we report that strains deficient in the ClpXP1P2 protease have, in addition to known long-period circadian rhythms, an exaggerated ability to synchronize with the external environment (reduced "jetlag") compared with WT strains. Deletion of the ClpX chaperone, but not the protease subunits ClpP1 or ClpP2, results in cell division defects in a manner that is dependent on the expression of a dusk-peaking factor. We propose that chaperone activities of ClpX are required to coordinate clock control of cell division whereas the protease activities of the ClpXP1P2 complex are required to maintain appropriate periodicity of the clock and its synchronization with the external environment.

circadian rhythms | ClpXP protease | cyanobacteria | cell division

Circadian rhythms, regulated by a 24-h biological clock, enable the coordination of biological activity over the course of the day and facilitate adaptation to daily environmental changes in diverse organisms (1). Cyanobacteria currently represent the only prokaryotic system for which the molecular details of the circadian clock have been elucidated. In *Synechococcus elongatus* PCC 7942, the premier model organism for the study of the cyanobacterial circadian clock, a core oscillator consisting of KaiA, KaiB, and KaiC regulates global patterns of gene expression (2, 3), compaction of the chromosome (4, 5), and the timing of cell division (6–8). Unlike the circadian oscillators found in eukaryotic systems, which employ a transcription–translation feedback loop, the cyanobacterial core oscillator functions posttranslationally. KaiC is an autokinase, autophosphatase, and ATPase; 24-h rhythms of KaiC phosphorylation and dephosphorylation on neighboring serine 431 and threonine 432 residues, facilitated via rhythmic associations with KaiA and KaiB, drive ∼24-h rhythms (9, 10). KaiA associates with KaiC to promote KaiC autophosphorylation during the day, and KaiB associates with the fully phosphorylated form of KaiC, as well as an autoinhibited conformation of KaiA, promoting the autophosphatase activity of KaiC at night. By dawn, KaiC has returned to the dephosphorylated state, and the cycle can begin anew (11, 12).

Rather than sense light directly, the clock proteins are sensitive to energy and redox-active metabolites that function as a day/night proxy to synchronize the circadian clock. Circadian input kinase A (CikA), a protein that plays major roles in both input to and output from the clock, and core oscillator component KaiA bind directly to the oxidized form of quinones, which signals the onset of darkness (13–15). KaiC is sensitive to the ATP/ADP ratio in the cell, which drops gradually during the course of the night (16). The ATP/ADP ratio serves to signal the duration of the nighttime portion of the cycle. The laboratory test for the oscillator's ability to synchronize with the environment in vivo is a phase-resetting assay, in which cells kept in conditions of constant light are exposed to a 5-h dark pulse. Depending on the time of day that this dark pulse is provided, the oscillator will respond in a predictable fashion by altering the phase of the circadian rhythm to peak either earlier (phase advance) or later (phase delay), or by having no effect on phase (17). CikA was determined to be a critical component to the input pathway because *cikA* mutants lack the ability to reset the phase of the rhythm after such a dark pulse (18).

The oscillator regulates clock-controlled activities via an output pathway consisting of histidine kinase SasA and cognate response regulator RpaA, as well as CikA, which functions as a phosphatase on RpaA (19, 20). Association with KaiC or the KaiABC ternary complex promotes the activities of SasA and CikA, respectively, resulting in rhythmic phosphorylation of RpaA (19), which in turn promotes global rhythms of gene expression via a transcriptional cascade (21). Moreover, the clock controls the timing of cell division. Cell division is inhibited for ∼6 h in the early subjective night (i.e., night portion of continuous light conditions) in a clock-dependent manner (6, 22). While the details of how the clock regulates cell division are not well understood, the current model proposes that RpaA regulates the expression of a factor or set of factors that inhibit the bacterial tubulin homolog, FtsZ, from forming the cytokinetic ring (7).

While rhythmic phosphorylation in the Kai-based oscillator can be reconstituted in vitro, without the need for de novo protein synthesis or degradation, the clock in vivo is subject to many additional layers of regulation. KaiB and KaiC exhibit protein abundance rhythms where peak levels are achieved just after dusk (23), and the over- or underexpression of any of the *kai* genes results in loss of rhythmicity (3, 24). Additionally, the subcellular localization patterns of KaiA and KaiC oscillate over the course of the day, with enhanced localization to the poles of

Significance

Protein degradation is critical for modulating cellular responses to the environment. Here, we show that proteome remodeling by ClpX, ClpP1, and ClpP2 controls circadian clock function in *Synechococcus elongatus* PCC 7942. The results suggest that protein degradation by the ClpXP1P2 protease is required to maintain normal periodicity of the circadian clock and to limit the temporal range over which the phase can be reset in response to darkness. Moreover, ClpX-mediated protein unfolding, independent of its functions with the ClpP protease subunits, is required to relieve a clock-induced cell division checkpoint. Together, the results suggest that the circadian clock and ClpX act together to time the window in every 24-h day in which cell division is inhibited.

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cells observed at night (25). Moreover, deletion or overexpression of the genes encoding the ClpXP protease results in circadian period lengthening (26, 27), suggesting a need for protein degradation in regulating the circadian clock in vivo.

Regulated proteolysis by ClpXP has been shown to be important for a wide variety of biological processes, including temporal programs, in various bacterial systems (28–31). The ClpX chaperone is responsible for substrate recognition and has an ATPase activity that supplies energy for unfolding substrates; the unfolded substrates are fed into the serine-type peptidase proteolytic core, comprising ClpP subunits (32). ClpX also is known to have ClpP-independent functions in which it can prevent protein aggregation and disassemble preformed complexes or aggregates (33). The *S. elongatus* genome includes one *clpX* homolog and three *clpP* peptidase paralogs (*clpP1*, *clpP2*, and *clpP3*). Earlier studies found that ClpP1 and ClpP2 form a proteolytic chamber guarded by ClpX; ClpP3 is proposed to interact with ClpR, which is similar to ClpP but lacks the catalytic triad necessary for proteolytic activity, and to be guarded by an alternative chaperone and ATPase, ClpC (34). Deletion or depletion of *clpX*, *clpP2*, or *clpP1* results in period lengthening as does overexpression of ClpP3 (26, 27), an essential gene (35). Each of the *clpP* paralogs is expressed independently of each other. The *clpP2*, *clpX* (*clpP2X*) operon is one of ∼100 transcripts that are directly regulated by RpaA (21), implicating roles for *clpP2* and *clpX* in clock function in vivo.

Here, we report that *clp* genes (*clpX*, *clpP1*, and *clpP2*) play previously unexpected roles in regulating the circadian clock in *S. elongatus*. In addition to known effects on modulating circadian period, our data suggest that the ClpXP protease, consisting of ClpX-ClpP1-ClpP2, governs the synchronization of the clock with the external environment. Specifically, ClpXP-mediated proteome changes that occur at night temper the range over which the oscillator phase can be reset. Moreover, the cell elongation phenotype of *clpX* mutants suggests that ClpX chaperone activity plays roles in regulating circadian gating of cell division in a manner that is independent of the peptidase activities. Taken together, the results suggest an intimate connection between the circadian clock and ClpXP-related activities, which are required for clock control of cell division and the ability to synchronize with the external environment.

