2012

# Proteolytic Regulation of CtrA, the Master Regulator of Cell Cycle in Caulobacter crescentus

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# PROTEOLYTIC REGULATION OF CTRA, THE MASTER REGULATOR OF CELL CYCLE IN *CAULOBACTER CRESCENTUS*

A Thesis Presented

by

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Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2012

Biochemistry

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ABSTRACT

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SEPTEMBER 2012

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Cell cycle progression in *Caulobacter crescentus* is absolutely dependent on the master regulator, CtrA. During cell cycle, *C. crescentus* undergoes a morphological change, moving from a motile swarmer cell to a stalked cell capable of replicating. CtrA plays a critical role in this progression, as it directly or indirectly controls the expression of at least 100 genes in the *C. crescentus* genome and represses replication in the swarmer cells (G1 phase). CtrA is degraded during the swarmer to stalk cell transition (G1 to S phase) and levels rise again in the predivisional stage. The goal of my thesis project is to explore how cyclic, regulated degradation of CtrA is controlled, a question that is still not fully understood. CtrA is known to bind to the origin of replication, thereby repressing replication, so I initially asked if DNA binding had an effect on CtrA stability. On its own, CtrA is readily degraded by the ClpXP protease, a AAA+ protease in *C. crescentus*. However, upon binding to double-stranded DNA containing proper binding sites, CtrA is unable to be degraded. Stabilization is dependent on DNA binding, as mutants of CtrA deficient in DNA binding show the same degradation regardless of addition of DNA, as does CtrA in the presence of a mutant origin sequence that lacks CtrA binding sites. DNA binding cannot be the only control of CtrA as some mechanism must signal CtrA to come off the DNA in order to be degraded. Based on this reasoning, I began exploring what other endogenous factors present in *C. crescentus* may play a role in the degradation of CtrA. The SciP protein has been shown to interact with CtrA and cause increased complex formation of CtrA with DNA. I found that SciP can enhance CtrA

stabilization in the presence of DNA and that SciP is a substrate of another AAA+ protease present in *C. crescentus*, known as Lon. Interestingly, the CtrA-SciP-DNA complex appears to protect SciP from Lon degradation. Looking closely at CtrA degradation in the presence of auxiliary factors suggests that higher order complex formation may be a mechanism of protecting critical cell cycle regulators from premature proteolysis. *In vivo* other factors are also known to be important for the regulated degradation of CtrA, but the overall control of this process is not well understood. In addition to my work *in vitro* studying CtrA degradation, I also looked at the regulation *in vivo*. Over-expression of a variant of CtrA that cannot be degraded, CtrA-DD, perturbs the cell cycle and leads to filamentation and a G2 arrest. This is likely due to the consequences of having too much CtrA around acting as a transcription factor and activating or repressing genes at inappropriate times during the cell cycle. Somewhat surprisingly, this does not affect the cells ability to progress through the G1-S and S-G2 portions of the cell cycle, so somehow the cells are able to clear CtrA-DD from the origin if it is bound there. The cells are, however, deficient in progressing past G2 and this leads to filamentation. Over-expression of the DNA-binding domain of CtrA alone shows a similar filamentation defect but no G2 arrest, suggesting that the full length non-degradable CtrA is detrimental for reasons including, but likely not limited to, its ability to bind DNA. Exogenously expressing other domains of CtrA may further elucidate the mechanism of its degradation *in vivo*.

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# CHAPTER 1

## INTRODUCTION

Cell cycle progression is a critically important and tightly controlled process in all cells. Cells must undergo several changes during this time, such as DNA replication, growth, and division. These changes are often accompanied by changes in protein levels due to changes in transcription or due to changes in proteolysis. During growth, several factors are present that can keep the cell in this state until the proper conditions for replication and division are met. These factors must be degraded once the cell enters the replication phase so that other proteins can signal DNA synthesis to begin. Once replication is complete, other proteins must be present to segregate the chromosomes and allow for proper cell division. Improper regulation of any of these factors can be detrimental to cell health and viability [10]. These changes could be relatively minor, such as filamentation defects, or could be more serious, ultimately leading to cell death. The focus of my thesis research has been on studying this delicate balancing act that comprises cell cycle progression in *Caulobacter crescentus*.

*C. crescentus* is a gram negative α-proteobacterium found in all fresh water sources [8]. It undergoes an obligate morphological shift during cell cycle. In the G1 phase, or initial growth phase, the *Caulobacter* cell is a swarmer, which possesses a flagellum and is capable of swimming around in search of nutrients. It is not, however, capable of replication when in this stage. Once proper nutrients are found, it can differentiate into a stalked cell (S or synthesis phase), shedding its flagellum and growing a stalk that allows for attachment to a surface. Only once it has undergone this transition can the *C. crescentus* cell replicate [1, 2].

The transition from G1-S phase is robust and accompanied by many changes within the cell. Importantly, during this transition many changes occur to the composition of the proteome. Proteolysis is critical during this stage because it ensures the complete and rapid clearance of proteins that are no longer necessary and may actually harm the cell if they remain around. *Caulobacter* contains several

important proteases responsible for accomplishing this rapid proteolysis [10]. One such protease is a AAA+ protease known as ClpXP. ClpXP is composed of the hexameric ClpX and the tetradecameric ClpP. ClpX is an unfoldase and is responsible for delivering substrates to ClpP, the peptidase, which then degrades them. One function of ClpXP is to clear the cell of polypeptides arising from faulty mRNA transcripts, which are marked with an ssrA-tag [3]. ClpXP also plays a role in the turnover of cell cycle regulators during cell cycle progression. Importantly, it is responsible for the degradation of a protein known as CtrA, often called the master regulator of cell cycle progression [3]. Another important AAA+ protease is Lon. Its main function is to degrade misfoldeded proteins, likely through the recognition of hydrophobic sequences that act as a tag and are hidden in properly folded proteins [7]. It also can play a role in degrading cell cycle regulators, such as SciP, which can affect CtrA activity.

CtrA is known to bind to the origin to inhibit replication and can also act as a transcriptional regulator of approximately 100 genes [6]. It is degraded by the ClpXP protease during the swarmer to stalked transition and levels are restored again in predivisional cells. Figure 1A shows the cell cycle of *C. crescentus*, including the Western blot following CtrA levels over time. The focus of my thesis work has been on attempting to elucidate the way in which the regulated degradation of CtrA is controlled. This is particularly difficult because the requirements for degradation *in vivo* do not match the requirements for degradation *in vitro*. *In vivo*, CtrA requires many factors in order to be efficiently degraded. For example, CpdR is known to be critical for degradation *in vivo* though it is unclear why this is the case [9]. Other important factors include RcdA and PopA, two proteins which appear to help localize CtrA so it can be efficiently degraded during the swarmer to stalk transition [5, 19]. However, *in vitro*, only ClpXP and adenosine triphosphate (ATP), which powers ClpXP, are required for degradation of CtrA. This poses challenges to identifying what factors are important for CtrA in the context of the cell. Since CtrA binds DNA, I initially explored if DNA played a role in CtrA degradation. It is known that several regulatory proteins found in other organisms bind DNA and cannot be proteolyzed when bound. One such

transcriptional regulator is ZntR, found in *E. coli*, which is capable of binding DNA and its degradation is inhibited when bound [16]. I found that DNA binding can inhibit CtrA degradation. Based on this I began exploring other factors that might play a role in CtrA degradation. Presumably other factors must be around to signal CtrA to dissociate from the DNA so that it can be recognized and degraded by ClpXP.

Study of CtrA led to the observation that while it is phosphorylated in both swarmer and predivisional cells, genes activated by CtrA are typically only transcribed in predivisional cells[6]. This led the Laub and Shapiro labs to investigate why this was the case. The Laub lab looked for genes that were coinherited, colocated, or coexpressed with *ctrA*. This led to the identification of a new gene, *sciP*, that encodes a 93 amino acid protein of previously unknown function. SciP is well conserved among a-proteobacteria, with orthologs being found in almost all a-proteobacteria and with most orthologs being 70% identical and 85% similar to the one found in *Caulobacter*. In *Caulobacter*, SciP was found to be capable of modulating CtrA activity. The Laub lab observed that depleting cells of SciP caused expression of genes activated by CtrA, while overexpression of SciP had the opposite effect. Genes repressed by CtrA were unaffected by either the overexpression or depletion of SciP. Depletion of SciP led to filamentation of cells, a common effect of perturbed CtrA activity. They found that SciP is also degraded during the swarmer-to-stalked transition [Figure 1A] and it is only found in swarmer cells [6]. This provided further evidence that SciP is likely functioning to inhibit CtrA's ability to activate genes in swarmer cells. Figure 1C provides a model for how this might occur during cell cycle.

