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## Regulated proteolysis of a transcription factor complex is critical to cell cycle progression in *Caulobacter crescentus*

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### Abstract

Cell cycle transitions are often triggered by the proteolysis of key regulatory proteins. In *Caulobacter crescentus*, the G1-S transition involves the degradation of an essential DNA-binding response regulator, CtrA, by the ClpXP protease. Here, we show that another critical cell cycle regulator, SciP, is also degraded during the G1-S transition, but by the Lon protease. SciP is a small protein that binds directly to CtrA and prevents it from activating target genes during G1. We demonstrate that SciP must be degraded during the G1-S transition so that cells can properly activate CtrA-dependent genes following DNA replication initiation and the reaccumulation of CtrA. These results indicate that like CtrA, SciP levels are tightly regulated during the *Caulobacter* cell cycle. In addition, we show that formation of a complex between CtrA and SciP at target promoters protects both proteins from their respective proteases. Degradation of either protein thus helps trigger the destruction of the other, facilitating a cooperative disassembly of the complex. Collectively, our results indicate that ClpXP and Lon each degrade a critical cell cycle regulator, helping to trigger the onset of S phase and prepare cells for the subsequent programs of gene expression critical to polar morphogenesis and cell division.

### Introduction

Protein degradation is an excellent and rapid mechanism to completely remove undesired proteins from cells. In bacteria, regulated proteolysis is particularly important when cells must drastically change their proteome composition without dilution by cell division. In the  $\alpha$ -proteobacterium *Caulobacter crescentus*, motile swarmer cells differentiate into sessile stalked cells at the same time they initiate DNA replication and transition from a G1 to S phase (Fig. 1A). Controlled proteolysis of many proteins is critical for this developmental and cell cycle transition and involves AAA+ proteases, oligomeric enzymes that harness ATP hydrolysis to power unfolding and degradation of specific substrates (Sauer & Baker, 2011). One of these AAA+ enzymes is the essential ClpXP protease (Jenal & Fuchs, 1998), which degrades a number of substrates including CtrA, a master cell cycle regulator. CtrA is a response regulator with a DNA-binding domain that enables it to regulate the transcription of nearly 100 genes (Quon *et al.*, 1996, Laub *et al.*, 2002). CtrA also binds directly to and silences the origin of replication in swarmer cells (Quon *et al.*, 1998). During the swarmer-to-stalked cell transition, the combined action of CtrA dephosphorylation and proteolysis

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ensures that active CtrA is eliminated, thus enabling the onset of DNA replication and S phase (Domian *et al.*, 1997) (Fig. 1A).

A complex circuit of regulatory proteins ensures the rapid elimination of CtrA specifically during the G1-S transition (Tsokos & Laub, 2012, Jenal, 2009). Because ClpXP is present throughout the cell cycle it was initially assumed that the protease was somehow activated to degrade CtrA at the appropriate time. However, CtrA proteolysis by ClpXP occurs *in vitro* at rates comparable to those observed *in vivo* (Chien *et al.*, 2007). Thus, the timing of CtrA degradation *in vivo* may result from a relief of inhibition rather than being promoted by an activator or adaptor. Notably, in swarmer cells, immediately before being degraded, CtrA is bound to DNA, including target promoters and the origin of replication. Whether binding to DNA influences CtrA degradation is currently unclear.

Although CtrA is abundant and phosphorylated in both swarmer and predivisional cells, most CtrA-activated genes are expressed predominantly during the predivisional stage (Laub *et al.*, 2002). These genes are not transcribed in swarmer cells because a small protein called SciP accumulates specifically in this cell type (Fig. 1A) and binds to CtrA to prevent it from activating target genes (Gora *et al.*, 2010, Tan *et al.*, 2010). SciP does not, however, prevent CtrA from binding DNA, so CtrA can still silence the origin of replication in swarmer cells. The elimination of SciP during the G1-S transition is likely critical to preparing cells for another round of CtrA-dependent gene expression later in the cell cycle. Consistently, cells forced to produce SciP at high levels in stalked and predivisional cells fail to activate CtrA target genes and consequently fail to divide or properly assemble polar organelles (Gora *et al.*, 2010).

The mechanisms regulating SciP abundance during the cell cycle are largely uncharacterized. Here, we demonstrate that SciP is degraded by the AAA+ protease Lon both *in vivo* and *in vitro*, and that synthesis of a non-degradable version of SciP leads to severe cell cycle defects. Further, we find that formation of a SciP:CtrA:DNA complex helps shield SciP and CtrA from degradation by Lon and ClpXP, respectively, in swarmer cells. These results suggest that the removal of each component during the G1-S transition may help drive degradation of the other, thereby accelerating the onset of S phase. Our results further highlight the prominent role played by regulated proteolysis in driving cell differentiation processes and cell cycle transitions in bacteria.

## Results

### SciP binds DNA nonspecifically when complexed with CtrA

SciP does not strongly bind DNA on its own, as judged by electrophoretic mobility assays, but does form a robust complex with DNA-bound CtrA (Gora *et al.*, 2010). Formation of this ternary complex requires DNA immediately adjacent to that bound by CtrA indicating that SciP likely contacts neighboring DNA directly when complexed with CtrA. Similar results were obtained with three different DNA probes suggesting that the SciP-DNA interaction is likely not highly sequence specific. However, SciP was subsequently suggested to bind DNA independently through recognition of a motif with consensus TGTCGCG (Tan *et al.*, 2010).

To test the functional significance of this motif, we examined its effect on SciP binding to a 50 bp fragment of the *pilA* promoter that binds SciP and CtrA together. We mutated the sequence GATCGCG, the closest match to the proposed SciP consensus, to GATTTTG (Fig. S1A). As expected, phosphorylated CtrA (CtrA~P) alone bound both the wild-type and mutant probes, producing nearly identical shifts (Fig. S1B-C). We then added purified SciP along with CtrA~P and observed a similar pattern of super-shifting with both probes (Fig.

S1B), suggesting that the mutated sequence is not required for a SciP:CtrA:DNA complex to form on this probe. This conclusion is consistent with our previous report that SciP and CtrA form a complex on a 50 bp probe taken from the *fliF* promoter that does not include a close match to the proposed motif (Gora et al., 2010).

To corroborate these results *in vivo*, we examined a  $P_{ctrA}$ -*lacZ* reporter that contains the P1 and P2 promoters of *ctrA* (Domian et al., 1999) and two instances of the proposed SciP motif (Fig. S1D). Cells harboring this reporter showed a significant decrease in *LacZ* activity when SciP was overproduced, even if the putative motif regions were mutated, individually or in combination (Fig. S1E). Thus, although SciP could have minor sequence preferences, it likely does not function through the binding of a specific motif. Instead, our findings support a model in which SciP binds both CtrA and the DNA in direct proximity to CtrA, with specificity of the ternary complex ultimately provided by CtrA. We do not, however, rule out that the proposed motif found in many CtrA-regulated genes (Tan et al., 2010) is recognized by another DNA-binding protein or by SciP in complex with a different partner under alternative conditions.

### Binding to DNA and SciP stabilizes CtrA against proteolysis *in vitro*

Because our data indicated that SciP and CtrA form a direct, ternary complex with DNA, we wondered whether complex formation would affect the stability of each protein. CtrA is stable in swarmer cells and then rapidly degraded during the swarmer-to-stalked cell transition (Domian et al., 1997). We hypothesized that DNA binding may contribute to the stabilization of CtrA, as seen with other transcription factors (Pruteanu et al., 2007). We first tested the effect of DNA binding on CtrA stability *in vitro* using a 25 bp DNA fragment derived from the *Caulobacter* origin of replication (*Cori*). Whereas CtrA alone was degraded rapidly by ClpXP, the inclusion of 5  $\mu$ M *Cori* DNA severely inhibited degradation (Fig. 2A). To assess inhibition quantitatively, we fused CtrA to GFP and measured initial rates of degradation by monitoring loss of GFP fluorescence. As expected, addition of the 25 bp *Cori* fragment inhibited GFP-CtrA degradation with a  $K_i$  of ~550 nM (Fig. S2), in agreement with previously measured dissociation constants of CtrA and DNA (Siam & Marczyński, 2000).