Results

***clpXP* Gene Products Are Required for Normal Circadian Function.** Of the three *clpP* paralogs present in the *S. elongatus* genome, *clpP2* is of particular interest as it is expressed as part of an operon with *clpX* (36) (Fig. 1*A*) and shares the most similarity (83%) with the highly studied ClpP from *Escherichia coli*, compared with 81% for ClpP1 and 80% for ClpP3. In accordance with previously published results, we found that inactivation of *clpX*, *clpP1*, or *clpP2* results in long-period rhythms of gene expression as monitored using a bioluminescence reporter (26, 27) (Fig. 1*B* and *SI Appendix*, Fig. S1*A*), corroborating roles for the *clp* gene products in regulating the circadian clock. These defects could be complemented by expressing the missing gene *in trans* from a neutral site (NS) in the chromosome (*SI Appendix*, Fig. S1*A*). A previous report suggested that the *clpP2X* operon of *S. elongatus* might be essential for viability as homogeneous segregation of mutant alleles in this polypoid organism could not be obtained (26). However, subsequent studies have found that *clpP2* and *clpX* are not essential (27, 35, 37). We took care to test transformants for homogeneous segregation of mutant alleles and for the appearance of potential second-site suppressors. Deletion of the *clpP2X* operon resulted in a long-period rhythm of gene expression; however, the effect of deleting both genes was not additive, suggesting that *clpP2* and *clpX* act in the same pathway with regard to modulating circadian period (Fig. 1*B* and *SI Appendix*, Fig. S1*A*). We were unable to obtain segregated *clpP1*, *clpP2* double mutants or *clpX*, *clpP1* double mutants; this outcome is consistent with a synthetic lethality in which one ClpP peptidase subunit can compensate for the other, but also suggests that ClpP1 and ClpP2 have functions independent of each other.

As regulated proteolysis has been shown to be important for modulating cellular responses to various environmental stimuli, we sought to determine whether the *clp* gene products are required for synchronization of the circadian clock with the external environment. Strains that lack *clpX* displayed enhanced resetting compared with WT (*SI Appendix*, Fig. S1 *B* and *C*), and strains lacking *clpP1* behaved similar to WT (*SI Appendix*, Fig. S1*D*). However, strains lacking *clpP2* had inconsistent resetting properties, in which about half of the samples behaved similar to WT and the other half failed to reset the rhythm after the dark pulse (*SI Appendix*, Fig. S1 *E* and *F*). A polar effect on downstream gene *clpX* is unlikely, as ClpX levels are unaltered in a Δ*clpP2* mutant background (Fig. 1*D*). We sought to determine roles *clpP2* might play in the pathway that resets the circadian clock by deleting both *clpP2* and *clpX*. Strikingly, we observed that deletion of the *clpP2X* operon results in an exaggerated hyper-resetting phenotype in which the mutant strains consistently reset the phase of the oscillation by 11.5 to 12 h after receiving a dark pulse, compared with WT strains that reset 4 to 5 h with the same dark pulse (Fig. 1*C*). The hyper-resetting phenotype can be complemented by expressing *clpP2X* from the native promoter in neutral site 1 (NS1) (Fig. 1*E*). Expression of *clpP2* or *clpX* individually in a Δ*clpP2X* background resulted in only partial suppression (Fig. 1*E*). The fact that this phenotype is observed only when both *clpP2* and *clpX* are deleted suggests potential cross-talk with other chaperones (ClpC) or peptidases (ClpP1 or ClpP3). The resetting phenotype observed by the Δ*clpP2X* strain was most pronounced for phase advances but was also observed to a lesser extent for phase delays (Fig. 1*F*). The hyper-resetting phenotype was not a side effect of the long circadian period of this strain because the Δ*clpX* mutant strain had a similarly long-period rhythm of gene expression but a more modest resetting phenotype (Fig. 1*F*). Taken together, these data suggest that changes in the cellular proteome are required to facilitate, and specifically to limit, the magnitude to which cells can reset the phase of the oscillator in response to environmental stimuli. We propose that a factor or set of factors, defined here as resetting factor (RF), that is necessary for resetting is targeted for degradation by the ClpX-ClpP1-ClpP2 protease.

Circadian and Environmental Regulation of ClpP2 Polar Localization. We tracked ClpP2 localization patterns over the course of the day to gain a more comprehensive understanding of how the Clp-protease system operates in the cell. As in other bacterial systems (38–40), ClpP2 localizes as a focus at or near one pole in *S. elongatus* (25). Despite a similar localization pattern to KaiA and KaiC, ClpP2 does not colocalize with the core oscillator components, suggesting that it does not serve as the mechanism by which KaiC localizes to the cell pole (25). Furthermore, ClpP2 polar localization was not dependent on the presence of *clpX* (*SI Appendix*, Fig. S2*A*). In a diel cycle, 12 h in light followed by 12 h in darkness (LD 12:12), ClpP2 localization oscillated, with low polar localization during the illuminated portions of the day and high at night in an otherwise WT background (Fig. 2*A*). Changes in ClpP2 localization were not a reflection of protein levels as abundance was equivalent at times where ClpP2 was diffuse and in a localized state (*SI Appendix*, Fig. S2*B*). However, this pattern was lost, with constitutively low polar localization observed, in either constant light conditions (Fig. 2*B*) or in a Δ*kaiC* mutant background in LD 12:12 (Fig. 2*A*). To determine if a dark stimulus is sufficient to induce ClpP2 localization, we entrained strains to two LD 12:12 cycles and then released into different light:dark cycles: L6:D12:L6 (Fig. 2*C*) or LD 6:6 (*SI Appendix*, Fig. S2*C*) and monitored ClpP2 localization in either a WT or in a Δ*kaiC* mutant background. We found that darkness is not sufficient to induce ClpP2 localization during the subjective day, but that light during