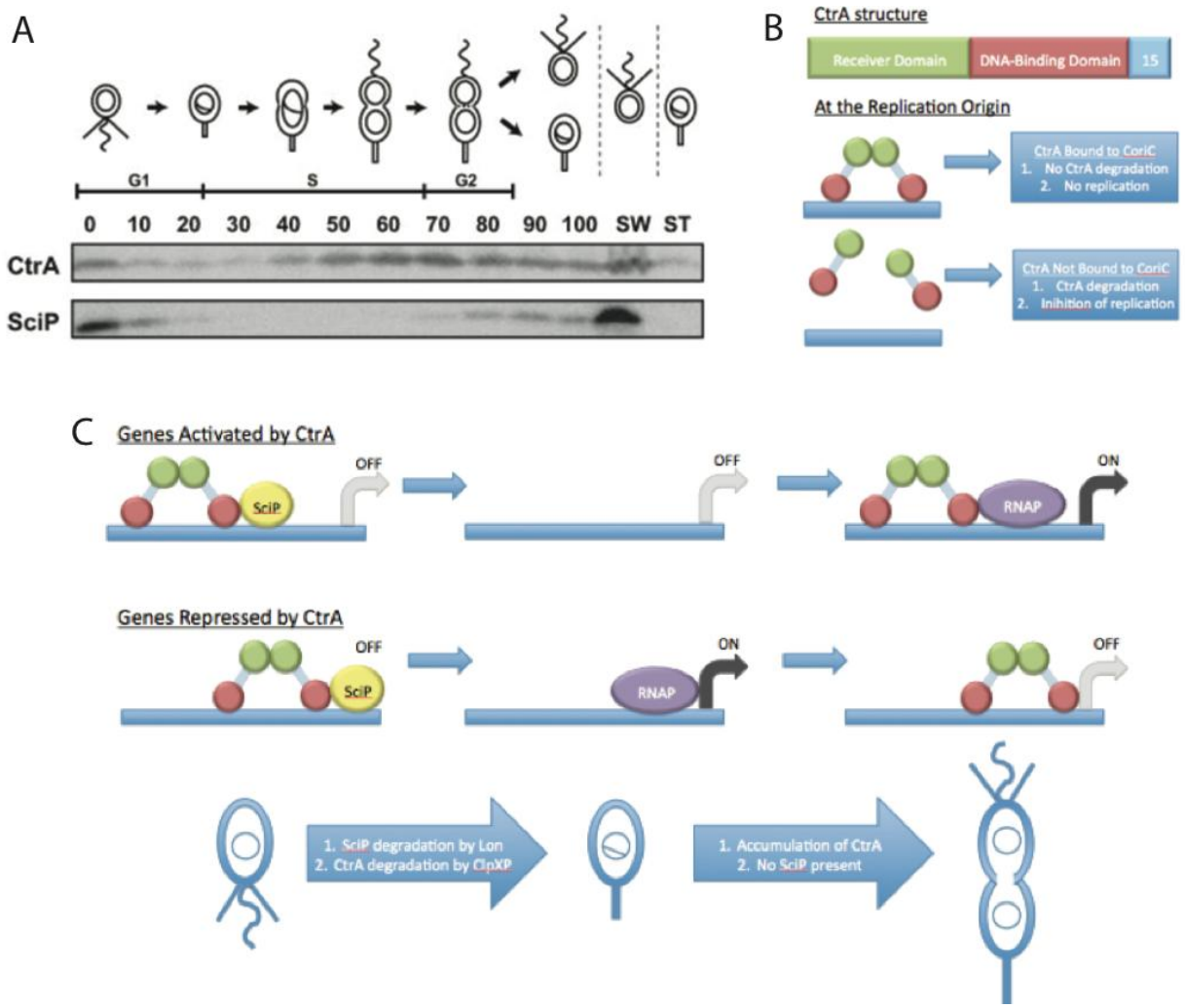


**Figure 1:** Function of CtrA and SciP. **A)** Cell cycle of *C. crescentus* with corresponding Westerns of CtrA and SciP levels. CtrA and SciP are both degraded at the swarmer to stalk transition. CtrA levels rise again in predivisional cells, while SciP levels do not. (From Gora, et al. 2010.) **B)** CtrA structure and binding at the origin. CtrA is composed of the receiver domain, the DNA-binding domain and the 15 residue degradation tag. At the origin, CtrA binds the DNA via the DNA-binding domain, blocking replication. CtrA must come off the DNA in order for proper proteolysis and cell cycle progression to occur. **C)** SciP functions as a regulator of CtrA. In genes activated by CtrA, SciP complexing with CtrA on the DNA turns these genes off, likely by preventing polymerase recruitment.

# CHAPTER 2

## CTRA DEGRADATION BY CLPXP IS INHIBITED BY DNA BINDING

I began my study of CtrA by exploring which parts of CtrA may play a role in its degradation by ClpXP. As shown above [Figure 1], CtrA has three main domains: the receiver domain, the DNA-binding domain, and a 15-residue degradation tag. In order to determine which parts were important for degradation, I made GFP-tagged versions of the various parts of CtrA. The proteins I tested were GFP-CtrA, GFP-RR15 (the receiver domain plus the 15-residue degradation tag), GFP15 (GFP with just the last 15 CtrA residues) and GFP-DBD (the DNA-binding domain, which contains the 15 residue degradation tag). The RR15, GFP15, and DBD proteins have been previously tested *in vivo* for their ability to be degraded. The results showed that while RR15 is degraded in a cyclic fashion, GFP15 and DBD are not, indicating that the receiver domain is required for regulated degradation [18]. Based on these results, I sought to test these constructs *in vitro* to test if there was a difference in their *in vitro* degradation that might explain the difference in *in vivo* degradation. I first tested the GFP-CtrA protein in a gel based degradation assay versus untagged full-length CtrA to ensure that the degradation was similar. GFP-tagged proteins can be excellent reporters for monitoring real-time degradation and to determine degradation kinetics but only if the GFP-tag does not affect protein degradation. After testing the tagged versus the untagged CtrA protein, I found that the degradation of both were almost identical [Figure 2A]. Based on this I moved forward with testing all the GFP-tagged proteins I had made in a fluorescence based degradation assay. As shown in Figure 2, the degradation kinetics of each construct are similar. The Vmax for each is almost identical, while the Km values differ. For the GFP-RR15 and the GFP15, the Km values are nearly twice as high as that for GFP-CtrA or GFP-DBD. The Vmax is the maximum rate at which the enzyme can degrade the substrate once it has engaged the substrate for degradation. This indicates how quickly the substrate is degraded by the enzyme, but does not indicate how well the enzyme can recognize the substrate for degradation. The Km, on the other hand, gives information

about how well the enzyme can recognize the substrate. The Km is defined at the substrate concentration at which the reaction rate is ½ of Vmax; a smaller Km indicates that the enzyme has high affinity for the substrate and so will reach the Vmax faster. The fluorescence data for the GFP-tagged constructs described above suggests that while all the fusion proteins can be efficiently degraded by ClpXP, they are not all recognized as well. The DNA-binding domain may play a small role in the recognition of CtrA for degradation. These results are interesting because it shows that the degradation kinetics of RR15 and GFP15 are not different *in vitro* even though their proteolysis *in vivo* is very different. This suggests that other factors must be present in the cell that must recognize the receiver domain in order for ClpXP to be able to degrade it in a cyclic fashion.