Previous work demonstrated that overproducing SciP leads to a small, but significant increase in CtrA stability (Gora et al., 2010). Therefore, we tested whether SciP affects CtrA degradation *in vitro*. Adding SciP to CtrA in the absence of DNA had little effect on CtrA degradation (Fig. 2B). We then tested the effects of adding SciP along with a 50 bp fragment of the *fliF* promoter that supports formation of a CtrA:SciP:DNA complex (Gora et al., 2010). In this case we observed a dramatic stabilization of CtrA (Fig. 2B-C). Notably, these experiments were done at a concentration of DNA that is insufficient, in the absence of SciP, to completely inhibit CtrA degradation (Fig. 2B-C), indicating a synergistic effect of SciP and DNA on CtrA stability. SciP also helped to block the degradation of CtrA bound to a 50 bp *pilA* fragment. However, SciP did not significantly affect proteolysis of CtrA bound to a 25 bp fragment of *Cori* (Fig. 2C) consistent with previous results, and those above, indicating that formation of a CtrA:SciP:DNA complex requires interactions of both CtrA and SciP with DNA (Gora et al., 2010).

A point mutant of SciP, R35A, which weakens the CtrA-SciP interaction and abrogates the ability of SciP to regulate CtrA *in vivo* (Gora et al., 2010), failed to stabilize CtrA against degradation *in vitro* when using the 50 bp *fliF* probe (Fig. 2D). We also tested another SciP mutant, R40A, which significantly reduces the growth defects arising from *sciP* overexpression, but is less deficient than SciP(R35A) in interacting with CtrA (Gora et al., 2010). With the 50 bp *fliF* probe, SciP(R40A) stabilized CtrA to a degree more similar to wild-type SciP than SciP(R35A) (Fig. 2D). However, with the *pilA* probe, which shows a

more dramatic stabilization of CtrA degradation in the presence of SciP (Fig 2C), we found that SciP(R40A) was worse than wild-type SciP in protecting CtrA from ClpXP degradation (Fig. 2E). Collectively, these results are consistent with prior observations indicating that the R35A variant is almost completely deficient in binding CtrA in a yeast two-hybrid assay, while the R40A variant is compromised to a lesser extent (Gora et al., 2010). Importantly, we also found that the stabilization of CtrA by wild-type SciP requires the CtrA recognition site in the DNA as a *pilA* probe harboring mutations in the CtrA binding sites failed to stabilize CtrA even in the presence of wild-type SciP (Fig. 2E). Taken together, our data indicate that SciP stabilizes the binding of CtrA to DNA, which, in turn, leads to the stabilization of CtrA against degradation by ClpXP.

### Cell cycle changes in SciP abundance are controlled post-transcriptionally

Our *in vitro* results suggested that changes in SciP abundance may influence the degradation of CtrA *in vivo*, helping to stabilize CtrA in swarmer cells. Notably, SciP is maximally abundant in swarmer cells and then rapidly disappears during the G1-to-S transition, coincident with the time of CtrA degradation (Gora et al., 2010, Tan et al., 2010). We therefore sought to determine the molecular mechanisms controlling SciP accumulation.

The transcription of *sciP* is cell cycle-regulated with peak *sciP* mRNA levels observed in predivisive cells (Chen *et al.*, 1986). To test whether regulated *sciP* transcription contributes to the swarmer-specific accumulation of SciP, we constructed a strain harboring a deletion of the native *sciP* and constitutively expressing *sciP* from the chromosomal *xytX* locus. Synchronized swarmer cells from this strain were isolated and released into rich medium containing xylose. We found that SciP levels were highest in swarmer cells and then decreased ~30 minutes post-synchronization, although SciP did not completely disappear, as it does in the wild type (Fig. 3A). Cells constitutively transcribing *sciP* also showed higher levels of SciP in predivisive cells than in the wild type.

Our results indicated that regulated *sciP* transcription has a modest effect on the cell cycle pattern of SciP abundance. However, SciP levels still dropped significantly during the G1-S transition suggesting that SciP may also be subject to regulated proteolysis. We therefore measured the stability of SciP using pulse-chase analysis of cells expressing *sciP* from a low-copy plasmid during growth in a minimal medium. Under these conditions, in which the cell cycle is ~140 minutes, SciP had a half-life of only ~23 minutes indicating it is a relatively unstable protein and likely being degraded during the G1-S transition when SciP levels drop (see Fig. 4A). Thus, we sought to identify the protease(s) responsible for SciP degradation.

### SciP is degraded by the Lon protease

Because SciP normally disappears during the G1-to-S transition, concurrent with ClpXP-dependent proteolysis of CtrA, we first investigated whether ClpXP also degrades SciP. As both ClpP and ClpX are essential for viability, we assayed SciP levels in cells depleted of ClpP. Strain UJ199 harbors a deletion of the native, chromosomal *clpP* gene with a single copy of *clpP* under control of the  $P_{xyt}$  promoter at the *xytX* locus (Jenal & Fuchs, 1998). This strain was grown in rich medium containing glucose for 6 hours (~four generations) to deplete ClpP. We then isolated swarmer cells and released them into glucose or xylose to repress or induce, respectively, *clpP*. We sampled cells every 30 minutes and examined both SciP and CtrA by immunoblotting (Fig. 3B). As expected, cells released into glucose failed to proteolyze CtrA, whereas cells released into xylose had almost completely eliminated CtrA after ~60 minutes. In contrast to CtrA, SciP levels dropped significantly in both the xylose and glucose samples by ~60 minutes, although levels remained slightly higher in glucose (Fig. 3B). These data indicate that cells lacking ClpP can still proteolyze SciP

during the G1-to-S transition. These data are also consistent with our results showing that purified ClpXP degrades CtrA three times faster than SciP when both are present at the same concentration *in vitro* (Fig. S3).

Because ClpP is the peptidase subunit for both ClpXP and the protease ClpAP, our data argue against the involvement of either protease in SciP proteolysis. Consistently, we found that SciP was also eliminated during the G1-to-S transition in synchronized  $\Delta clpA$  cells (Fig. 3C).

To test the role of HslUV, another ATP-dependent protease (Rohrwild *et al.*, 1996), we synchronized a strain harboring a transposon within the *hslV* coding region, *hslV::Tn5*, and monitored SciP levels by Western blotting. SciP was completely eliminated during the G1-S transition, indicating that HslUV likely does not degrade SciP (Fig. 3C).

We next measured SciP abundance in cells lacking the protease Lon (Wright *et al.*, 1996). We synchronized  $\Delta lon$  cells and monitored both SciP and CtrA levels during cell cycle progression. Whereas CtrA was cleared by 30 minutes post-synchronization, SciP was still present after 30 minutes and maintained throughout the cell cycle (Fig. 3C). Further, pulse-chase analyses on mixed populations of cells indicated that SciP was significantly stabilized in  $\Delta lon$  cells compared to the wild type (Fig. 4A). Taken together, these data strongly suggest that SciP is degraded by Lon *in vivo*.

We then tested the ability of *C. crescentus* Lon to proteolyze SciP *in vitro*. Purified SciP was rapidly degraded by Lon *in vitro*, with a half-life of ~12 minutes (Fig. 4B). These findings support the conclusion that SciP is a direct substrate for Lon in *Caulobacter*. Although SciP was stabilized most significantly during the G1-S transition in  $\Delta lon$  cells, levels still decreased modestly during cell cycle progression in this strain (Fig. 3C), indicating that other proteases may degrade SciP, but at lower rates.

### A C-terminal tag interferes with SciP proteolysis

To test the importance of regulated SciP degradation to the pattern of SciP abundance and cell cycle progression, we sought to create a proteolytically stable version of SciP. Because energy-dependent proteases often rely on terminal sequences for substrate recognition (Chien *et al.*, 2007, Domian *et al.*, 1997, Griffith *et al.*, 2004, Rood *et al.*, 2012, Shah & Wolf, 2006), we tagged the N- and C-terminus of SciP with an M2 epitope. We transformed wild-type cells with low copy plasmids expressing *sciP*, *M2-sciP*, or *sciP-M2* under the control of  $P_{xyI}$ . Mixed populations of cells were grown in rich medium supplemented with glucose to repress the plasmid copy of *sciP*, synchronized, and released into medium supplemented with xylose to induce expression. Samples were collected every 15 minutes for SciP immunoblot analysis (Fig. 4C). Note that we did not induce expression of the plasmid-borne copies of *sciP* prior to synchronization, as pre-induction decreased the yield of swarmer cells for the *sciP-M2* strain during synchronization; hence, SciP levels during early time points reflect the effects of transcriptional induction and proteolysis.