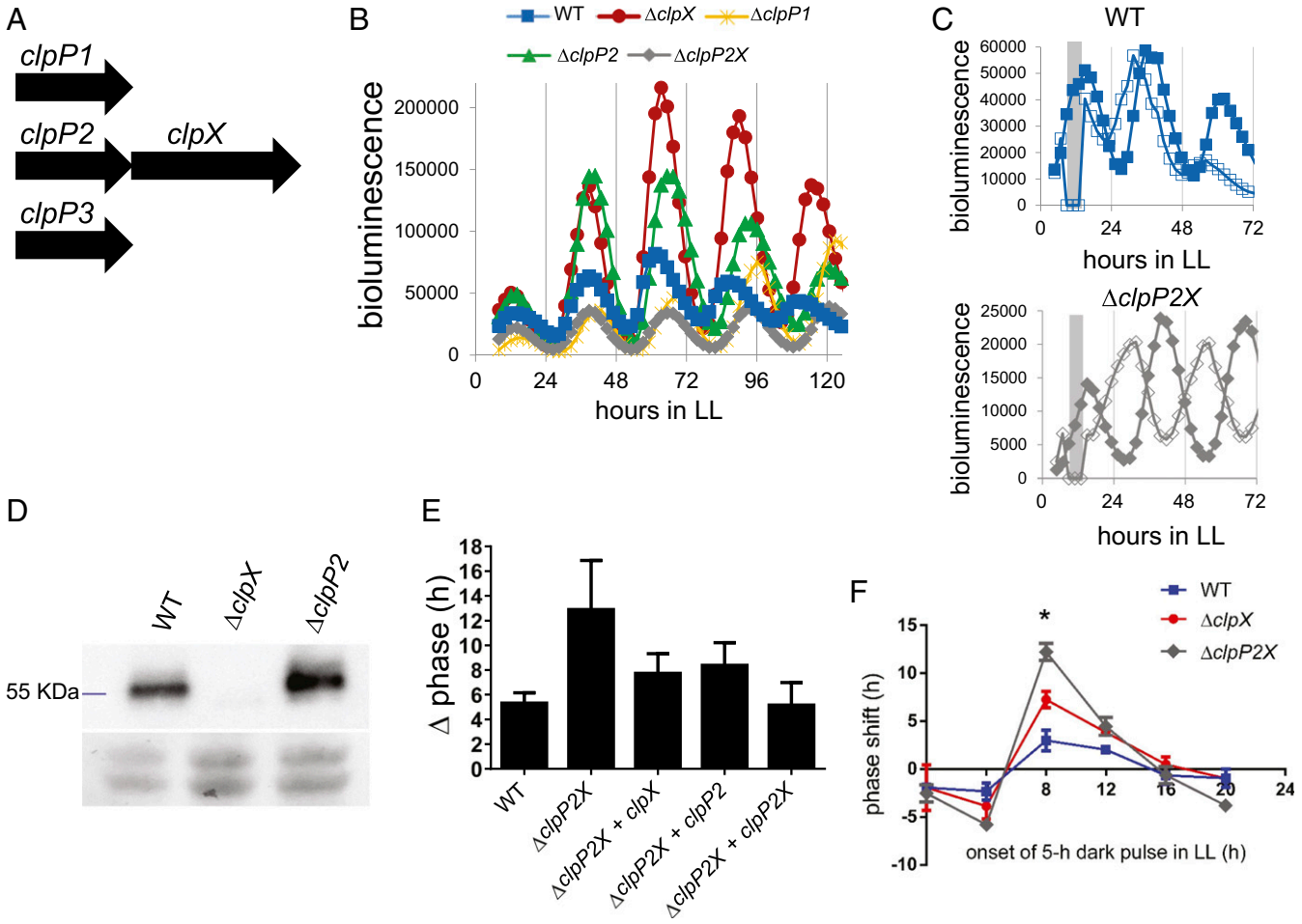


Fig. 1. Disruption of *clpX*, *clpP1*, or *clpP2* results in altered circadian phenotypes. (*A*) Schematic of gene organization for *clpX* and the three *clpP* paralogs in *S. elongatus* PCC 7942. (*B*) Bioluminescence output represented as counts per second from strains carrying P_{kaiB}-*luc* reporter. LL in *x* axis refers to constant light. Deletion of *clpX*, *clpP1*, *clpP2*, or the *clpP2X* operon results in long-period rhythms of gene expression compared with WT AMC1825; precise periods are reported in *SI Appendix*, Fig. S1*A*. (*C*) Bioluminescence output from strains carrying P_{kaiB}-*luc* reporter demonstrate that deletion of the *clpP2X* operon (*Bottom*) enables a 10-h phase shift of the circadian peak of gene expression, compared with the WT (AMC1825) 4-h shift (*Top*), in response to exposure to darkness for 5 h (shaded bar) after 8 h in constant light. (*D*) α-ClpX immunoblot demonstrates that ClpX (49.8 kDa) protein levels are not affected in a Δ*clpP2* mutant background compared with WT (AMC1825) (*Top*). (*Bottom*) Coomassie blue-stained gel loaded with the same samples. (*E*) Expression of *clpP2X* from a neutral site in the chromosome can complement the hyper-resetting phenotype of the Δ*clpP2X* mutant. Expression of either *clpX* or *clpP2* alone only partially suppresses the hyper-resetting phenotype, elicited in response to exposure to darkness for 5 h commencing after 8 h in constant light. WT is AMC2036. (*F*) Phase response curve for WT (AMC2036), Δ*clpX* and Δ*clpP2X* mutants. Strains are exposed to darkness for 5 h, commencing at the indicated time after the first day in constant light. Deletion of the *clpP2X* operon results in enhanced resetting for both phase advances and delays relative to WT whereas loss of *clpX* moderately enhances phase advances but not delays. Significance of the phase change in each strain at 8 h was calculated by Student's *t* test ($n = 4$). *$P < 0.05$ for all comparisons.

the subjective night is sufficient to eliminate ClpP2 polar localization. While it remains to be determined whether ClpP2 at the pole represents an active or inactive complex, taken together, these data suggest that the polar localization of ClpP2 is subject to regulation both by the circadian clock and external signals, also referred to as external coincidence (41).

ClpX Regulates Circadian Gating of Cell Division. In addition to defects in the circadian clock, *clpX* mutant cells, but not *clpP* mutants, displayed a cell division defect, where an elongated cell morphology was observed (Fig. 3*A*) (27). Although *clpX* is downstream of *clpP2* in a *clpP2X* operon, the distinct cell division phenotypes of their mutant alleles confirm *clpX* expression in a *clpP2* mutant background and further support the notion that *clpX* can be expressed independent of *clpP2* (37). Perhaps *clpP* mutants do not display a cell division defect because of redundancy, such that multiple *clpP* paralogs would need to be deleted before a cell division phenotype is observed. Alternatively, ClpP paralogs may function independently and are not required to regulate cell division as mutation of *clpP1* or *clpP2* individually resulted in circadian period defects (Fig. 1*B*). It was intriguing that *clpX* mutant cells have defects in both circadian periodicity, as well as cell division, as these phenotypes are observed in other clock mutant strains. In particular, strains that lack *cikA* have short-period rhythms of gene expression and elongated cell morphology (7, 42). While the details regarding clock control of cell division are not well understood, current models propose that the clock inhibits cell division by targeting the bacterial tubulin FtsZ, required to form the cytokinetic or Z-ring. The loss of CikA, leading to elevated levels of active, phosphorylated RpaA, extends the window during which the circadian clock inhibits cell division via inhibition of FtsZ, resulting in elongated cells (7, 21). This inhibition is proposed to occur via stoichiometric inhibition of FtsZ because a second of *ftsZ* expressed from the *S. elongatus* chromosome can rescue the cell morphology defect of a *cikA* mutant strain (7).