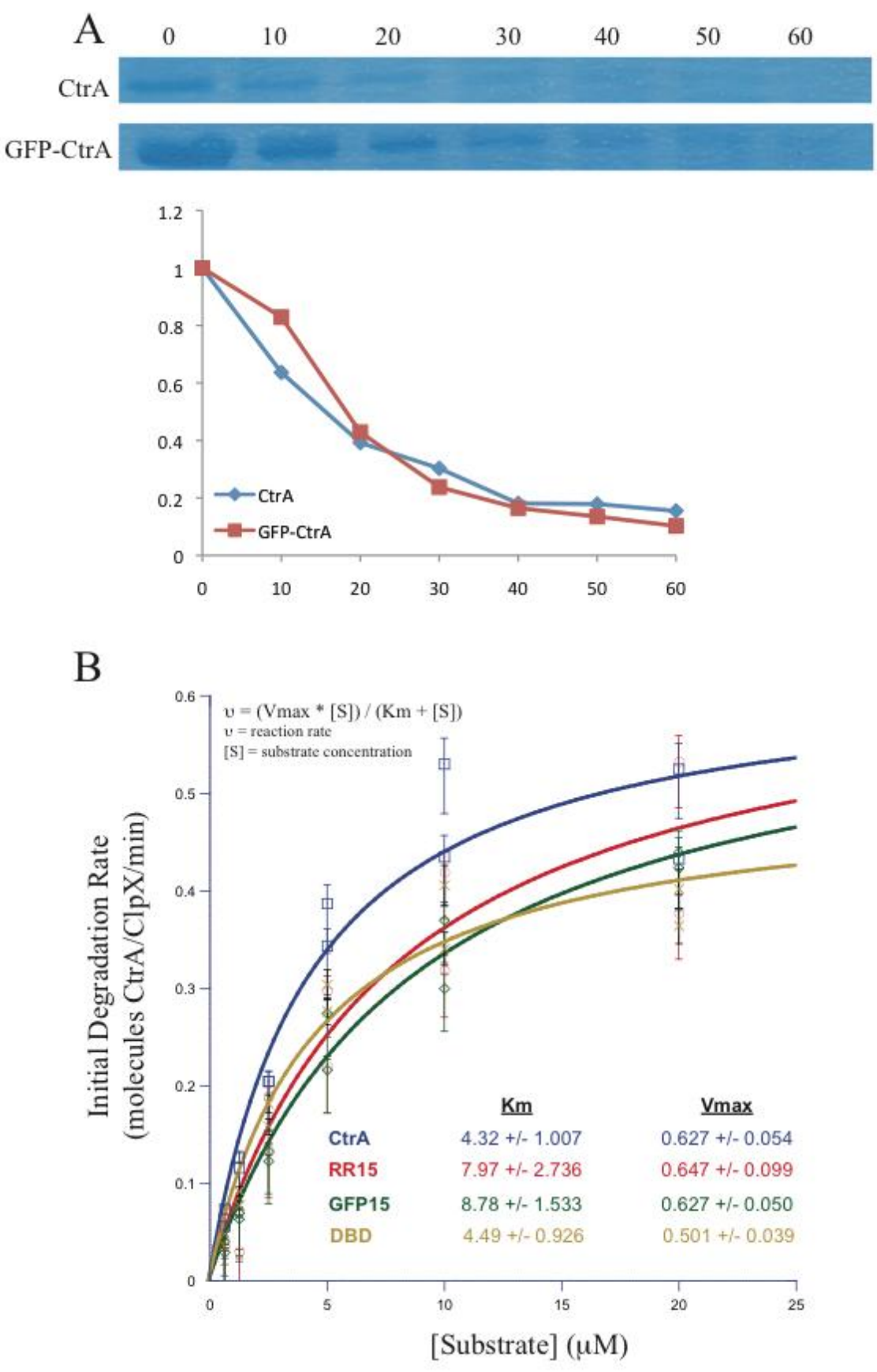


**Figure 2:** Degradation of GFP-tagged domains of CtrA. **A)** GFP-CtrA is degraded at the same rate as untagged CtrA. 5uM used for both proteins. **B)** Degradation curves for GFP-CtrA, GFP-RR15, and GFP15, and GFP-DBD. Curves are fit to the Michaelis-Menton equation shown at the top left of the graph. All four have very similar Vmax values but the Km for GFP-RR15 and GFP15 is roughly twice that of GFP-CtrA or GFP-DBD, indicating that the DNA binding domain has a potentially small but nonessential effect on ClpXP recognition.

Based on the fact that CtrA binds to DNA, I began looking at if DNA binding played a role in CtrA degradation. Since CtrA is known to bind to DNA containing the proper DNA binding sites, it seemed reasonable that this binding may play a role in its degradation. This hypothesis was also supported by the fact that several other known regulators bind DNA and are not degraded when bound. Using both gel-based and fluorescence-based degradation assays I explored whether DNA affected CtrA degradation. I found that DNA binding did indeed stabilize CtrA, preventing its degradation by ClpXP. Figure 3A shows the results of a fluorescence based assay using 50nM CtrA and 5uM CoriC. CoriC is a 25bp fragment of the origin containing the TTAA-N7-TTAA binding site for CtrA. The stabilization of CtrA in the presence of DNA is directly due to CtrA's ability to bind to the DNA. Using a similar CoriC fragment as described above, we mutated the TTAA half site to CAGG, which CtrA cannot bind to. In assays containing this mutant CoriC, CtrA was not stabilized, as shown in Figure 3D, along with the sequences of both the mutant and wild-type CoriC fragments. This fluorescence assay was run using 5uM CtrA and 5uM CoriC or CoriC mutant. I also tested fluorescently tagged mutants of CtrA that were deficient in DNA binding. These mutants, T213A and R198A, both occur in the DNA binding domain and prevent CtrA from binding. Previous studies in proteins similar to CtrA found that these residues in the DNA-binding domain appear critical for the protein's interaction with DNA and mutating them to alanine abolishes their ability to bind DNA. Mutating either the R201 to A or the T194 to A in the DNA-binding domain of the PhoB transcriptional regulator prevented it from binding to the DNA [15]. In the response regulator KdpE, a PhoB-like protein, mutating R193, R200, or T215 to A prevented DNA binding [14]. Mutating the T19 to A in the DNA-binding domain of ZntR also prevented it from binding to DNA and as a result prevented it from being stabilized in the presence of DNA [16]. This provided evidence that the T213 and R198 residues in CtrA were critical for interaction with DNA and mutating them would interfere with DNA binding. As expected, neither of these mutants in CtrA is stabilized in the presence of DNA, further showing that DNA-binding is important for the observed stabilization [Figure 3D].

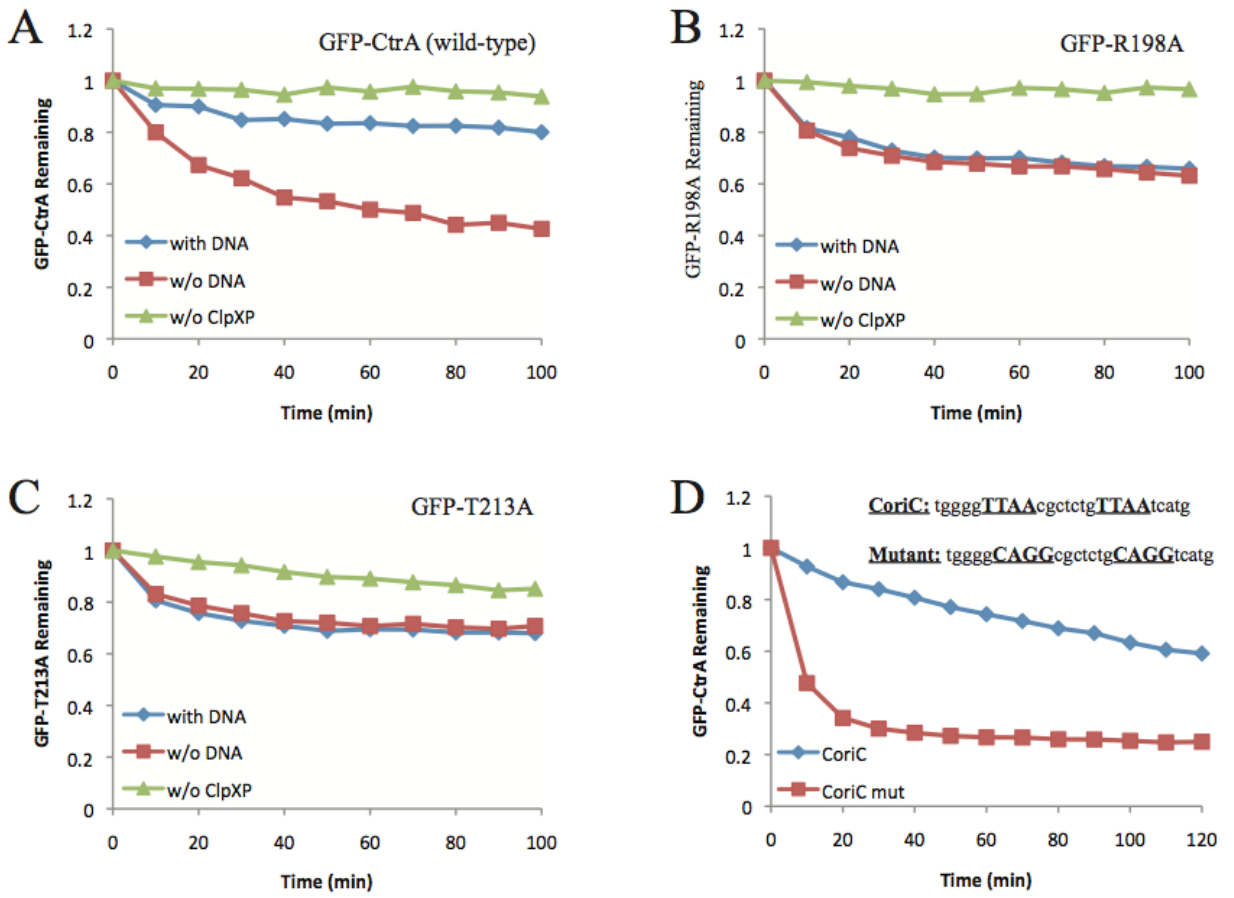


**Figure 3:** Degradation of GFP-tagged mutants of CtrA. All values have been normalized. **A)** GFP-CtrA in the presence or absence of DNA. CtrA in the presence of DNA is not degraded by ClpXP. 50nM GFP-CtrA and 5uM DNA used. **B)** GFP-R198A in the presence or absence of DNA. R198A is unable to bind DNA and is not stabilized in the presence of DNA. 50nM GFP-R198A and 5uM DNA used. **C)** GFP-T213A in the presence and absence of DNA. T213A is also not stabilized by DNA because it is deficient in DNA binding. **D)** GFP-CtrA in the presence of wild-type or mutant CoriC. In the mutant, all the binding sites for CtrA are mutated, as shown in the inset in the top graph. Binding sites are in caps. CtrA is not stabilized in the presence of mutant CoriC, providing further evidence that DNA-binding plays a role in CtrA stabilization. 500nM GFP-CtrA and 5uM DNA used.