For cells expressing untagged *sciP* from a low-copy plasmid, along with the chromosomal *sciP*, we found that SciP levels were highest in swarmer cells, dropped dramatically by ~30–45 minutes post-synchronization, and then accumulated again during later stages of the cell cycle (Fig. 4C), a pattern similar to that seen when the only copy of *sciP* is expressed from the chromosomal *xyIX* locus (Fig. 3A). By contrast, for cells producing N-terminally tagged SciP, we found that SciP accumulated during the early stages of the cell cycle with slightly higher levels of SciP at the 30 and 45 minute time points compared to cells producing untagged SciP (Fig. 4C). A similar, but more pronounced effect was seen in cells expressing *sciP-M2*, with high levels of SciP-M2 detectable throughout the time course examined (Fig.



4C), compared to cells expressing untagged *sciP*, and particularly when compared to wild-type cells. Our results indicate that a C-terminal M2 epitope likely stabilizes SciP by interfering with its proteolysis by Lon.

We assayed the *in vivo* stability of epitope-tagged SciP using pulse-chase analyses of mixed populations of cells grown in minimal medium. For cells producing M2-SciP or SciP-M2, we measured half-lives of 36 and 58 minutes, respectively, compared to 23 minutes for the untagged protein (Fig. 4A). We also found that the degradation of SciP-M2 by Lon *in vitro* was dramatically slowed compared to degradation of the wild-type SciP (Fig. 4B). The C-terminal tag likely did not stabilize SciP by interfering with its function as purified SciP-M2 was still capable of complexing with CtrA *in vitro* (Fig. S3) and SciP-M2 could still down-regulate CtrA-dependent genes *in vivo* (see below). Together, these results support the conclusion that Lon recognizes SciP through an exposed C-terminus.

### SciP and CtrA reciprocally stabilize each other

The formation of a CtrA:SciP:DNA complex can protect CtrA from degradation by ClpXP *in vitro* (Fig. 2), and overproducing SciP stabilizes CtrA *in vivo* (Gora et al., 2010). We hypothesized that SciP may similarly be protected from Lon-dependent degradation by interacting with CtrA at target promoters. Indeed, we found that SciP degradation *in vitro* was significantly slower in the presence of CtrA and a 50 bp *fliF* fragment (Fig. 5A-B). This stabilization was dependent on complex formation as excess DNA or CtrA alone did not alter SciP degradation by Lon. Moreover, mutants of SciP that do not interact with CtrA were susceptible to Lon degradation even when both CtrA and DNA were included (Fig. 5C). We also found that SciP was not eliminated as rapidly during the G1-S transition in  $\Delta cpdR$  cells in which CtrA remains stable throughout the cell cycle (Biondi *et al.*, 2006, Iniesta *et al.*, 2006); SciP was still abundant 30 minutes post-synchronization in  $\Delta cpdR$  cells but not in wild-type cells (Fig. 3A, C). This result is consistent with a model in which CtrA normally stabilizes SciP; consequently,  $\Delta cpdR$  cells, which do not properly clear CtrA during the G1-S transition, do not clear SciP as efficiently. Taken together, these findings suggest that SciP and CtrA reciprocally stabilize each other against degradation by Lon and ClpXP, respectively, when assembled into a DNA-bound complex. Conversely, proteolysis of each may accelerate degradation of the other during the G1-S transition, contributing to the robustness and irreversibility of this cell cycle transition.

### Regulated proteolysis of SciP is necessary for proper cell cycle progression

To further investigate the phenotypic consequences of producing protease-insensitive SciP, we expressed *sciP-M2* from a low-copy plasmid under the control of a xylose-inducible promoter in otherwise wild-type cells. This strain was grown in rich medium supplemented with glucose and then shifted to medium containing xylose, leading to a modest growth defect relative to a strain maintained in glucose (Fig. 6A). After four hours, cells were also moderately filamentous and flow cytometry analysis indicated that cells had accumulated up to four chromosomes per cell, consistent with a defect in cell division (Fig. 6B). In contrast to *sciP-M2*, cells expressing untagged *sciP* or *M2-sciP* from the same low-copy plasmid exhibited no significant defects in growth, morphology, or chromosome content (data not shown).

The phenotypic consequences of producing SciP-M2 from a low-copy plasmid are reminiscent of those previously reported for cells producing M2-SciP from the same promoter but on a high-copy plasmid (Gora et al., 2010). To further compare the phenotypes of these two strains, we examined global changes in gene expression in each strain using DNA microarrays. Wild-type cells harboring the low-copy *sciP-M2* expression plasmid were induced with xylose for 4 hours and mRNA was then isolated and compared to mRNA

from identically treated wild-type cells using whole genome microarrays. We directly compared this expression profile to those obtained previously for cells expressing *M2-sciP* from a high-copy plasmid, and for *ctrA<sup>ts</sup>* and *cckA<sup>ts</sup>* strains grown at a restrictive temperature (Gora et al., 2010). For all four strains, we observed a significant down-regulation of CtrA-activated genes (Fig. 6C).

Based on the DNA microarrays, we found that the expression of *sciP-M2* from a low-copy vector led to ~3-fold higher *sciP* mRNA relative to wild-type cells, whereas expression from a high-copy vector resulted in an ~18-fold increase. However, the down-regulation of CtrA target genes was comparable in magnitude in each case (Fig. 6C), consistent with our conclusions that a C-terminal M2 tag stabilizes SciP. These data support a model in which SciP is normally proteolyzed at the G1-S transition and that a failure to degrade SciP during this transition (Fig. 4C) prevents the subsequent activation of CtrA-dependent genes in predivisive cells, thereby disrupting cell cycle progression.

## Discussion

### Cell cycle regulation of SciP

SciP plays a critical role during the *Caulobacter* cell cycle by helping to restrict CtrA-dependent gene expression to the G1, or swarmer cell, phase (Gora et al., 2010, Tan et al., 2010). SciP is not, however, essential for viability as cells harboring a deletion of *sciP* can be generated (Gora et al., 2010); moreover, resequencing of the genome of the  $\Delta sciP$  strain did not identify any additional mutations relative to the parent strain CB15N (data not shown). Nevertheless, cells lacking SciP show a range of severe phenotypes, underscoring its importance as a cell cycle regulator. SciP is maximally abundant in swarmer cells and must be eliminated before cells reach the predivisive stage when CtrA is required to activate an arsenal of genes critical to morphogenesis and cell division (Laub et al., 2002). Regulated transcription and proteolysis work together to ensure SciP is abundant principally in swarmer cells. The transcription of *sciP* is cell cycle-regulated and directly activated by CtrA leading to maximal expression of *sciP* in predivisive cells (Chen et al., 1986, Gora et al., 2010, Tan et al., 2010). SciP protein does not, however, accumulate to high levels until after cell division, and then accumulates almost exclusively in the daughter swarmer cell, indicating post-transcriptional control of SciP (Gora et al., 2010). Moreover, cells constitutively expressing *sciP* still show cell cycle-dependent changes in SciP protein levels (Fig. 4C), suggesting other levels of regulation. Here, we demonstrated that SciP is likely rapidly degraded during the swarmer-to-stalked cell, or G1-S, transition, by the protease Lon (Fig. 7). Proteolysis allows cells to irreversibly clear SciP, priming cells for a new round of CtrA-dependent gene expression following DNA replication initiation.

### Regulated proteolysis by Lon

SciP is the first direct substrate of Lon identified in *Caulobacter*. Genetic studies have previously implicated Lon in the degradation of the cell-cycle methyltransferase CcrM (Wright et al., 1996), but whether Lon directly degrades CcrM has not been established. Nevertheless, the Lon protease clearly plays an important role in the degradation of both CcrM and SciP, and probably other cell cycle regulators. Consistent with such a multifaceted role, cells lacking Lon exhibit a variety of cell cycle phenotypes, including moderate cellular filamentation and the accumulation of up to six chromosomes per cell, as judged by flow cytometry (Wright et al., 1996). These phenotypes are similar to those resulting from the expression of non-degradable *sciP-M2* in wild-type cells suggesting that a failure to properly clear SciP at the G1-to-S transition may contribute to some aspects of the pleiotropic effects of a *lon* mutant.