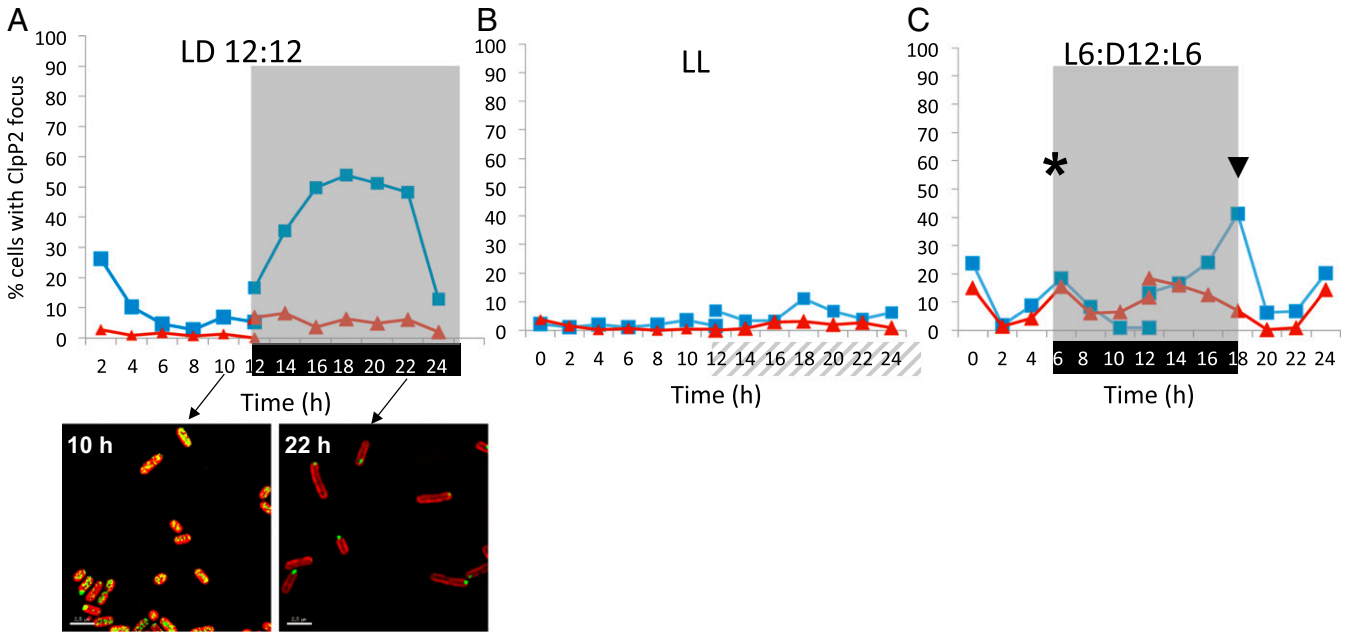


Fig. 2. ClpP2 polar localization is subject to circadian and diel regulation. The percentage of cells in the population that have a ClpP2 polar focus (*y* axis) is plotted as a function of time (*x* axis) in WT (AMC2466, blue squares) and in a Δ*kaiC* mutant background (red triangles). Cells were grown and entrained to two cycles of LD 12:12 and then released into (*A*) LD 12:12, dark (indicated by shaded box); fluorescent micrographs of cells expressing ClpP2-YFP (green) demonstrate ClpP2 localization at indicated time points 10 h after lights on or 22 h after lights on (10 h in the dark). Autofluorescence is shown in red. (Scale bars: 2.5 μm.) (*B*) LL, constant light; subjective night indicated by hatched box. (*C*) L6:D12:L6, 6 h light, followed by 12 h of dark and 6 h of light. * denotes when the lights were turned off; ClpP2 localization is not induced by a dark pulse in the subjective day but rather begins to increase as it would in the LD 12:12 condition. ▼marks when the lights were turned on, demonstrating that light during the subjective night is sufficient to eliminate ClpP2 polar localization.

FtsZ is a known target of the ClpXP protease in *E. coli* (43); it is possible that deletion of *clpX* results in the stabilization and aggregation of FtsZ, resulting in the elongated cell morphology we observed. Others have shown previously that gross overexpression of FtsZ, more than the levels required to suppress a Δ*cikA* mutant, in *S. elongatus* results in elongated cells (44). To investigate the mechanism by which ClpX affects cell division in *S. elongatus*, we determined FtsZ localization in strains lacking *clpX*. A full-length fluorescent fusion to FtsZ (45) was expressed either as the sole copy of FtsZ (1× *ftsZ*) or in addition to the endogenous gene (2× *ftsZ*). In the 1× *ftsZ* background, *clpX* mutant cells were elongated, FtsZ was localized in patchy clusters throughout the cell, and Z-rings were seen less often than in an otherwise WT background (Fig. 3*B*). In a 2× *ftsZ* background, WT cells displayed the expected short-cell phenotype (7), and the cell elongation typical of *clpX* mutant cells was suppressed; normal cell morphology was observed, although Z-ring formation was not quite normal, as off-center and askew rings could be observed (Fig. 3*B*). While the cell morphology phenotype of a *clpX* mutant was reversed by 2× expression of *ftsZ*, long-period rhythms of gene expression were still observed (*SI Appendix*, Fig. S3), supporting the notion that the circadian clock functions independently of cell division. Taken together, our data suggest that the stabilization of FtsZ is not causing the elongated cell morphology of a Δ*clpX* mutant, but rather that ClpX, potentially via interactions with ClpP peptidase subunits, inhibits a factor that inhibits FtsZ ring formation.

To test whether the pathway by which ClpX regulates cell division intersects with the clock-controlled pathway, we inactivated *clpX* in a variety of clock mutant backgrounds. While a *clpX*, *kaiC* double mutant exhibited elongated cells, deletion of either *kaiA* or *rpaA* in conjunction with *clpX* restored normal cell morphology (Fig. 4*A*). These results indicate that the clock and ClpX converge on a factor or set of factors to regulate cell division; however, it was unclear why deletion of *kaiC* is not sufficient to suppress the cell morphology of a *clpX* mutant as it suppresses the phenotype caused by deletion of *cikA* (7). To investigate potential roles of KaiC in ClpX-regulated control of cell division, we expressed KaiC phosphomimetics that represent each of the KaiC phospho-states in a *clpX* mutant background (*SI Appendix*, Fig. S4*A*). Intriguingly, expression of $kaiC^{ET}$, but none of the other KaiC phosphomimetics, suppressed the cell morphology defect of a *clpX* mutant (Fig. 4*B* and *SI Appendix*, Fig. S4*B*). $KaiC^{ET}$, which mimics KaiC phosphorylated on serine 431, occurring three fourths of the way through the KaiC phosphorylation cycle, represents the active signaling state of the clock (46). KaiC, phosphorylated on serine 431, is associated with the formation of the KaiABC nighttime complex, which engages CikA, activating CikA's phosphatase activity, via association with KaiB, and resulting in the dephosphorylation of RpaA (47). Expression of $KaiC^{ET}$, loss of *kaiA*, or loss of *rpaA* results in high constitutive expression of dawn-peaking genes, with expression levels occurring near the peak of a normal oscillation; conversely, dusk-peaking genes exhibit low constitutive expression, with expression levels occurring near the normal trough (46). Taken together, these results suggest that the clock and ClpX converge to regulate the expression and activity of a dusk-peaking gene(s) that functions to inhibit cell division, via FtsZ.

Peptidase Activity of ClpP1/ClpP2 Is Required to Modulate Resetting but Not Cell Division. We tested whether ClpX control of cell division occurs via association with the ClpP peptidase by expressing active-site mutant alleles of *clpP1* and *clpP2*. In *E. coli* mutation of the active site, serine to alanine results in a ClpP variant, referred to as $ClpP^{TRAP}$, that retains proteins translocated into its chamber by ClpX but is incapable of degrading them (28, 48). Expression of $clpP1^{TRAP}$ or $clpP2^{TRAP}$ alleles complemented the long-period phenotypes caused by loss of *clpP1* or *clpP2*, respectively (*SI Appendix*, Fig. S5 *A* and *B*). However, expression of both $clpP1^{TRAP}$ and $clpP2^{TRAP}$, as the only copies of *clpP1* or *clpP2* in the cell, phenocopied the long-period rhythms of gene expression exhibited by loss of either gene alone (Fig. 5*A*), indicating that proteolytic