CtrA is degraded efficiently *in vitro* by the ClpXP protease in the absence of other factors. The 15 amino acid degradation tag of CtrA appears to be sufficient for this degradation, as the GFP-RR15 and GFP15 are both degraded with Vmax rates similar to that of GFP-CtrA. The differences in Km values between GFP-RR15 and GFP15 compared to GFP-CtrA and GFP-DBD indicate that the RR15 and 15-residue tag might not be recognized as efficiently as the full length CtrA or DBD by ClpXP. Furthermore this indicates that the DNA-binding domain may play a small role in recognition by the protease. DNA-binding by CtrA plays a role in its stability *in vitro*. In the presence of DNA, CtrA degradation is inhibited. This inhibition is directly related to CtrA's ability to bind DNA. CtrA in the presence of CoriC with mutated binding sites is not stabilized, nor are mutants of CtrA that are deficient in DNA binding. DNA-binding clearly plays a role in CtrA degradation *in vitro* and may be one of the many controls on CtrA degradation *in vivo*. However, DNA-binding cannot be the only control. As mentioned above, many other factors are required for degradation *in vivo*. Also, another study showed that DNA-binding is likely not playing a large role in the cells. They tested the receiver domain, the receiver domain plus the degradation tag, and the DNA-binding domain (which contains the degradation tag) *in vivo* to see if these proteins were cyclically degraded. RR was not degraded at all, nor was the DBD. However, the RR15 was degraded in a regulated fashion similar to full-length CtrA even though RR15 cannot bind DNA [18]. This means that other signals must work to cause the regulated degradation of CtrA in *Caulobacter*. This led me to investigate other factors that might be important for regulated degradation which, as stated above, led me to study SciP.

# CHAPTER 3

## CTRA STABILIZATION IS ENHANCED BY SCIP

Using both gel based and fluorescence *in vitro* assays, I explored the role of the SciP protein in the regulation of CtrA degradation. Previous data had shown that SciP interacts with CtrA, based on yeast two-hyrbid data. Also, overexpression of SciP caused stabilization of CtrA *in vivo* [6]. This suggested that the interaction between CtrA and SciP is functioning to stabilize CtrA. In the previous chapter I had shown that CtrA is stabilized by DNA but the concentration of DNA must be high enough to promote binding. In the assays in this chapter, the DNA is used at a concentration that is not sufficient by itself to stabilize CtrA. However, in a complex with CtrA and DNA of at least 50bp, SciP can stabilize CtrA [Figures 4 and 5]. While SciP alone is not enough to stabilize CtrA, in the presence of DNA, addition of SciP further stabilizes CtrA [Figure 4]. This is due to SciP forming a complex with CtrA and DNA. The DNA must be of sufficient length for this complex to form. In the presence of 25bp DNA, the further stabilization by SciP was not observed; in the presence of 50bp DNA, the complex was able to form and further protect CtrA from proteolysis. The ability of CtrA to form a complex in the presence of 50bp DNA but not 25bp DNA was previously shown by our collaborators in the Laub lab using *in vitro* gel-shift mobility assays [6]. Adding CtrA to a fragment of DNA containing the proper binding sites caused a shift in DNA mobility. Adding SciP to that mix in the presence of 50bp DNA caused an even greater shift in mobility, suggesting that all three were forming a complex. This super-shift was not observed in the presence of only 25bp DNA. The length of DNA appears to be playing some role in stabilization but is not dependent on the specific number of base pairs. The 25bp DNA is simply not long enough for SciP to form the interactions with both CtrA and DNA required to form the stabilizing complex. The 50bp DNA is long enough for this complex to form; a super-shift corresponding to the complex is seen and CtrA is protected from degradation. Mutants of SciP show differences in their ability to form this complex, in

accordance with the yeast two-hybrid data [6]. The SciP mutant R35A in completely unable to form this complex while the R40A mutant is closer to wild-type in its ability to form this complex [figure 5D].

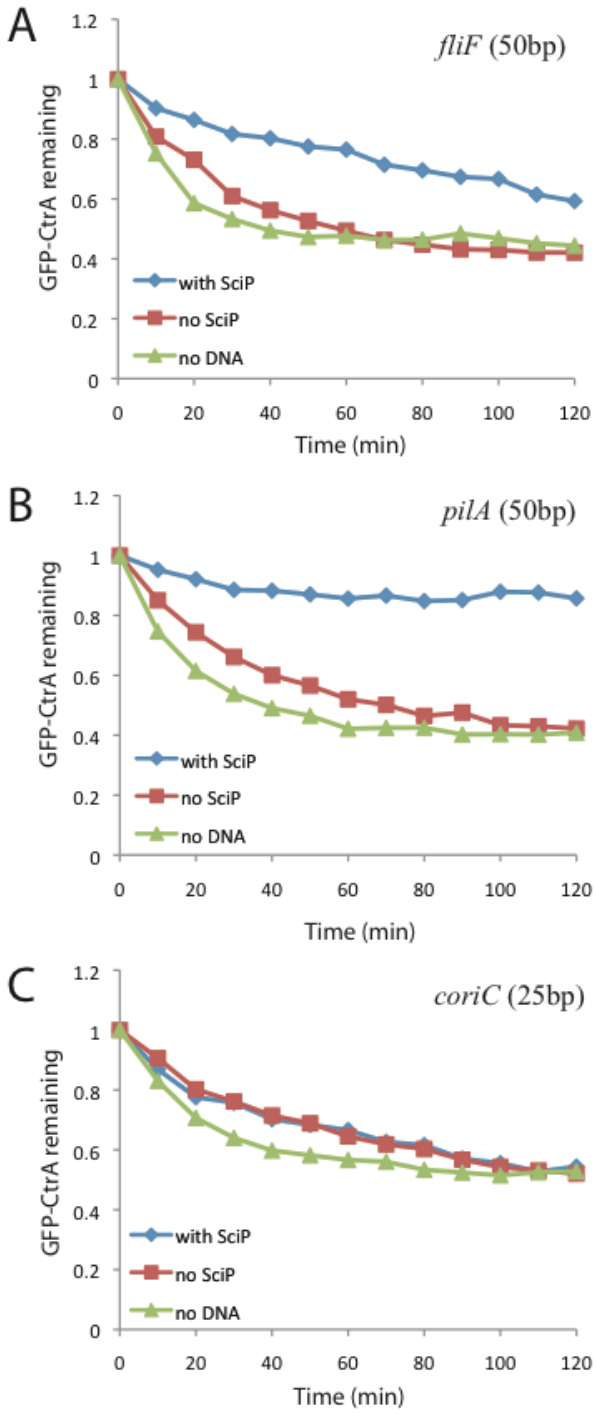


**Figure 4:** Degradation of GFP-CtrA with DNA and SciP. **A)** GFP-CtrA in the presence of *fliF* (50bp) and SciP. SciP further stabilizes CtrA in the presence of *fliF*. SciP alone does not stabilize CtrA **B)** GFP-CtrA in the presence of *pilA* (50bp) and SciP. CtrA is stabilized by SciP and *pilA* more than by just *pilA*. **C)** GFP-CtrA in the presence of *CoriC* (25bp) and SciP. CtrA is not stabilized in any of these reactions.