How is Lon activity regulated? Lon is present at relatively constant levels throughout the cell cycle suggesting that its activity is not regulated through changes in abundance (Wright et al., 1996). Hence, in the case of CcrM, Lon was suggested to be constitutively active throughout the cell cycle, with an increase in *ccrM* transcription in predivisive cells leading to an accumulation of CcrM by exceeding the proteolytic capacity of Lon. In contrast, we found that SciP levels drop rapidly during the G1-S transition when there is no concurrent change in *sciP* transcription, suggesting that the specific activity of Lon could increase during this stage of the cell cycle. Polyphosphate can stimulate Lon activity in *E. coli* (Kuroda et al., 2001), and could function similarly in *Caulobacter*, although a recent study suggested that polyphosphate actually slows the G1-S transition in *Caulobacter* (Boutte et al., 2012). However, another cofactor could, in principle, accumulate during the G1-S transition to activate Lon and stimulate SciP degradation. Alternatively, although Lon recognizes SciP directly *in vitro*, an adapter protein could accumulate during the G1-S transition *in vivo* that further improves degradation. The binding of SciP to CtrA and target promoters may also help shield SciP from degradation in swarmer cells (Fig. 5). Finally, it is also possible that differential localization of Lon during the cell cycle may contribute to changes in its activity, as suggested for ClpXP (Iniesta et al., 2006).

Lon is a AAA+ protease that is widely conserved throughout the bacterial kingdom. In *E. coli*, Lon is responsible for degrading unfolded proteins as well as several key regulators, including the transcription factor SoxS and the cell division inhibitor Sula (Melderen & Aertsen, 2009, Mizusawa & Gottesman, 1983, Griffith et al., 2004). Although the rules for Lon substrate specificity are still poorly defined, Lon is thought to recognize hydrophobic patches in unfolded proteins and the N- or C-terminus of the substrates SoxS and Sula, respectively (Shah & Wolf, 2006, Ishii et al., 2000, Gur & Sauer, 2008). The stabilization of SciP by the addition of a C-terminal M2 epitope (DYKDDDDK) strongly suggests that the free C-terminus of SciP (RTTRIQQYR) is recognized by Lon in *Caulobacter*. Alternatively, addition of the M2 epitope may block degradation by masking electrostatic interactions with the C-terminus that are needed for recognition of SciP by Lon, or by forcing the C-terminus to adopt a non-native secondary structure.

### Disassembly of the CtrA-SciP-DNA complex during the G1-S transition

SciP interacts directly with CtrA bound at target promoters (Fig. 7). Although SciP has a recognition helix, it binds DNA only weakly on its own, requiring CtrA to form more stable contacts with DNA. Thus, SciP likely does not recognize and bind specific sites on the chromosome like canonical transcription factors do, and instead is mainly recruited to the promoters of CtrA-regulated genes through its direct interaction with CtrA. In this sense, SciP functions as a cofactor, or regulator, of CtrA.

The formation of a complex between SciP and CtrA is critical for downregulating CtrA-activated genes in swarmer cells. This complex also promotes the stability of both proteins by protecting each from degradation by their respective protease (Figs. 2, 5). Their mutually enforced stabilization likely means that the degradation of either protein will facilitate degradation of the other (Fig. 7). Hence, the complex may be rapidly disassembled and degraded in a more switch-like fashion. Which protein gets degraded first is not yet clear. Notably though, SciP did not disappear as rapidly, or to the same extent, in  $\Delta cpdR$  cells in which CtrA is not properly degraded at the G1-S transition (Fig. 3). Having SciP degradation contingent on the degradation of CtrA could be beneficial as it would help prevent a scenario where the premature removal of SciP allows CtrA to inappropriately activate gene expression in swarmer cells.

Mutual protection from degradation will, of course, only apply to CtrA and SciP proteins that are bound in a complex on DNA. The binding of CtrA and SciP is strongly promoted by

their binding to DNA that contains a CtrA binding site (Fig. 2E). However, CtrA and SciP may also bind at other sites in the chromosome, even transiently, and such ternary complexes could also protect both proteins from degradation. Nevertheless, given their high concentrations in swarmer cells, not all SciP and CtrA will be complexed, and the unassociated proteins would remain susceptible to proteases. However, additional mechanisms exist that ensure CtrA is rapidly degraded only during the G1-S transition (Abel *et al.*, 2011), and similar mechanisms could exist for SciP as well.

In sum, our results indicate that *Caulobacter* cells rely heavily on regulated proteolysis of a transcription factor complex to drive the G1-S transition. The degradation of CtrA by ClpXP enables a new round of DNA replication to initiate, while the degradation of SciP by Lon ultimately primes the cell for CtrA-dependent gene expression following replication initiation and the reaccumulation of CtrA.

## Experimental procedures

### Bacterial growth conditions

Strains used are listed in Table S1. *Caulobacter* strains were grown in rich (PYE) or minimal (M2G) media and supplemented with 0.2% glucose or 0.3% xylose, as indicated. ML1750 was grown in PYE supplemented with xylose, synchronized, and released into PYE supplemented with glucose or xylose. ML1990, ML1991, and ML1992 were grown in PYE supplemented with glucose and oxytetracycline, synchronized, and released into PYE with glucose or xylose, as indicated, and oxytetracycline. UJ838, UJ199, LS2382,  $\Delta cpdR$ , and *hsIV::tn5* were grown in PYE. UJ199 was depleted and synchronized as previously reported (Jenal & Fuchs, 1998). All synchronies were performed in a 50% Percoll gradient in 50 mL Falcon tubes.

### Cloning, mutagenesis, and strain construction

Primers used for strain construction are listed in Table S2. To construct the plasmid pENTR- $P_{xyI}$ -*sciP* the  $P_{xyI}$  promoter was amplified from pHXM-DEST using the primers  $P_{xyI}$ \_F and  $P_{xyI}$ \_R, *sciP* was amplified from genomic DNA using the primers *sciP*\_F and *sciP*\_R, and the two pieces were fused together by splice overlap extension PCR. The fused construct was cloned into pENTR through TOPO cloning (Invitrogen). The plasmid pENTR- $P_{xyI}$ -*sciP*-M2 was constructed by TOPO cloning a PCR fragment amplified from pLX-*sciP* using the primers  $P_{xyI}$ \_F and *sciP*\_M2\_R. The low-copy plasmids with  $P_{xyI}$ -*sciP* (pLX-*sciP*) and  $P_{xyI}$ -*sciP*-M2 (pLX-*sciP*-M2) were constructed by LR recombination of pENTR- $P_{xyI}$ -*sciP* and pENTR- $P_{xyI}$ -*sciP*-M2 into the destination vector pMR20-DEST as described previously (Skerker *et al.*, 2005). The low-copy plasmid with  $P_{xyI}$ -M2-*sciP* (pLXM-*sciP*) was constructed by LR recombination of pENTR-*sciP* into pLXM-DEST. Expression plasmids were electroporated into CB15N and  $\Delta lon$  electrocompetent cells.

The pBAD-*lon* plasmid was constructed by digesting pBAD33-*lon* with NdeI and SbfI and ligating in a PCR product containing *C. crescentus lon* amplified from CB15N genomic DNA using the primers *lon*\_F and *lon*\_R and digested with NdeI and SbfI.

Constructs for production of SciP variants were constructed by LR recombination of pENTR-*sciP* variants into pHIS-DEST (Skerker *et al.*, 2005). A His<sub>6</sub>-tagged GFP-CtrA (pHIS-GFP-CtrA) was constructed by amplifying *ctrA* from genomic DNA using primers (CtrAforRI and CtrArevXhoI) to install EcoRI and XhoI sites flanking *ctrA*. After purification and restriction, the insert was used to replace *pdeA* in vector pENTR-GFP-PdeA (Rood *et al.*, 2012) generating pENTR-GFP-CtrA. An expression construct was generated by LR recombination of pENTR-GFP-CtrA into pHIS-DEST (Skerker *et al.*, 2005).