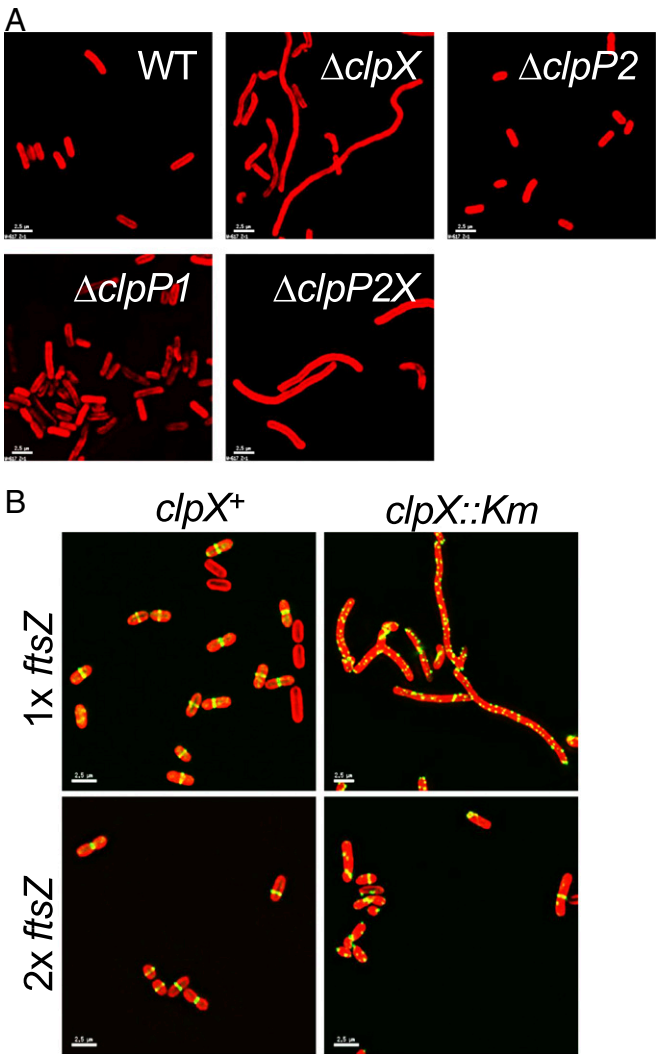


Fig. 3. Cell morphology of *clp* mutant cells. (*A*) Fluorescence micrographs showing autofluorescence (red) of WT (AMC1825) and *clp* mutant cells. (*B*) Fluorescence micrographs of cells expressing YFP-FtsZ (yellow) as either the only copy of *ftsZ* (1× *ftsZ*, *Top* row) or in addition to the endogenous copy (2× *ftsZ*, *Bottom* row) in either WT or *clpX*-disrupted cells. (Scale bars: 2.5 μm.)

activity in at least one of these ClpP isoforms is necessary and sufficient for normal periodicity. While we were unable to obtain completely segregated mutants deleted for both *clpP1* and *clpP2*, expression of either ClpP1^{TRAP} or ClpP2^{TRAP} alone allowed the double *clpP1*, *clpP2* double mutant to be constructed. This result suggests that a complex comprising ClpP1 and/or ClpP2 is essential for a function that does not require proteolytic activity. Long-period rhythms of gene expression were observed when either ClpP1^{TRAP} or ClpP2^{TRAP} alone was expressed in a Δ*clpP1*Δ*clpP2* mutant background (*SI Appendix*, Fig. S5*C*), reinforcing the conclusion that proteolytic activity is required by at least one of the ClpP1/ClpP2 proteins to support normal circadian period. Using Strep-tagged variants of either ClpP1 or ClpP2, we confirmed that ClpP1 and ClpP2 physically interact, such that ClpP2 or ClpP2^{TRAP} can pull down ClpP1 and vice versa (Fig. 5*B*). Strep tags do not interfere with the function of ClpP1 or ClpP2 as the tagged full-length fusion proteins complement, supporting WT rhythms of gene expression (*SI Appendix*, Fig. S5*A*). These results suggest that ClpP1 and ClpP2, as well as TRAP variants, interact to form mixed complexes. In contrast to the robust ClpP1–ClpP2 interaction, ClpP1 and ClpP2 weakly associated with ClpP3

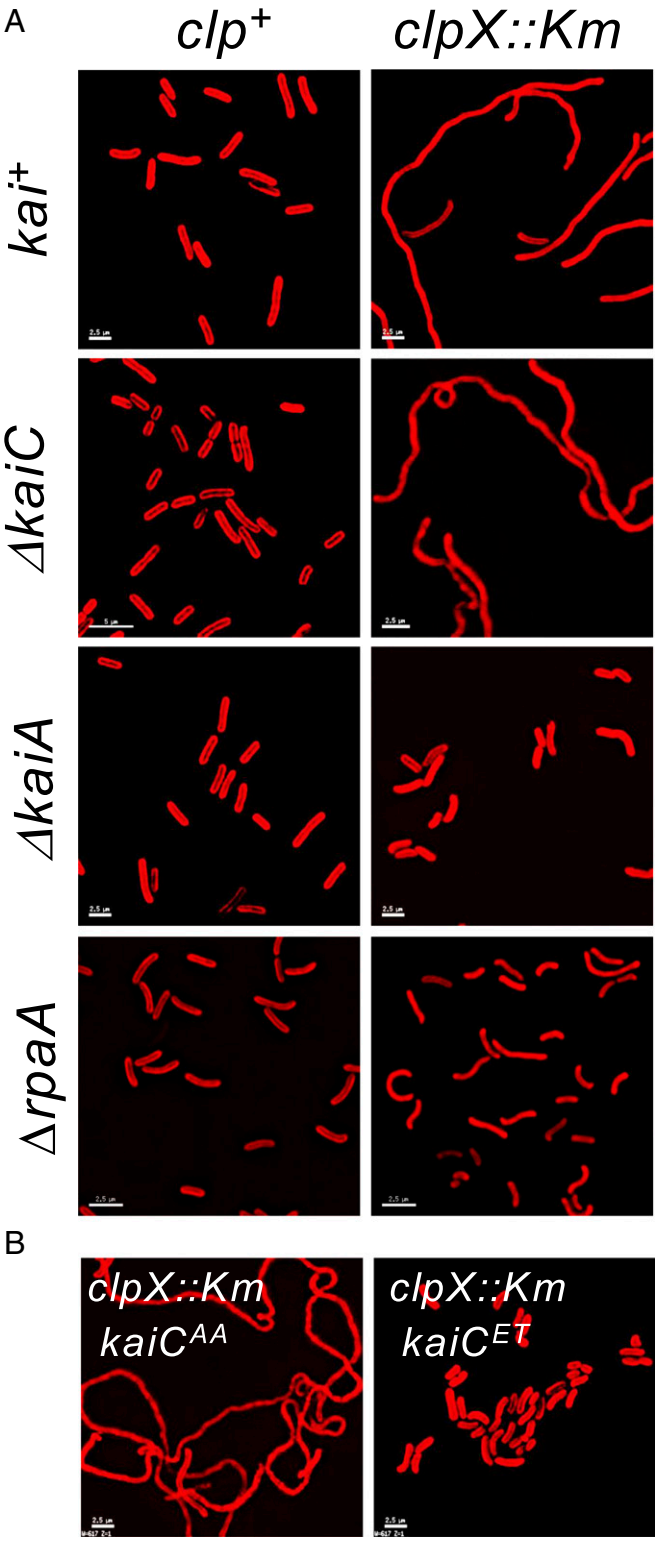
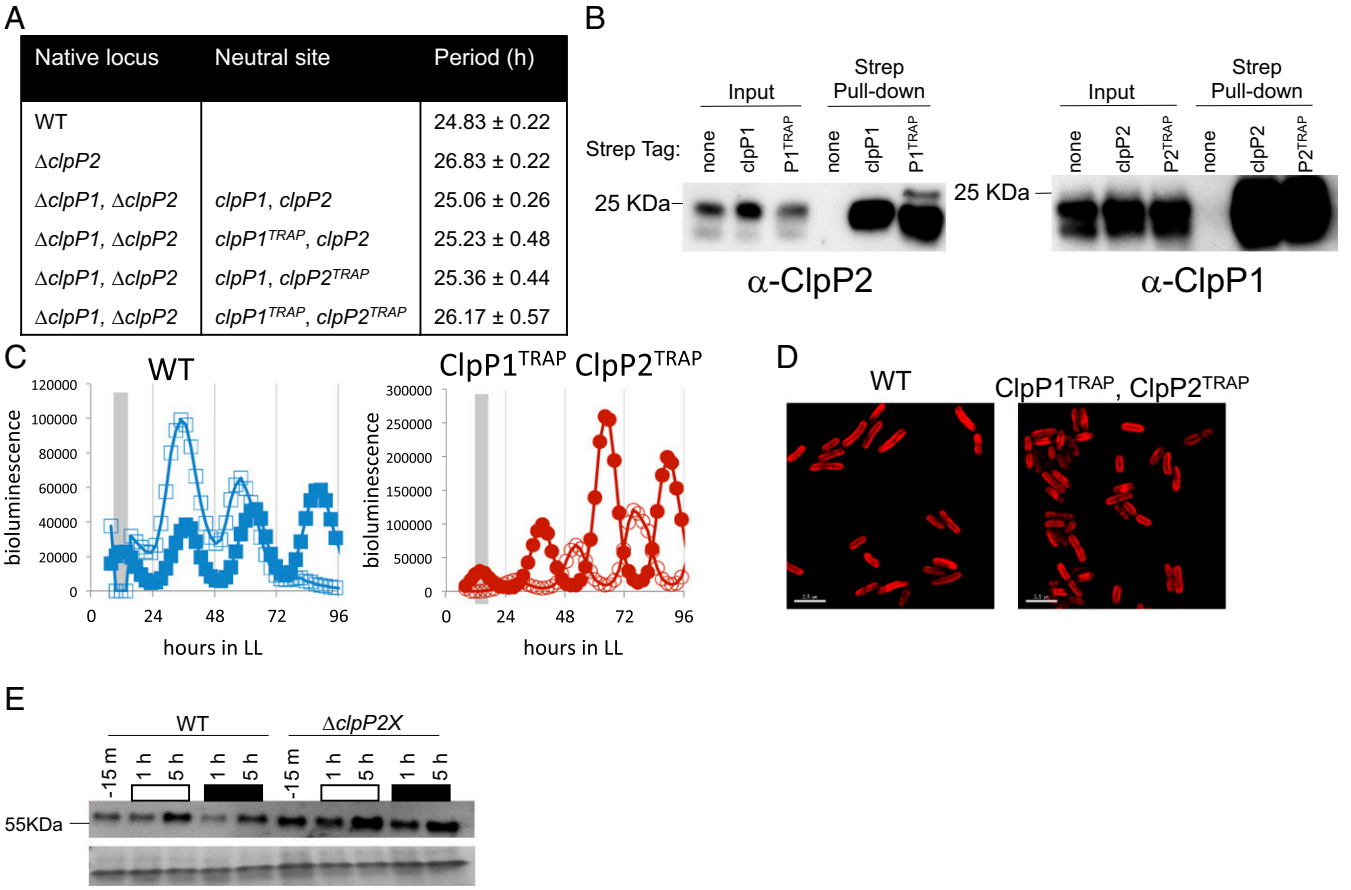