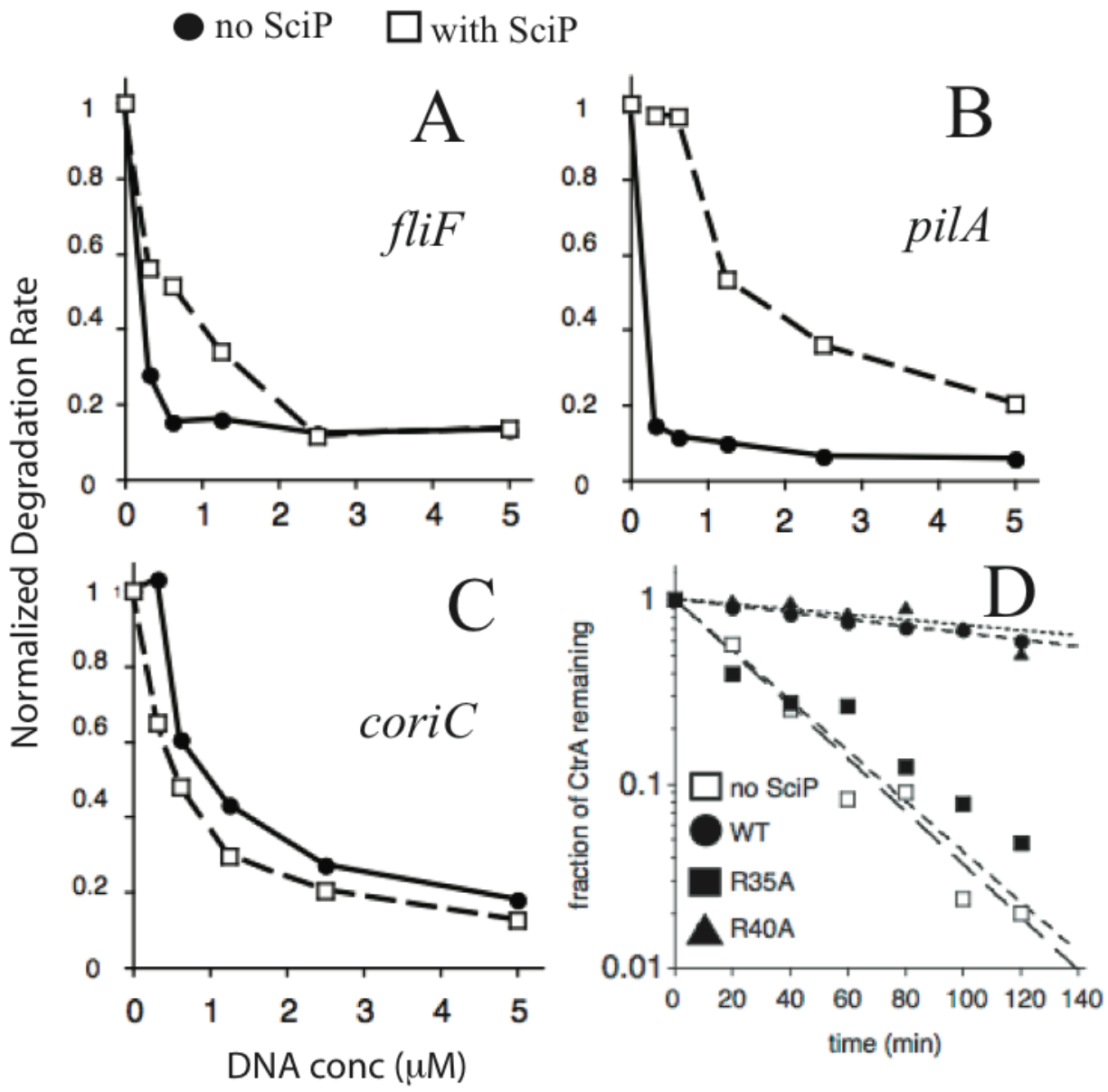


**Figure 5:** Degradation of GFP-CtrA with DNA and SciP or mutants. **A-C)** GFP-CtrA stabilization in the presence of 50bp *fliF* or *pilA* or 25bp CoriC in the presence or absence of SciP. 50nM GFP-CtrA and 5uM SciP used. **D)** CtrA stabilization in the presence of SciP or mutants. Mutants of SciP are not able to stabilize CtrA as well. R40A is closest to wild-type, while the others do not stabilize CtrA at all. 5uM of CtrA, DNA and SciP or mutants used.

As shown in the previous chapter, DNA binding can stabilize CtrA, protecting it from proteolysis by ClpXP. In this chapter I have shown that SciP not only interacts with CtrA in the presence of DNA but that this interaction can stabilize CtrA even more than DNA alone can. At low concentrations of DNA, CtrA is not stabilized. Once SciP is added under the same conditions, however, CtrA is stabilized. This suggests that SciP, CtrA, and DNA can form a tight complex that is capable of protecting CtrA from proteolysis, even under conditions in which CtrA would not be stabilized if SciP was not present. This could be an important mechanism of how CtrA is regulated in the *Caulobacter* cell. SciP may be one factor that keeps CtrA tightly bound to the DNA when cells are in the swarmer state so that CtrA cannot be improperly proteolyzed by ClpXP. Since one of CtrA's functions is to bind to the origin of replication and block its initiation, tight control of CtrA proteolysis is required to ensure that cells do not prematurely initiate DNA replication. This is also important because replication uses a lot of energy that the cell cannot afford to waste by initiating replication at inappropriate times. SciP also functions to modulate CtrA's transcriptional regulator activity. Most genes activated by CtrA are only activated in predivisional cells and not in swarmer cells even though CtrA is present in both. SciP appears to be at least one factor that ensures that CtrA-induced genes are not turned on in swarmer cells. The observed *in vitro* complex formation is likely the mechanism through which SciP acts to interact with CtrA and prevent it from turning on genes in swarmer cells. This also would explain why SciP is present only in swarmer cells and gets degraded at the swarmer to stalked transition. Based on my work with how the CtrA-DNA-SciP complex affected CtrA, I then began looking at the SciP protein itself and how this complex may affect SciP. The fact that the R35A mutant of SciP cannot stabilize CtrA shows that this residue is likely important for the interaction between CtrA and SciP when DNA is present. R40 may be important but not mandatory for the interaction, as the R40A mutant can still stabilize CtrA. R35 appears to be critical for the interaction, since mutating it completely abolished the SciP's ability to stabilize CtrA in the CtrA-DNA-SciP complex.

## CHAPTER 4

## SCIP DEGRADATION BY LON IS INHIBITED BY CTRA AND DNA

After looking at the effect SciP had on CtrA, I began looking at the SciP protein itself. I found that it is efficiently degraded by the Lon protease. Efficient degradation is dependent on both the N and C termini, as tagging either end with an M2-tag drastically reduced the degradation rate. However, the C-terminus appears to be of particular importance, as tagging it almost completely eliminated degradation by Lon [Figure 6]. Interestingly, though SciP is not a particularly stable protein, it is not efficiently degraded by other known proteases in the cell, such as ClpXP or ClpAP [manuscript in progress].

Based on my work studying how the CtrA-DNA-SciP complex affects CtrA degradation, I decided to also explore if the complex had an effect on SciP degradation. Using *in vitro* gel based degradation assays, I was able to determine that the complex does appear to stabilize SciP. Neither CtrA nor *fliF* DNA alone are enough to stabilize SciP. This indicates that SciP is not interacting with either of these components alone. This is also supported by previously published data using DNA mobility shift assays, which showed that there was no shift in DNA mobility when SciP alone was added in concentrations up to 1uM [6]. However, when all three components are mixed together, SciP degradation by Lon is inhibited [Figure 7]. To ensure that this was actually due to complex formation and was not specific to the use of *fliF* DNA, I also tested this with *pilA* and *CoriC*. SciP in the presence of CtrA and *pilA* is stabilized as expected. SciP in the presence of CtrA and CoriC also appeared slightly stabilized. This was surprising because in the study of the complex's effect on CtrA, the 25bp CoriC was not sufficient to stabilize CtrA. It may be that with a the shorter fragment of DNA a weaker complex is able to form, one which does not prevent the recognition of CtrA by ClpXP but does prevent SciP recognition by Lon. The additional length of DNA may be required to form a more stable complex in which both proteins are stabilized and thus unable to be degraded by their respective proteases.