Plasmids harboring the *ctrA* promoter were derived from *pctrA290* (Domian et al., 1999). Mutations were introduced using the primers listed in Table S2 to create *pctrA290-mut1*, *pctrA290-mut2*, *pctrA290-mut1+mut2*. Plasmids were transformed into ML1748 to create ML1994-ML1997.

DNA templates containing CtrA binding sites were generated by mixing equal concentrations of oligonucleotides derived from the origin of replication or from promoters of CtrA-regulated genes. Pairs of complementary oligonucleotides, listed in Table S2, were heated at 95 °C for 10 minutes in 100 mM KCl and cooled slowly to facilitate annealing.

### Immunoblots

Cell pellets were resuspended in the equivalent of 50  $\mu$ L 1 X SDS sample buffer per 1 mL of OD 0.15 culture. 10  $\mu$ L of lysate was resolved on a 12% gel (Bio-Rad) run at 130 V for 1 hr at room temperature. Samples were transferred to PVDF membrane and probed as described previously (Gora et al., 2010).

### Flow cytometry

Samples were analyzed as described previously (Chen *et al.*, 2009).

### Pulse-chase analyses

SciP stability was assayed as previously described (Gora et al., 2010) with the following modifications. The various *sciP* alleles in strains ML1990, ML1991, ML1992, and ML1993 were induced through the addition of xylose for 30 minutes immediately before the pulse-chase. Samples were immunoprecipitated using 2  $\mu$ L of SciP anti-sera for 2 hrs at 4 °C.

### DNA microarray analysis

Microarray analyses were conducted as previously reported using custom Agilent oligo arrays (Gora et al., 2010). Expression experiments were performed in duplicate and results averaged.

### Protein purifications

For purifications of SciP from *E. coli*, overnight cultures were diluted into LB with ampicillin (100  $\mu$ g/ml), grown for 2–3 hours at 37 °C, and then induced with 0.4 mM IPTG. After 3 hours, cells were spun down at 5000 rpm for 15 minutes. Pellets were resuspended in lysis buffer (50mM Tris pH 8.0, 300mM NaCl, 10mM imidazole, 10% glycerol, 5mM  $\beta$ -mercaptoethanol) and frozen at –20 °C. Cells were then thawed on ice before being lysed by high pressure disruption using a Microfluidizer (Microfluidics, Newton, MA). Following clarification by centrifugation at 15,000 *g*, lysate was applied to a 1 mL Ni-NTA column pre-equilibrated with lysis buffer at room temperature. The column was washed with 20 volumes of lysis buffer, followed by elution with 6 column volumes of elution buffer (50mM Tris pH 8.0, 300mM NaCl, 300mM imidazole, 10% glycerol, and 5mM  $\beta$ -mercaptoethanol). Purity was assessed by SDS-PAGE / Coomassie staining and samples containing the desired protein were pooled, concentrated, and aliquoted before being frozen at –20 °C. ClpX, ClpP, and CtrA were purified as previously described (Chien et al., 2007) (Rood et al., 2012). *Caulobacter* Lon was expressed from plasmid pBAD-*lon* in a  $\Delta$ *Lon E. coli* strain (ER2566) and purified following the protocol for purification of *E. coli* Lon (Gur & Sauer, 2009).

### *In vitro* degradation assays

Protein degradation was monitored using SDS-PAGE as before (Rood et al., 2012). For ClpXP degradation reactions, the concentrations were as follows, unless otherwise noted:

0.2  $\mu\text{M}$  ClpX<sub>6</sub>, 0.4  $\mu\text{M}$  ClpP<sub>14</sub>, 4 mM ATP, 75  $\mu\text{g}/\text{mL}$  creatine kinase, 5 mM creatine phosphate in H-buffer (25mM HEPES-KOH pH 7.4, 100 mM KCl, 10% glycerol, 10mM MgCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol). For Lon degradation reactions, concentrations of the components were the same as above, but with 0.5  $\mu\text{M}$  Lon hexamer as the protease and Lon degradation buffer (25 mM Tris pH 8.0, 100 mM KCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT). Proteolysis was also monitored by fluorescence, as previously described (Rood et al., 2012). Degradation of a GFP-CtrA fusion was measured via loss of fluorescence over time at 30 °C. Final concentrations in these assays were: 0.2  $\mu\text{M}$  ClpX<sub>6</sub>, 0.4  $\mu\text{M}$  ClpP<sub>14</sub>, 4 mM ATP, 75  $\mu\text{g}/\text{mL}$  creatine kinase, 5 mM creatine phosphate, 50 nM GFP-CtrA, and 1  $\mu\text{M}$  SciP. Concentrations of DNA were varied over the ranges reported in the figures. Typical reaction volumes were 20  $\mu\text{L}$  and were monitored with a Spectramax M5 (Molecular Devices) plate reader with 440 nm / 510 nm excitation / emission settings and an emission cutoff set at 495 nm.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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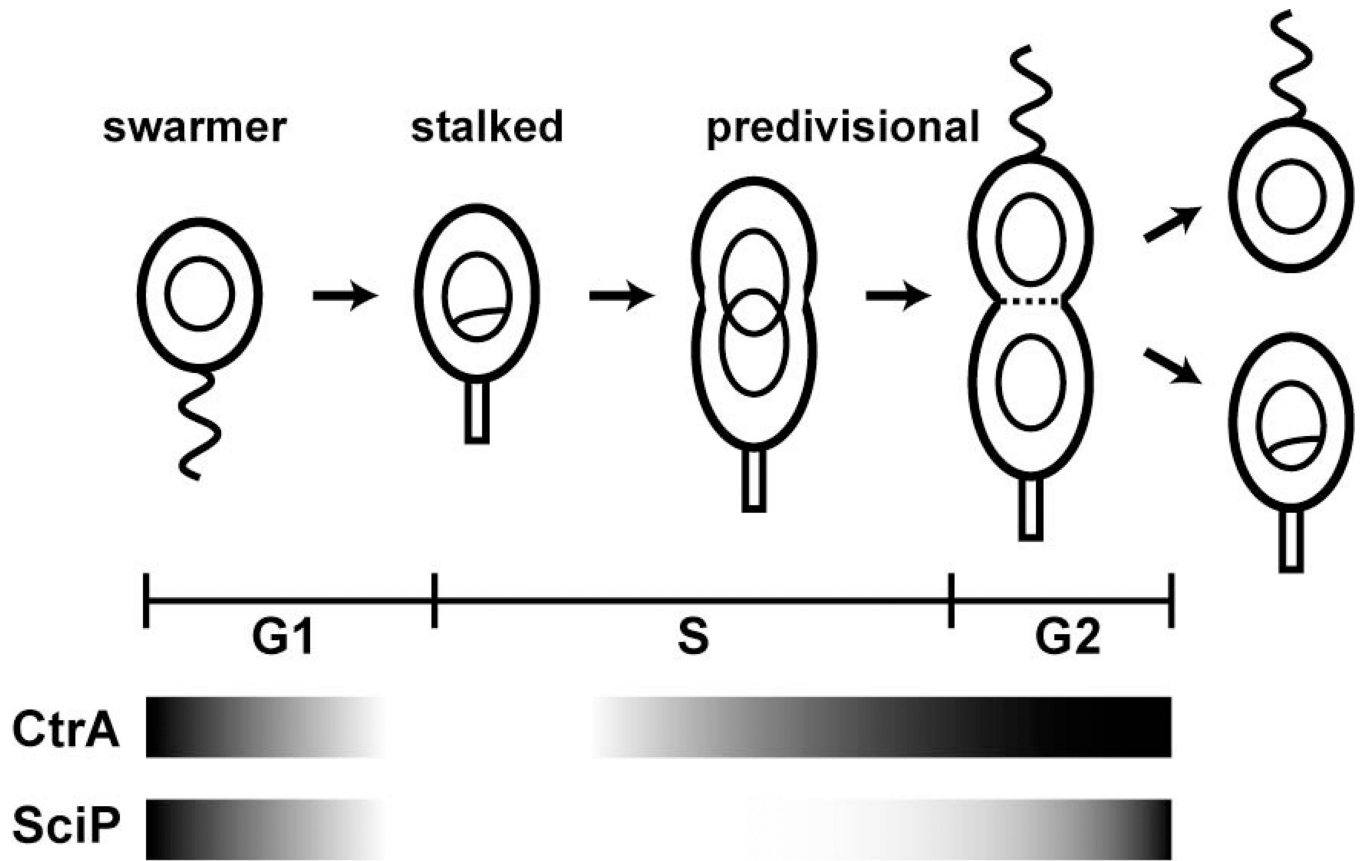
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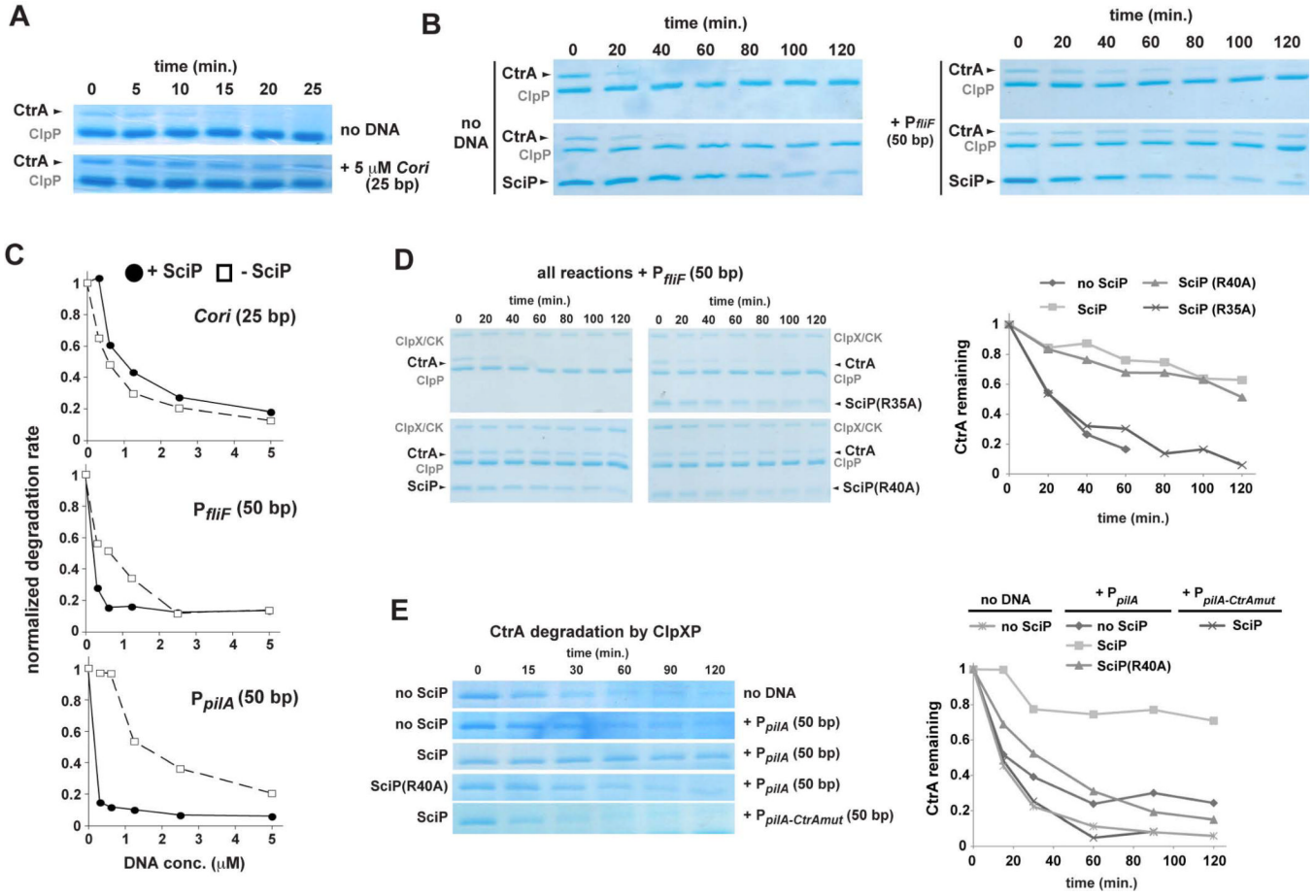
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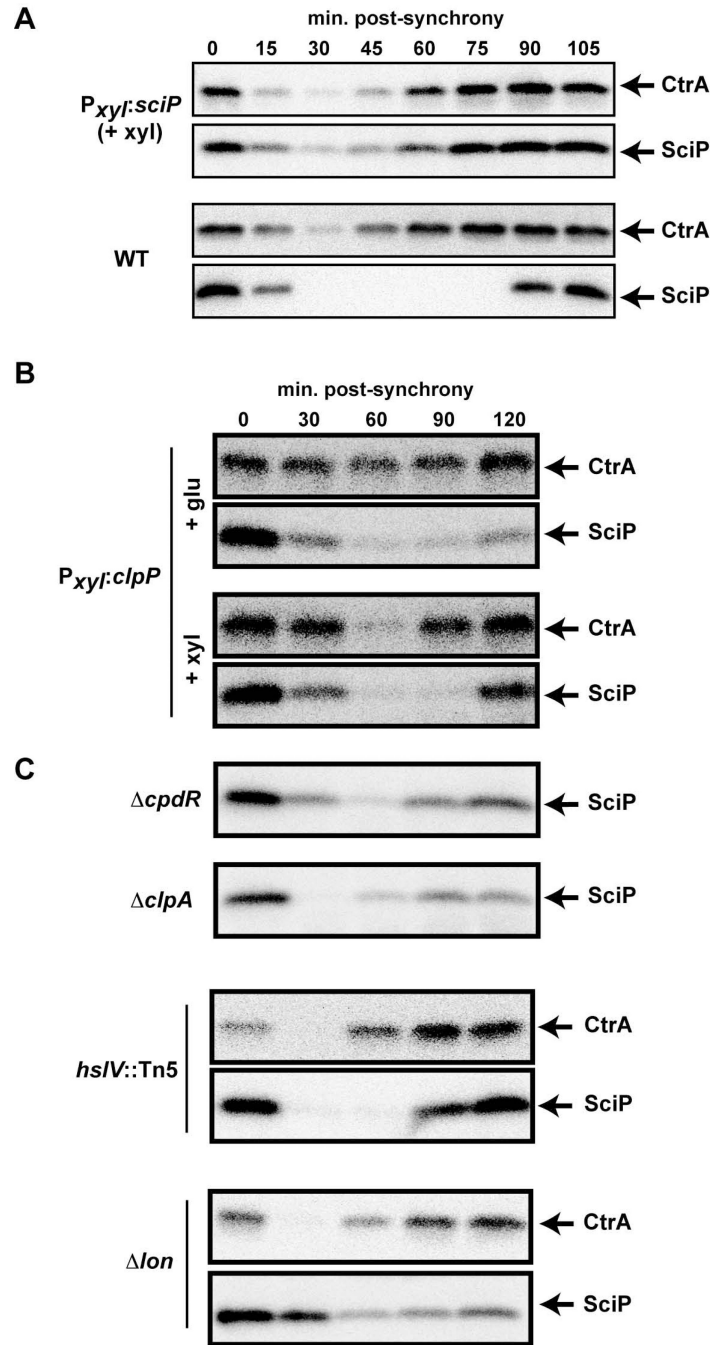


**Figure 1. Schematic of the *Caulobacter* cell cycle**  
 The timing of CtrA and SciP accumulation is indicated with black bars.



**Figure 2. CtrA is protected from ClpXP degradation when bound to DNA**

(A) Time courses of 1  $\mu\text{M}$  CtrA degradation by ClpXP in the presence or absence of 5  $\mu\text{M}$  DNA containing a CtrA binding site derived from *Cori* (25 bp in length). (B) Time courses of 1  $\mu\text{M}$  CtrA degradation by ClpXP in the presence of 1  $\mu\text{M}$  *PflIF* (50 bp fragment) and 5  $\mu\text{M}$  SciP, as indicated. (C) Degradation of GFP-CtrA was measured as a function of DNA concentration for fragments from *Cori*, *PflIF*, and *PpilA* with or without 1  $\mu\text{M}$  SciP. (D) Time courses of CtrA degradation (3.2  $\mu\text{M}$ ) by purified ClpXP in the presence of 5  $\mu\text{M}$  wild-type or mutant SciP and 5  $\mu\text{M}$  *PflIF*. Quantification of CtrA levels over time are shown on the right. (E) Degradation of CtrA (0.5  $\mu\text{M}$ ) by ClpXP with wild-type (*PpilA*) or mutant (*PpilA-CtrAmut*) probe at 10  $\mu\text{M}$  (when added) and SciP or SciP(R40A) at 10  $\mu\text{M}$ . Quantification of CtrA levels over time are shown on the right.