Fig. 4. Reduced expression of dusk-peaking genes can suppress the cell morphology defect of *clpX* mutant cells. Fluorescence micrographs showing autofluorescence (red) of (*A*) *clpX* disruption alone or in combination with Δ*kaiC*, Δ*kaiA*, or Δ*rpaA*. WT strain is AMC1825. (*B*) KaiC phosphomimetic mutants AA or ET expressed in a *clpX* mutant background. (Scale bars: 2.5 μm.)



Native locus	Neutral site	Period (h)
WT		24.83 ± 0.22
Δ*clpP2*		26.83 ± 0.22
Δ*clpP1*, Δ*clpP2*	*clpP1*, *clpP2*	25.06 ± 0.26
Δ*clpP1*, Δ*clpP2*	$clpP1^{TRAP}$, *clpP2*	25.23 ± 0.48
Δ*clpP1*, Δ*clpP2*	*clpP1*, $clpP2^{TRAP}$	25.36 ± 0.44
Δ*clpP1*, Δ*clpP2*	$clpP1^{TRAP}$, $clpP2^{TRAP}$	26.17 ± 0.57

Fig. 5. Protease function of ClpP1 or ClpP2 is required for normal circadian functions. (*A*) Circadian period analysis of strains carrying a P_{kaiB}-*luc* reporter. (*B*) Strep-tag pull down experiments. (*Left*) α-ClpP2 immunoblot of strains that express either no epitope tag (none), ClpP1-Strep, or $ClpP1^{TRAP}$-Strep; input represents whole cell extracts (1%) that were incubated with the Strep-Tactin XT–coated magnetic beads, showing that ClpP2 is present in lysates. Strep-pull down lanes show that ClpP2 (26.3 kDa) is retained only if ClpP1-Strep or $ClpP1^{TRAP}$-Strep is present. (*Right*) Demonstrates that ClpP1 (21.7 kDa) can copurify with either ClpP2-Strep or $ClpP2^{TRAP}$-Strep, but not if ClpP2 is not Strep-tagged (none); 8% of the reaction is loaded in each Strep-pull down lane. (*C*) Bioluminescence output, in counts per second, from strains carrying P_{kaiB}-*luc* reporter that received a 5-h resetting dark pulse (shaded bar) 8 h after release into constant light (open symbols) compared with cells kept in constant light (filled symbols). LL in *x* axis refers to constant light. WT samples (*Left*) shift the circadian peak of gene expression by 3.9 h while strains expressing both $ClpP1^{TRAP}$ and $ClpP2^{TRAP}$ phase shift by 11.6 h. (*D*) Fluorescence micrographs showing autofluorescence (red) of WT (AMC2036) and strains expressing $ClpP1^{TRAP}$ and $ClpP2^{TRAP}$ (AMC2474). (Scale bars: 2.5 μm.) (*E*) α-KaiC immunoblot (*Top*) of WT (AMC2036) and Δ*clpP2X* mutant cells that were entrained to two cycles of LD 12:12. Samples were taken from duplicate cultures on the first day after release into LL either 15 min before (−15 m) the dark pulse administered at 8 h into LL, 1 h or 5 h either into the dark pulse (dark bars) or in cells kept in conditions of constant light (open bars). KaiC (58 kDa) protein levels are 2× (1 h) and 1.4× (5 h) elevated in Δ*clpP2X* background compared with WT during a resetting dark pulse. *Bottom* shows Coomassie blue-stained gels loaded with the same samples.

(*SI Appendix*, Fig. S5*D*), suggesting that, while ClpP1 and ClpP2 function primarily together, there is likely cross-talk with components of the ClpC-ClpP3-ClpR protease. Protease activity of the ClpP1–ClpP2 complex is involved in the normal limitation of phase resetting because expression of $ClpP1^{TRAP}$, $ClpP2^{TRAP}$ as the only copies of ClpP1/ClpP2 showed a similar hyper-resetting phenotype to that of the Δ*clpP2X* mutant, altering the phase by 11.5 h in response to a dark pulse compared with 4.5 h in WT (Fig. 5*C*). However, the $ClpP1^{TRAP}$, $ClpP2^{TRAP}$ strain displayed normal cell morphology (Fig. 5*D*), suggesting that it is not the protease activity but rather the chaperone functions of ClpX that are required to regulate cell division. Together, these results suggest that ClpX plays three distinct roles in regulating circadian phenomena. First, ClpX functions in the ClpX-ClpP1-ClpP2 protease required for maintaining normal circadian periodicity. Secondly, ClpX acts specifically with ClpP2 to modulate the range of phase resetting in response to environmental stimuli that allow the clock to entrain with the environment. Lastly, ClpX, independent of its association with ClpP, removes a dusk-peaking inhibitor of FtsZ.