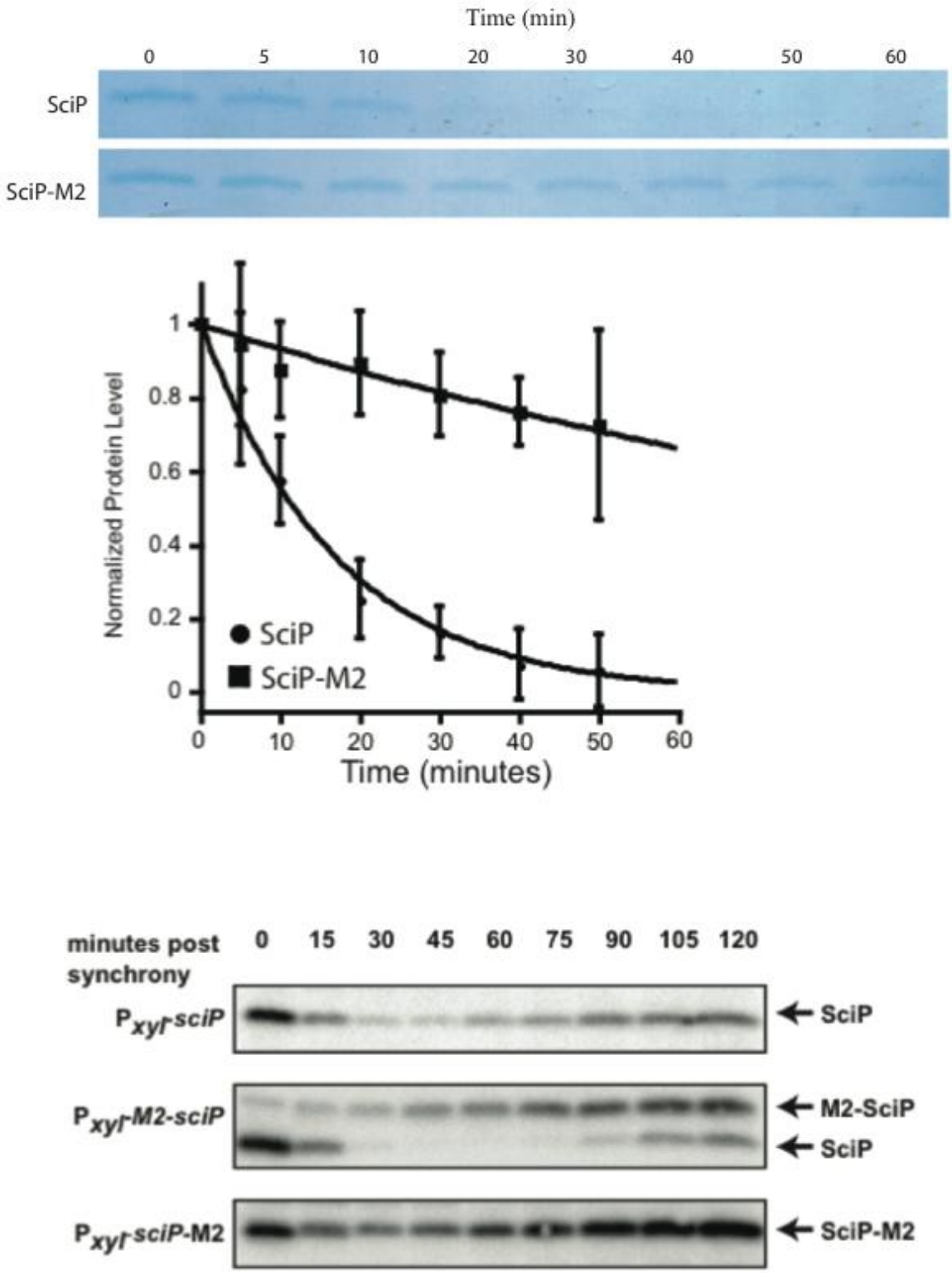


**Figure 6:** Degradation of SciP and SciP-M2 by Lon. Adding a C-terminal M2 tag almost completely abolishes degradation of SciP by Lon, while an N-terminal M2 tag slows degradation by Lon. This indicates that SciP recognition and degradation by Lon is critically dependent on the C-terminus and somewhat dependent on the N-terminus. [manuscript in progress]

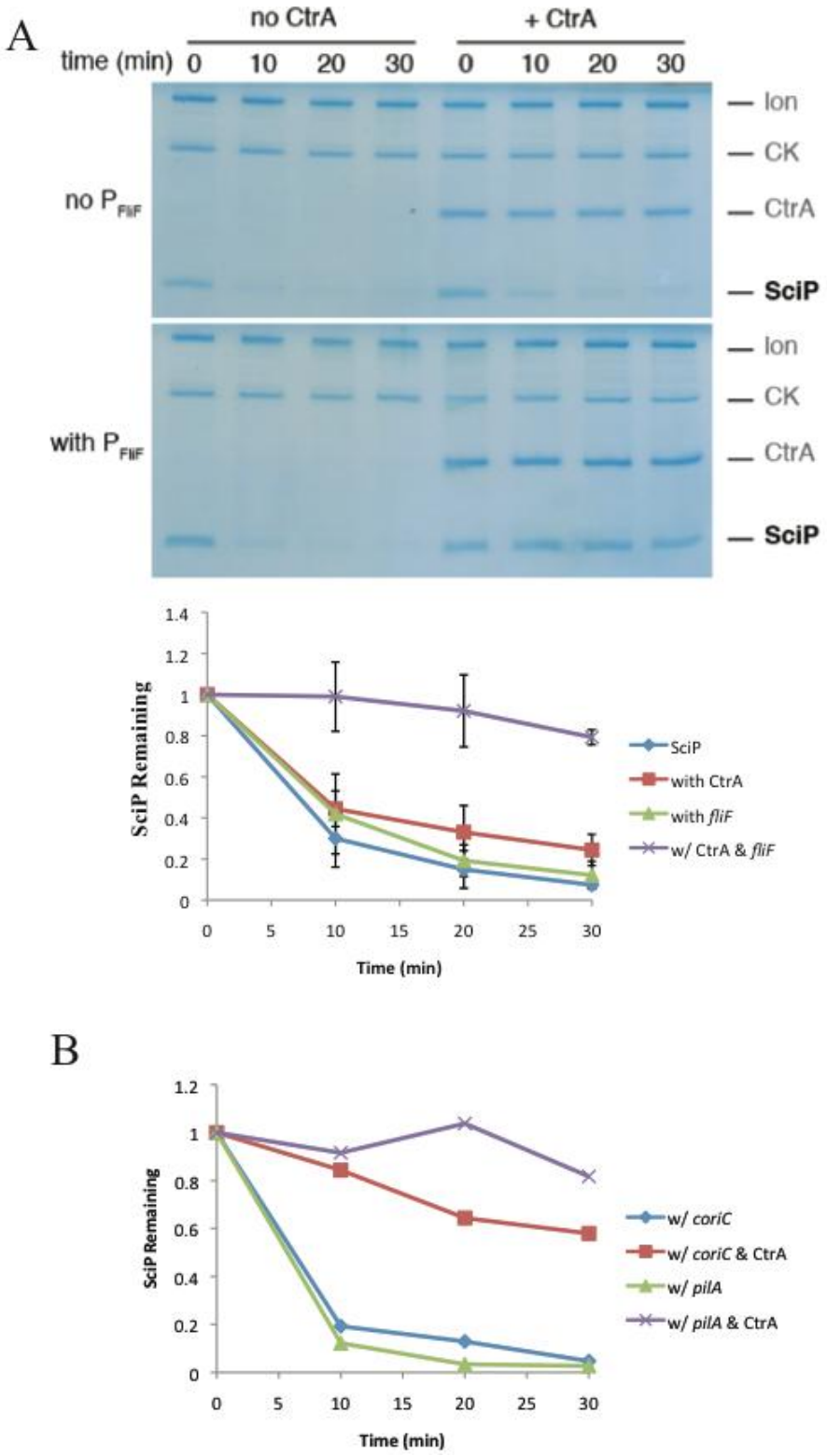


**Figure 7:** Stabilization of SciP in presence of CtrA and DNA. **A)** SciP is not stabilized in the presence of only CtrA or *fliF* DNA. In the presence of both, however, SciP is stabilized. **B)** SciP stabilization is due to the formation of the complex between SciP, CtrA, and DNA.

Mutants of SciP show varying degrees of stabilization by the presence of the DNA-CtrA-SciP complex. Previous data has shown that in yeast two-hybrid assays the R35A and R40A mutants of SciP differed from wild-type in their ability to interact with CtrA. The R35A was completely unable to interact, as evidenced by a complete lack of growth of the yeast. The R40A was only slightly deficient in its interaction, shown by growth of cells that was less robust than in the SciP-CtrA interaction [6]. I tested these constructs *in vitro* and found that my results matched those from the previous yeast two-hybrids. R40A closely resembles wild-type SciP. It is able to interact with CtrA and DNA to form a complex that prevents its degradation by Lon. The other mutants tested, however, are completely deficient in their ability to form these complexes. This indicates that the R35 and E57 residues of SciP are critical for the interaction in the CtrA-DNA-SciP complex. The R40 residue is not crucial, as mutating it does not completely abolish interaction. However, having the unmutated R40 residue does aid in the interaction.