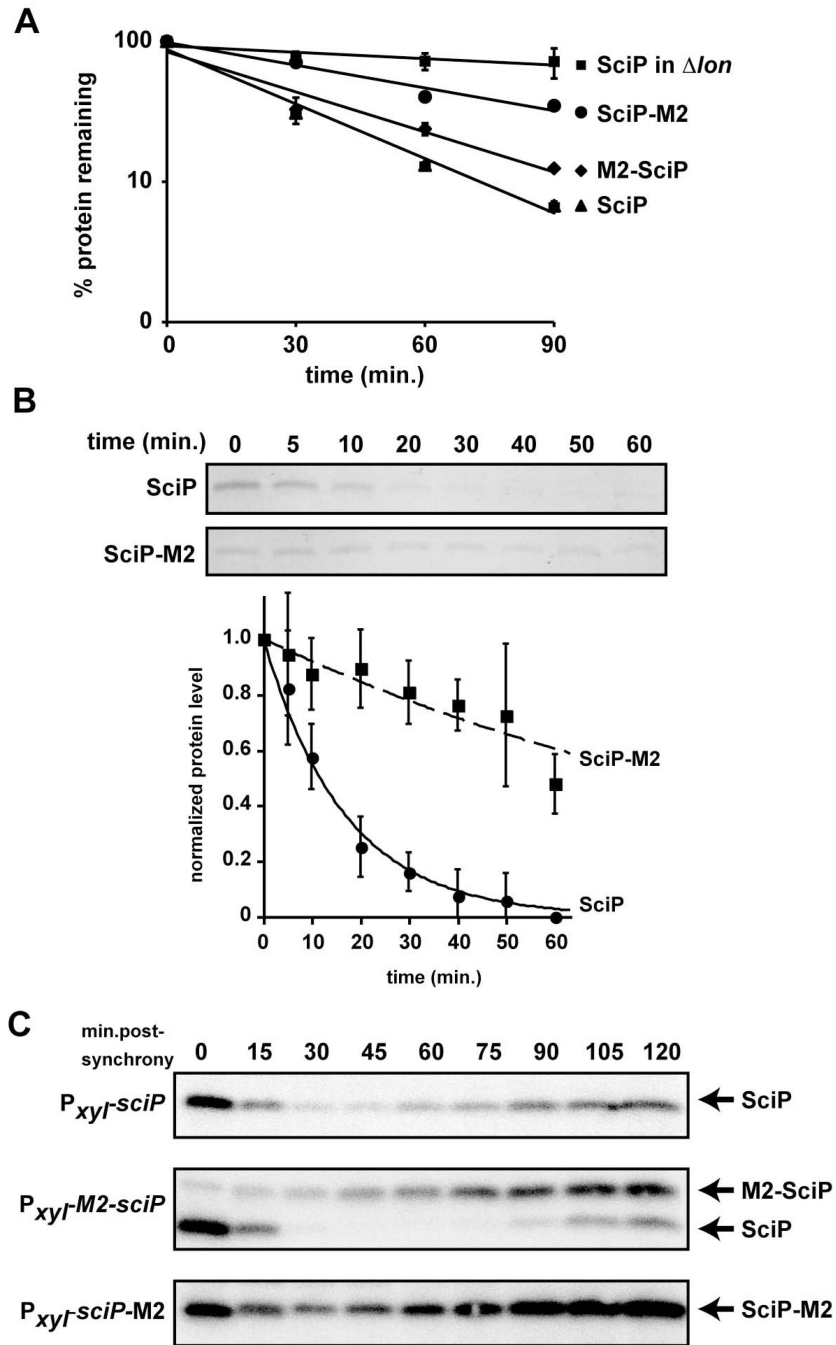


**Figure 3. SciP is an unstable protein whose degradation *in vivo* depends on Lon**

(A) Swarmer cells expressing *sciP* constitutively (cells harboring  $P_{xyI}::sciP$  and grown in the presence of xylose) were isolated and allowed to proceed synchronously through a single cell cycle. SciP and CtrA levels were assessed using Western blotting at the time points indicated (top blots) and compared to synchronized wild-type cells. (B) A *clpP* depletion strain was grown in rich medium with xylose and then washed and resuspended in rich medium with glucose for 6 hours to deplete ClpP. Cells were then synchronized and released into rich media with glucose or xylose, as indicated, and samples were taken at 30 minute intervals for Western blot analysis with CtrA and SciP antisera. (C) Cultures of  $\Delta cpdR$ ,  $\Delta clpA$ ,  $hsIV::Tn5$ , and  $\Delta lon$  were each synchronized and cells released into fresh media

with samples taken every 30 minutes for Western blot analysis of CtrA and SciP levels, as indicated.