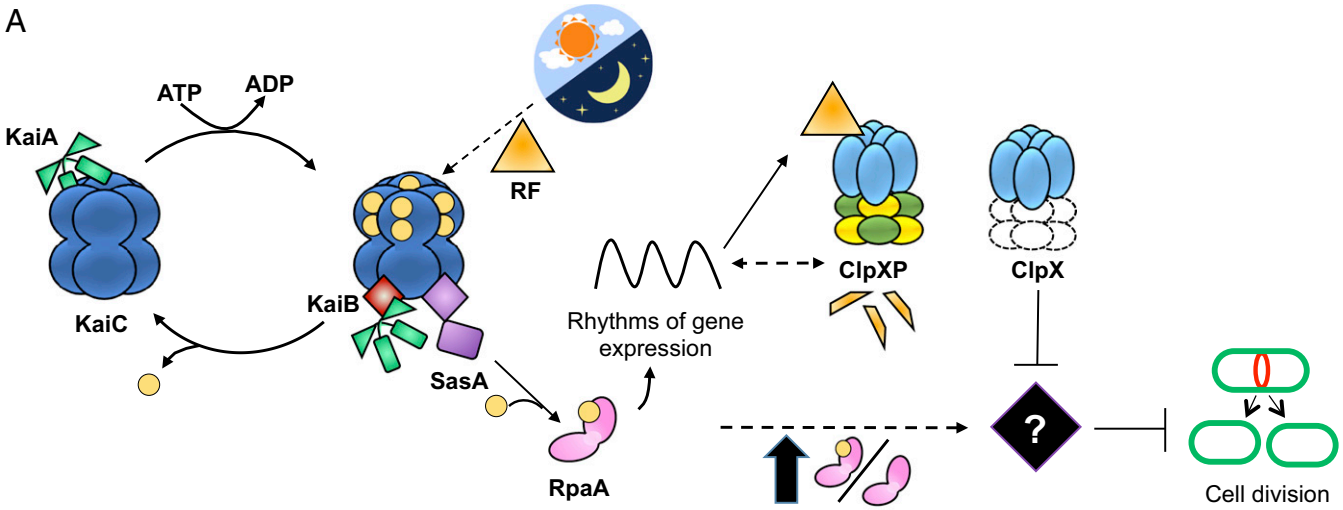
Rhythms of protein abundance have been reported for the oscillator components (14, 23), and the physiological relevance of these rhythms has not been explored. To determine whether one or more of the Kai proteins could, itself, be RF, we tested the phenotypes of mild overexpression of each to see if they mimic those of loss of *clpP2X*. We found that an extra copy of either *kaiA* or *kaiC*, expressed from a neutral site, resulted in mild period lengthening (*SI Appendix*, Fig. S5*E*) although the effect with *kaiA* may not be significant. In contrast, an extra copy of *kaiB* caused period shortening (49). We then compared KaiA and KaiC protein levels in WT and Δ*clpP2X* mutant backgrounds during a dark pulse. While there was no significant difference in KaiA protein levels between the WT and mutant backgrounds, KaiC was twice as high in the Δ*clpP2X* mutant 1 h into the 5 h resetting dark pulse and 1.4 times that in WT 5 h into the dark pulse (Fig. 5*E*), suggesting that KaiC is indeed a target of the ClpXP protease.

Discussion

The Kai-based oscillator can function in vitro without the need for protein synthesis and degradation, which has led to a focus on the fundamental timekeeping mechanism that is regulated by the ordered phosphorylation events on KaiC and progressive protein–protein interactions with KaiA and KaiB. Protein degradation, a

critical component of eukaryotic oscillations via transcription–translation feedback loops, has received little attention in bacteria. However, the cyanobacterial clock system is more complicated in vivo than in the in vitro reconstituted system. We present evidence that ClpX, ClpP1, and ClpP2 play important and previously unexpected roles in regulating the circadian clock in cyanobacteria beyond known period effects and separate out the contributions of these three proteins to specific phenotypes. We propose that the Clp-protease is involved in a program of circadian-regulated proteolysis, in which proteins are targeted for degradation by the ClpX–P1–P2 complex at specific circadian times, as well as in response to a dark pulse. Specifically, we propose that ClpX–P1–P2 is necessary to keep levels of resetting factor (RF) low and that conditions that inhibit RF degradation lead to elevated RF levels and enable hyper-resetting. Additionally, we propose a role for ClpX in regulating circadian control of cell division in a protease-independent pathway, in which the chaperone is needed to relieve the action of a clock-controlled inhibitor of cell division (Fig. 6*A*).

Our work shows that protein degradation by the ClpXP protease is required to fine tune the circadian clock and to constrain the pathway that resets the oscillator for entrainment with the external environment. In natural situations the clock resets by only minutes on a daily basis and would never require a multihour jump in phase. While mutants that cannot reset the oscillator have been described, such as a *cikA* null mutant and the *kaiC-pr1* point mutant (18, 50), here we report a mutant that can reset the oscillation to a greater extent than does WT. We propose that hyper-resetting is enabled by high levels of RF, achieved by either loss of both ClpP2 and ClpX, or when ClpP1 and ClpP2 are both expressed as inactivated variants, but not when ClpX or ClpP2 is individually lost (Fig. 6*A*). This pattern suggests that, while ClpX-ClpP2 function together with ClpP1, it is the ClpX-ClpP2 functions that are likely to contribute



B

Genotype	Phenotype	Resetting Factor (RF)	Model
WT	constrained	Normal range	ClpXP1P2 degrades RF
Δ*clpX*	partially constrained	High levels	ClpP1 brings ClpC to partially clear RF
Δ*clpP2*	+/-	Borderline level WT/ too low	RF is cleared by ClpP1 more efficiently, perhaps via association with ClpP3
clpP1^{TRAP}, *clpP2*^{TRAP}	Hyper-reset	Very high	RF not degraded, ClpXP jammed
Δ*clpP2X*	Hyper-reset	Very high	ClpP1-ClpC not sufficient to clear RF

Fig. 6. Model for roles for the *clpXP* gene products in regulating the circadian clock and cell division. (*A*) The ClpXP protease consisting of ClpX (blue), ClpP1 (yellow), and ClpP2 (green) subunits is required for the proper timing of the circadian clock, as well as its ability to synchronize with the external environment. The circadian clock (KaiA, KaiB, KaiC, and SasA) regulates the levels of RpaA phosphorylation (for simplicity, the clock-associated RpaA phosphatase CikA is not shown). When RpaA-P is high, dusk-peaking genes are activated, and dawn-peaking genes are repressed. *clpX* and *clpP2* expression is directly regulated by RpaA. In turn, ClpXP are important to maintain periodicity of the circadian clock. RF is required to allow the clock to synchronize with the external environment. ClpXP is required to keep levels of RF low. In the absence of a functional ClpXP protease, RF levels increase and extend the range over which the oscillator can reset. ClpX chaperone activity, independent of protease activities, is required for circadian control of cell division. ClpP1 and ClpP2 may still associate with ClpX as deletion of both is lethal, perhaps functioning as a scaffold rather than a protease. The circadian clock regulates the expression of a dusk-peaking factor that inhibits cell division for a period of time in every 24-h cycle. While the clock determines the onset of this inhibition, ClpX refolds or unfolds and inhibits this factor, determining the offset of this inhibition. Together, these processes precisely determine when, in every 24-h day, cell division is specifically inhibited. (*B*) Table describing how the loss or alteration of ClpX, ClpP1, or ClpP2 leads to changes in RF and the pathway that resets the circadian oscillator.