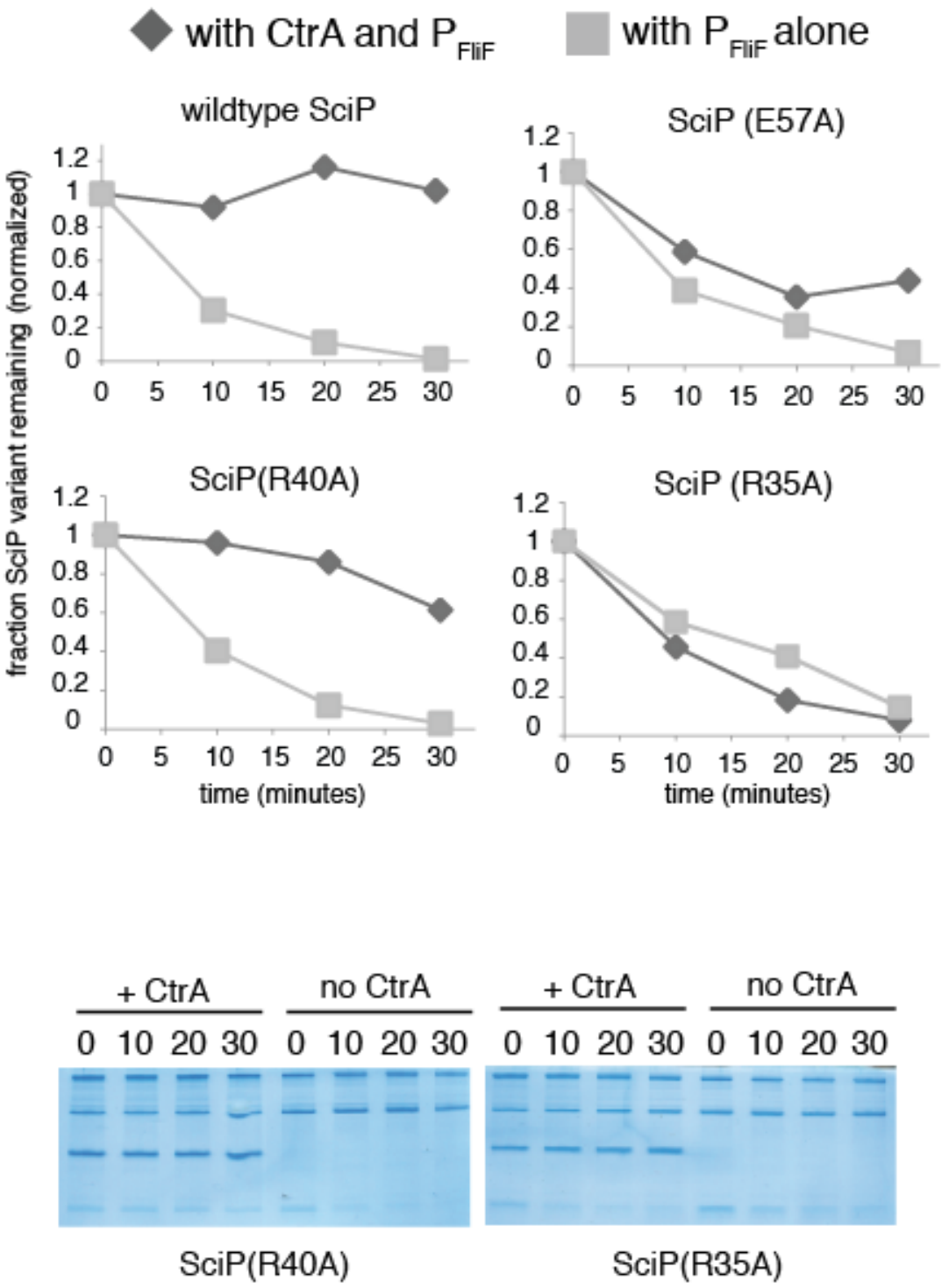


**Figure 8:** Degradation of SciP mutants with CtrA and DNA. Wild-type SciP is stabilized in the presence of CtrA and DNA but not in the presence of just DNA. Mutants of SciP show differential stabilization when in the presence of CtrA. R40A most closely mimics wild-type SciP while the other two mutants are completely deficient in the ability to form a complex with CtrA and DNA and are thus degraded by Lon regardless of other components present.

SciP has been shown to interact with CtrA and DNA in order to further inhibit the degradation of CtrA by ClpXP. Interestingly, CtrA and DNA have a similar affect on SciP. CtrA and SciP are also degraded by independent proteases in the cell. CtrA cannot be degraded by Lon and SciP cannot be degraded by ClpXP. Mutants of SciP are differentially deficient in their ability to form these complexes, highlighting key residues responsible for this interaction. R35 and E57 are critical for the interaction between SciP and CtrA in this complex, while R40 is not.

The ability of proteins to form stabilizing complexes which require multiple proteases to disassemble may be a more general mechanism that cells use to ensure that critical cell cycle regulators are only degraded at appropriate times during the cell cycle. These complexes can ensure that proteins are protected from proteolysis until the proper signals are given that the cell must proceed through the cell cyle. The ability of multiple proteases to degrade the complex can also ensure that proteolysis is rapid and robust when it comes time for these proteins to be degraded.

While these *in vitro* results were interesting and pointed to a mechanism of regulation for both CtrA and SciP, they did not fully elucidate CtrA regulation *in vivo*. In *Caulobacter* it is known that many other factors are required for efficient degradation of CtrA at the proper point in cell cycle, as stated above. These factors include CpdR, PopA, RcdA, and others that may not be known yet. This regulation is hard to recreate *in vitro* due to the fact that CtrA is degraded by ClpXP *in vitro* without the need for other factors. Because of this, I began studying CtrA regulation *in vivo* in addition to my work *in vitro*.

# CHAPTER 5

## *IN VIVO* CONSEQUENCES OF CTRA MISREGULATION

In addition to exploring the *in vitro* aspects of CtrA degradation, I also explored the *in vivo* characteristics of its regulation. *In vitro*, all parts of CtrA appear to be degraded similarly and are efficiently degraded in the absence of factors other than ClpXP. *In vivo* however the process is more complicated. CpdR is known to be critical for CtrA degradation *in vivo* as ΔCpdR cells do not degrade CtrA [9]. Other proteins, such as PopA and RcdA, are also known to be important, likely due to the role they play in localizing CtrA to the cell pole during the swarmer-to-stalk transition [5, 19]. In addition, not all parts of CtrA are degraded equally well *in vivo* as they are *in vitro*. Previous data has shown that while RR15 is degraded in a cell cycle dependent fashion like wild-type CtrA, RR alone and DBD (which contains the 15 residue degradation tag) are not degraded [18]. This brings up new questions of how CtrA degradation is regulated *in vivo*. While *in vitro* DNA binding appears to play a role, it clearly cannot have a major role in regulated proteolysis, since the RR15 cannot bind to DNA but gets degraded cyclically while the DBD can bind DNA but does not get degraded cyclically.

To begin my exploration of CtrA degradation *in vivo*, I used two strains of *Caulobacter*, both harboring a xylose-inducible plasmid. The first strain had a copy of wild-type CtrA while the second had a copy of CtrA-DD, a non-degradable version of CtrA [3]. Cells over-expressing CtrA were expected to look similar to wild-type cells without any plasmids since the protein can still be degraded normally. However, cells over-expressing CtrA-DD were expected to arrest in G1, since CtrA-DD cannot be cleared from the cells and is presumably always bound to the DNA, preventing replication. I used unsynchronized cells in this experiment, induced them with xylose then took time points every 90 minutes, the typical doubling time of *C. crescentus*. I ran samples on the flow cytometer to monitor the DNA content of each culture over the time course and found that the results were unexpected [Figure 9]. Cells over-expressing CtrA-DD actually appeared to arrest in G2 rather than G1.