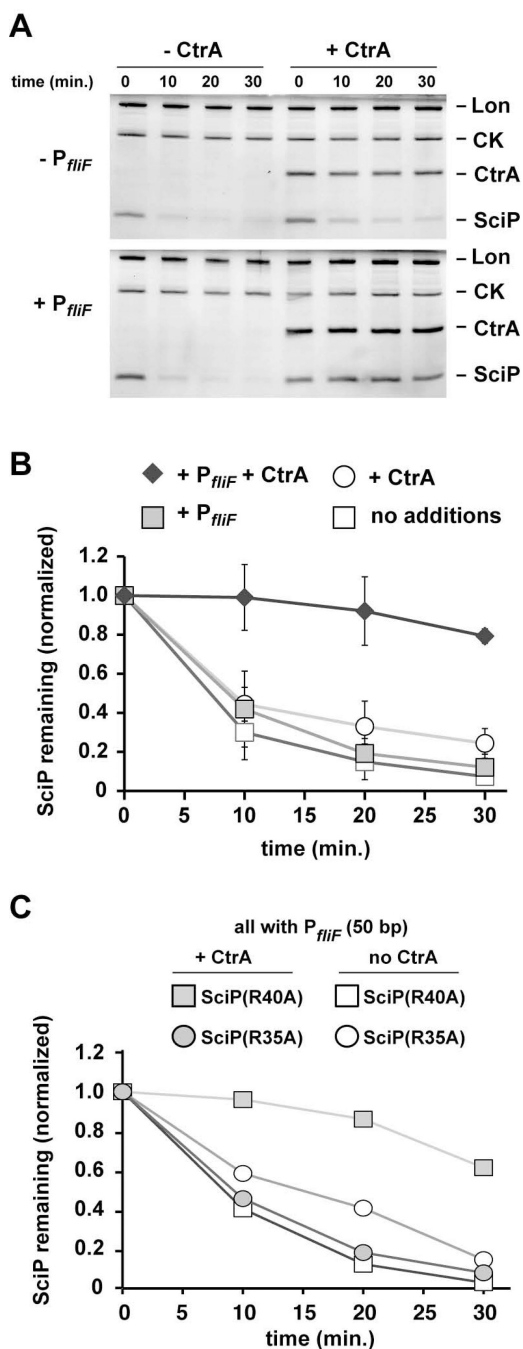




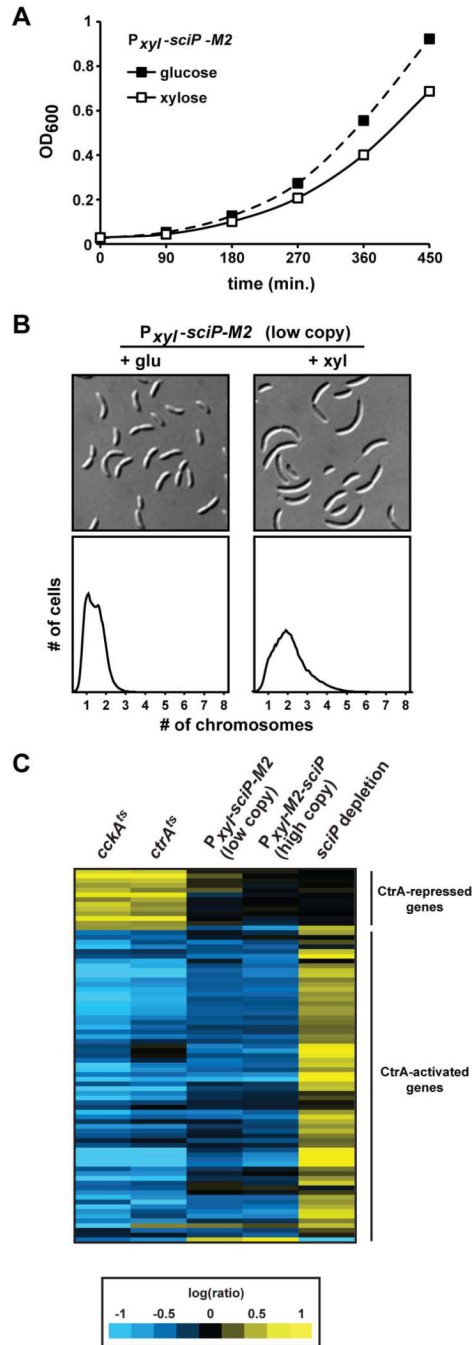
**Figure 4. A C-terminal tag on SciP disrupts degradation by Lon**

(A) Pulse-chase analysis of SciP stability in the strains indicated. Wild type or  $\Delta lon$  cells producing the indicated version of SciP from a xylose-inducible promoter on a low-copy plasmid were grown in minimal media and pre-induced with xylose for 30 minutes. Each experiment was performed in duplicate or triplicate and error bars indicate standard error of the mean. (B) Time courses of degradation of SciP (0.5  $\mu$ M) or SciP-M2 (1  $\mu$ M; note that purified SciP-M2 stains poorly) by purified *C. crescentus* Lon (0.5  $\mu$ M). Graph indicates averages of triplicate experiments fit to single exponential decay curves; error bars are standard deviations. (C) Cells producing untagged or M2-tagged versions of SciP from a xylose-inducible promoter were grown in the presence of glucose, synchronized, and then

released into fresh medium containing xylose. Samples were taken every 15 minutes for Western blotting with an anti-SciP antibody. Note that the linker between M2 and SciP is shorter when the epitope tag is C-terminal to SciP leading SciP-M2 to run closer to native SciP.

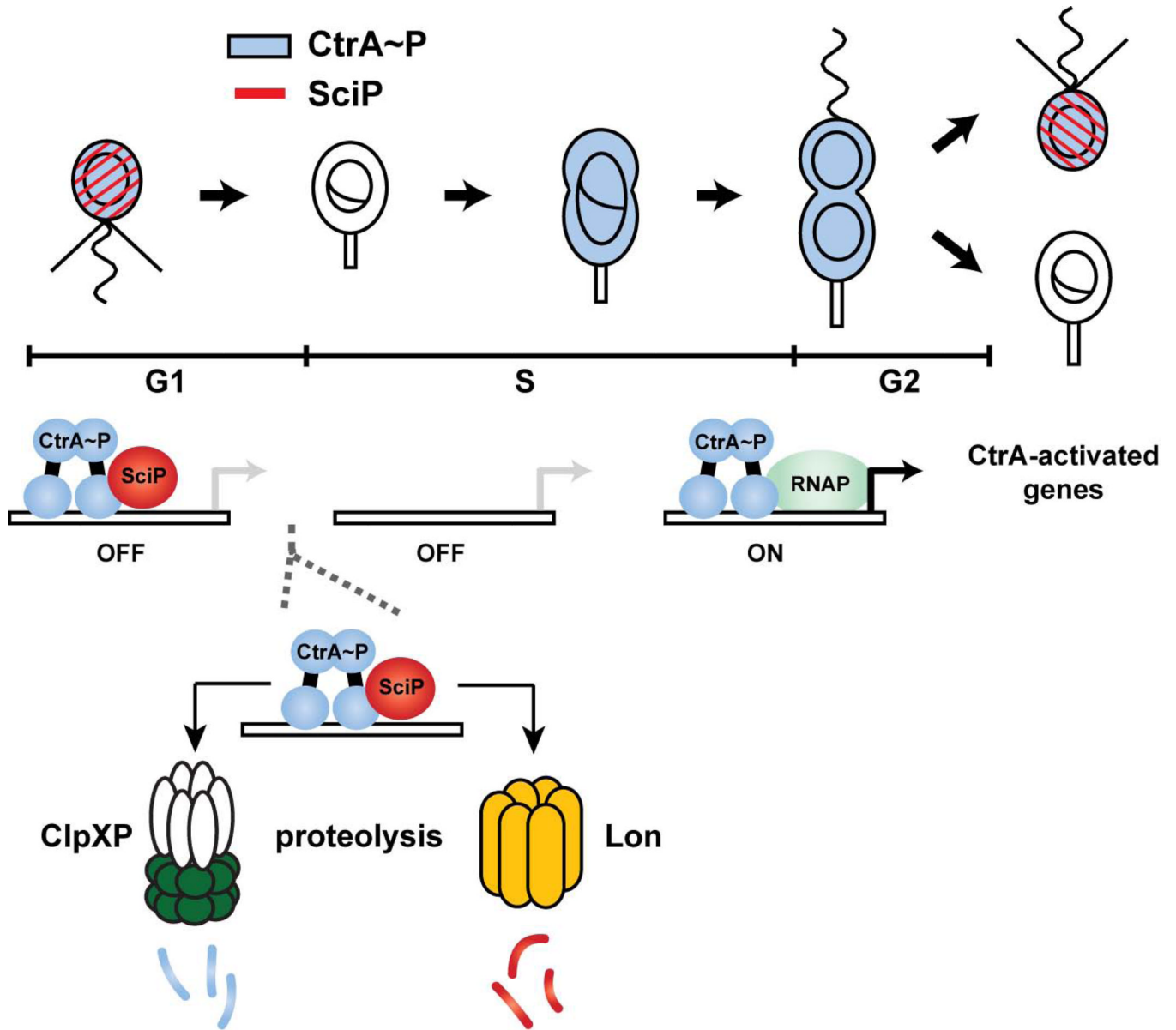


**Figure 5. Degradation of SciP by Lon is inhibited by formation of a SciP:CtrA:DNA complex (A-B)** Time courses of SciP degradation by Lon. CtrA and a  $P_{fiiF}$  probe were added, as indicated. CK is creatine kinase used for ATP regeneration. Graph indicates averages from time courses performed in triplicate; error bars are standard deviations. **(C)** Time courses of degradation of SciP mutants by purified Lon in the presence of  $P_{fiiF}$  fragment with or without CtrA, as indicated. When noted, SciP variants, CtrA, and  $P_{fiiF}$  are present at 5  $\mu$ M. Lon is present at 0.5  $\mu$ M.



### Figure 6. Stabilization of SciP affects cell cycle progression

(A) Growth curve for cells harboring a low-copy plasmid with *sciP-M2* driven by a xylose-inducible promoter and grown in the presence of glucose or xylose. (B) DIC micrographs and flow cytometry profiles for cells from panel A. (C) The expression levels of CtrA-regulated genes were measured by DNA microarrays in cells induced to produce SciP-M2 from a low-copy plasmid relative to an uninduced control. Ratios for each gene were compared to those generated previously for *cckA<sup>ts</sup>* and *ctrA<sup>ts</sup>* strains grown at a restrictive temperature for 2 hours, a strain producing M2-SciP from a high-copy plasmid, and a *sciP* depletion strain grown in restrictive conditions (Gora et al., 2010). Log expression ratios are represented as colors based on the legend shown.



**Figure 7. Regulated proteolysis of CtrA and SciP during the *Caulobacter* cell cycle**

A schematic of the *Caulobacter* cell cycle is shown along with the approximate timing of G1, S, and G2 phases. The abundances of phosphorylated CtrA (CtrA~P) and SciP during the cell cycle are indicated by blue shading and red stripes, respectively. In swarmer cells, SciP binds phosphorylated CtrA to block CtrA-activated gene expression. During the G1-S transition, CtrA~P and SciP are degraded by ClpXP and Lon, respectively. The subsequent accumulation of phosphorylated CtrA, but not SciP, in predivisive cells enables the activation of CtrA target genes.