specifically to the hyper-resetting phenotype. We hypothesize that, when ClpP1is missing, ClpP3 can substitute and function with ClpP2 to keep RF levels low. When ClpX is missing, ClpP1 can recruit ClpC (34), and the ClpC–P1–P2 complex partially clears RF, leading to mildly elevated RF levels and enhanced resetting. The loss of ClpP2 results in inconsistent resetting properties, suggesting that RF levels are on the borderline between normal and too low. One possible explanation is that ClpP1, via associations with ClpP3, may be more efficient at degrading RF (Fig. 6*B*). RF represents a factor or set of factors required mechanistically for resetting. While KaiC is a target of the ClpXP protease during a resetting dark pulse that contributes the resetting potential, it is likely that other factors contribute as well as mild *kaiC* overexpression does not mimic the phenotypes exhibited by a Δ*clpP2X* mutant in terms of magnitude. In particular, we propose that RF levels are likely to peak around 5 to 8 h after dawn as this is when *S. elongatus* is capable of the largest phase shift in response to a dark pulse.

ClpP2 exhibits a diel rhythm of polar localization, with enhanced polar foci observed at night. This localization is regulated both by the circadian clock and environmental cues. The observation that dark pulses are not sufficient to induce ClpP2 localization during the subjective day and ClpP2 localization does not oscillate in a *kaiC* mutant background suggests circadian control. However, ClpP2 localization does not oscillate in constant light conditions, suggesting that a diel cycle is required to drive ClpP2 localization rhythms. This requirement contrasts with KaiC localization rhythms, which persist in constant conditions (25). The ClpXP protease localizes to cell poles in other bacterial systems (39, 40, 51). Identification of the environmental cues that are sensed may reveal insights into the role of the localization pattern. Regardless, the dual regulation by the clock and the environment suggests external coincidence regulation, in which ClpP2 will localize to the cell pole only when specific phases of the internal biological clock and the external environment coincide.

We also present evidence that ClpX plays distinct roles in regulating circadian period and cell division. The findings are consistent with the expected role of ClpX as the molecular chaperone required for substrate recognition and delivery of target proteins to the ClpP peptidase chamber. However, our results also suggest a role for ClpX, independent of its roles with ClpP2, in regulating circadian gating of cell division. Although the protease activities are not required for ClpX control of cell division, the fact that the *clpP1*, *clpP2* double mutant could not be constructed suggests an essential nonproteolytic function where perhaps ClpP1 and ClpP2 serve scaffolding purposes. Microarray analysis suggests that, while *clpP2* and *clpX* are expressed as part of an operon, *clpP2* message peaks 8 h before those of *clpX* (52, 53), suggesting that either independent transcription or specific message degradation enables ClpX to continue to accumulate after *clpP* expression wanes. Such a mechanism might enable ClpX to function without ClpP2 during specific times of the day. We propose that the circadian clock regulates a circadian block on cell division via the expression of a dusk-peaking gene, which functions to inhibit FtsZ ring formation, and that ClpX refolds or unfolds this factor, relieving the repression and allowing cell division to continue (Fig. 6*A*). Thus, the circadian clock, which determines the onset of the cell division block, and ClpX, which determines the offset of the block, work together to precisely determine when, in every 24-h day, cell division is specifically inhibited.

Materials and Methods

Bacterial Strains, Growth Conditions, and DNA Manipulations. Plasmids and *E. coli* and *S. elongatus* PCC 7942 stains are described in *SI Appendix*, Tables S1 and S2. *S. elongatus* strains were grown as previously described (54). Plasmids were constructed using the GeneArt Seamless Cloning and Assembly Kit (Life Technologies) and the CYANO-VECTOR assembly portal (55) as previously described (55). Mutant alleles were constructed using the QuikChange (Stratagene) protocol, and clones were verified by DNA sequencing. Homogeneous segregation of alleles was confirmed by PCR for all knock-out and disruption alleles. Three neutral sites in the chromosome (NS1, NS2, and NS3) are used for extopic expression in *S. elongatus* (25).

Circadian Bioluminescence Monitoring. Bioluminescence from *S. elongatus* strains expressing a P_{kaiBC}–*luc* reporter was monitored at 30 °C under constant-light conditions after two entrainment cycles in LD 12:12 to synchronize the population as previously described (56). Data were analyzed with the Biological Rhythms Analysis Software System import and analysis program using Microsoft Excel (millar.bio.ed.ac.uk/Downloads.html). To assay phase-resetting ability, one of a pair of duplicate plates of strains was transferred to the dark for 5 h at the indicated time after entry into constant light conditions and replaced after the dark pulse.

Immunoblot Analysis. Whole-cell extract preparation and immunoblot analysis were performed as previously described (57). Equal amounts of total protein from whole-cell extracts (10 μg for Clp proteins and 5 μg for KaiC and Strep-tagged proteins) were separated by SDS/PAGE (10%), transferred to a poly(vinylidene difluoride) (PVDF) membrane, and blocked with 2.5% wt/vol nonfat dry milk/Tris-buffered saline plus 0.1% Tween 20 (TBS-T). Membranes were probed with α-ClpX, α-ClpP1, α-ClpP2 (37), or α-KaiC at 1:10,000 in blocking buffer, followed by several washes in TBS-T, and probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Calbiochem) for Clp proteins or HRP-conjugated goat anti-Chicken (Aves Labs) for KaiC at 1:10,000 in blocking buffer. Detection of Strep-tagged proteins was performed with α-Strep tag (QIAGEN) according to the manufacturer's protocol. Chemiluminescent detection was performed using Pierce Super Signal West Femto detection reagents (Thermo Scientific).

Fluorescence Microscopy and Image Analysis. Cells were placed on a pad of 1.2% agarose in BG-11 medium and covered with a coverslip. Microscopy was performed with a DeltaVision Core system (Applied Precision) with a WeatherStation attached to an Olympus IX71 inverted microscope and an Olympus Plan Apochromat 100× objective at 30 °C with tetramethyl rhodamine isocyanate (TRITC) (EX555/EM617) and YFP (EX500/EM535) filter settings. Images were captured using a CoolSnap HQ CCD camera (Photometrics) and deconvolved using the SoftWorx imaging program (Applied Precision). Exposure times were limited to conditions under which we did not observe fluorescence from WT strains in the YFP channels to limit bleed-through from thylakoid fluorescence (45). For analysis of ClpP2 localization in time-course experiments, aliquots of cells were taken at designated time points and fixed directly in BG-11 growth medium with a final concentration of 2.4% (vol/vol) paraformaldehyde (Electron Microscopy Sciences) in 30 mM $NaPO_4$ buffer (pH 7.5) for 20 min at room temperature before they were moved to 4 °C. Images were colorized in SoftWorx and then transferred to Photoshop (Adobe) for figure assembly. ClpP2 foci tracking was performed as previously described (25).

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