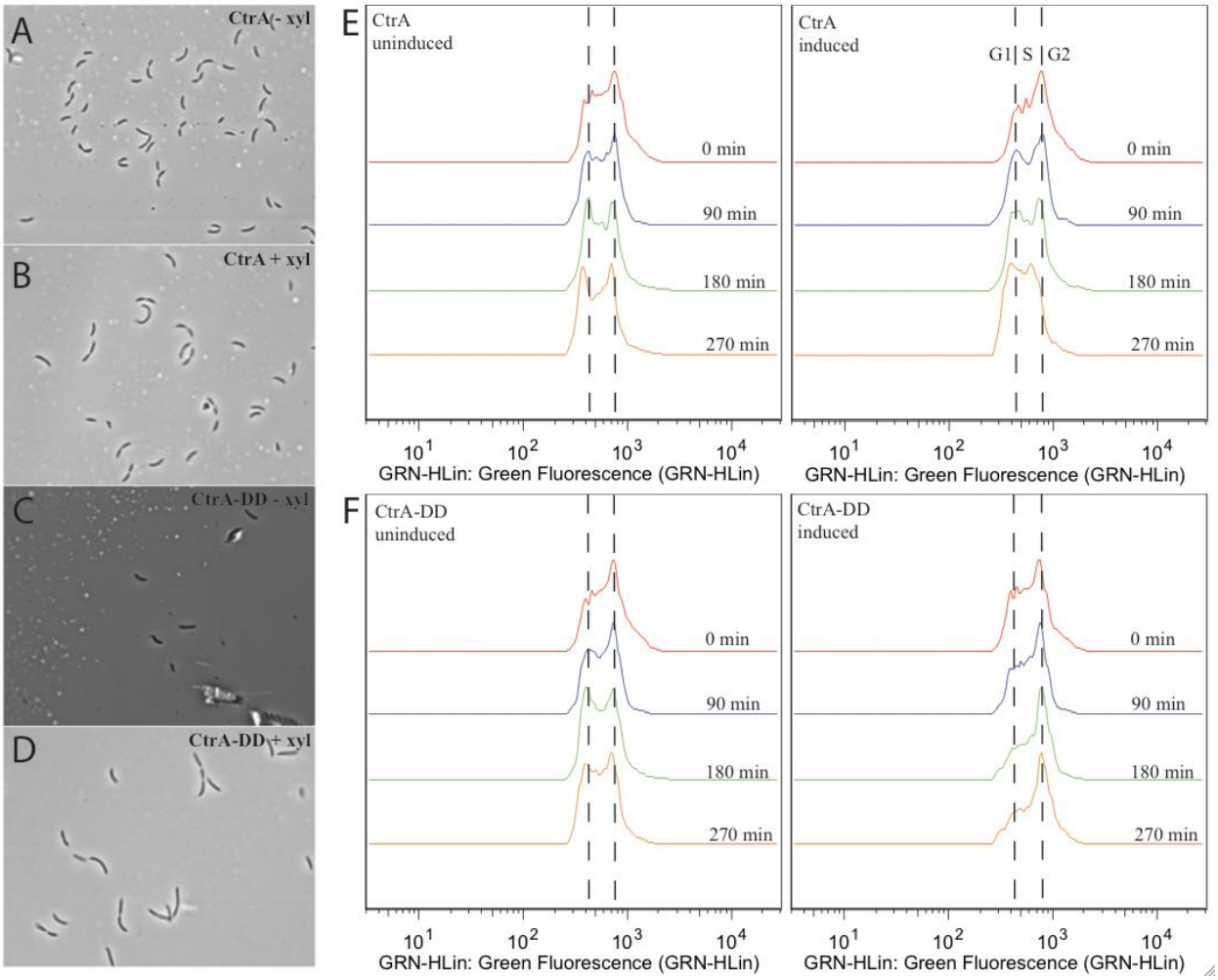


**Figure 9:** Over-expression of CtrA and CtrA-DD. Microscopy images taken 270 minutes post induction with 0.2% xylose. **A)** Cells harboring CtrA on a xylose-inducible plasmid under non-inducing conditions. **B)** Cells over-expressing CtrA. No changes in cell morphology are observed. **C)** Cells harboring a xylose-inducible plasmid with CtrA-DD under non-inducing conditions. **D)** Cells over-expressing CtrA-DD. Cells are becoming straighter and filamentous. **E)** FACS data from CtrA expressing strain. As expected, asynchronous cells show populations in G1, S, and G2 states. CtrA over-expression does not affect the FACS profile. **F)** FACS data from CtrA-DD expressing strain. Cells over-expressing CtrA-DD arrest in the G2 state, not the G1 state as expected.

However, since the cells I used were asynchronous, I hypothesized that this arrest might be due to CtrA's varied roles in the cell. This effect may just be a cumulative effect of CtrA activating and repressing genes in pre-divisional cells and repressing replication in swarmer cells. To test if this was the case, I grew up new cultures and synchronized them. I hypothesized that synchronized cells might show more of a G1 arrest since I could follow one population of swarmer cells through the cell cycle rather

than looking at a mixed population. In this experiment, I grew the cells and induced them for an hour with xylose before starting the synchrony. I then took time points over the course of 90 minutes, to capture one entire cell cycle. The results, shown in Figure 10, were again unexpected. Rather than arresting in G1, the cells over-expressing CtrA-DD seemed to progress through the cell cycle almost as well as the cells over-expressing CtrA. There may be a slight defect in the speed at which they progress through the cell cycle, but they appear to still go all the way through the cell cycle.

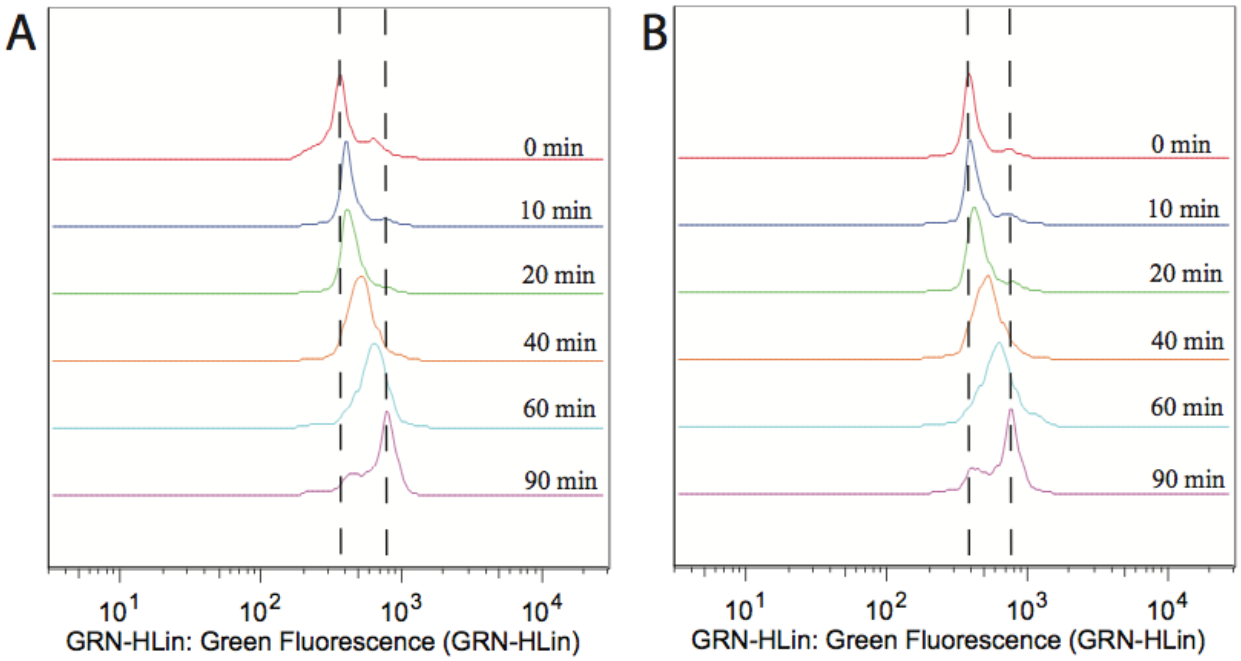


**Figure 10:** FACS profiles of synchronized cells. **A)** Cells over-expressing CtrA progress through the cell cycle as expected. The peak shifts over from 1N to 2N over the time course, with a small population at 90 minutes showing 1N content, indicating that they have already divided. **B)** Cells over-expressing CtrA-DD show a similar FACS profile.

These results were slightly unexpected at first, but perhaps not too surprising. In the synchronized experiment, I only induced for 1 hour with xylose. There may not have been enough CtrA-DD present to elicit much of an effect on cell cycle progression.

I then began to look at why cells over-expressing CtrA-DD had arrested in G2 in the asynchronous population [Figure 9]. I used four other strains. Two were ΔCpdR strains harboring a

xylose inducible plasmid to express either CtrA or CtrA-DD. It is known that *in vivo* CpdR is required for CtrA degradation [9]. I also tested two other strains; one expressing wild-type CtrA and the other expressing only the DNA-binding domain of CtrA, both off of xylose inducible plasmids. The purpose of the DBD strain was to determine why the CtrA-DD cells become filamentous when the protein is expressed. There are two possibilities. The first is that the CtrA-DD is always bound to the DNA and that is what is causing the cells to become filamentous. The other possibility is that the inability of CtrA-DD to be degraded, even if it is not bound to DNA, is what causes the cells to be filamentous. If the strain expressing DBD showed a similar phenotype and G2 arrest, then it was likely that the reason CtrA-DD over-expression is so detrimental to the cells is because it is binding to the DNA and not coming off. The results can be found in Figure 11 below. DBD cells show filamentation but do not arrest in G2. CtrA over-expression in ΔCpdR cells shows a G2 arrest in addition to filamentation, similar to what was seen above for CtrA-DD over-expression in wild-type cells. This indicates that the defects observed for CtrA-DD over-expression are likely due to the build-up of CtrA-DD. Even if it is not bound to DNA, like the DBD would be, it is still detrimental. ΔCpdR cells over-expressing CtrA-DD show the strongest defects. They become extremely filamentous and arrest in G2. This could be a combinatorial effect of lacking CpdR, which is important for regulation of other cellular proteins, and of having too much CtrA around. In these cells endogenous CtrA cannot be degraded since the cells lack CpdR and the pool of CtrA-DD continues to build up and cannot be degraded even if it comes into contact with ClpXP. This pool of CtrA and CtrA-DD could then be around regulating genes at inappropriate times in cell cycle, leading to the observed defects.

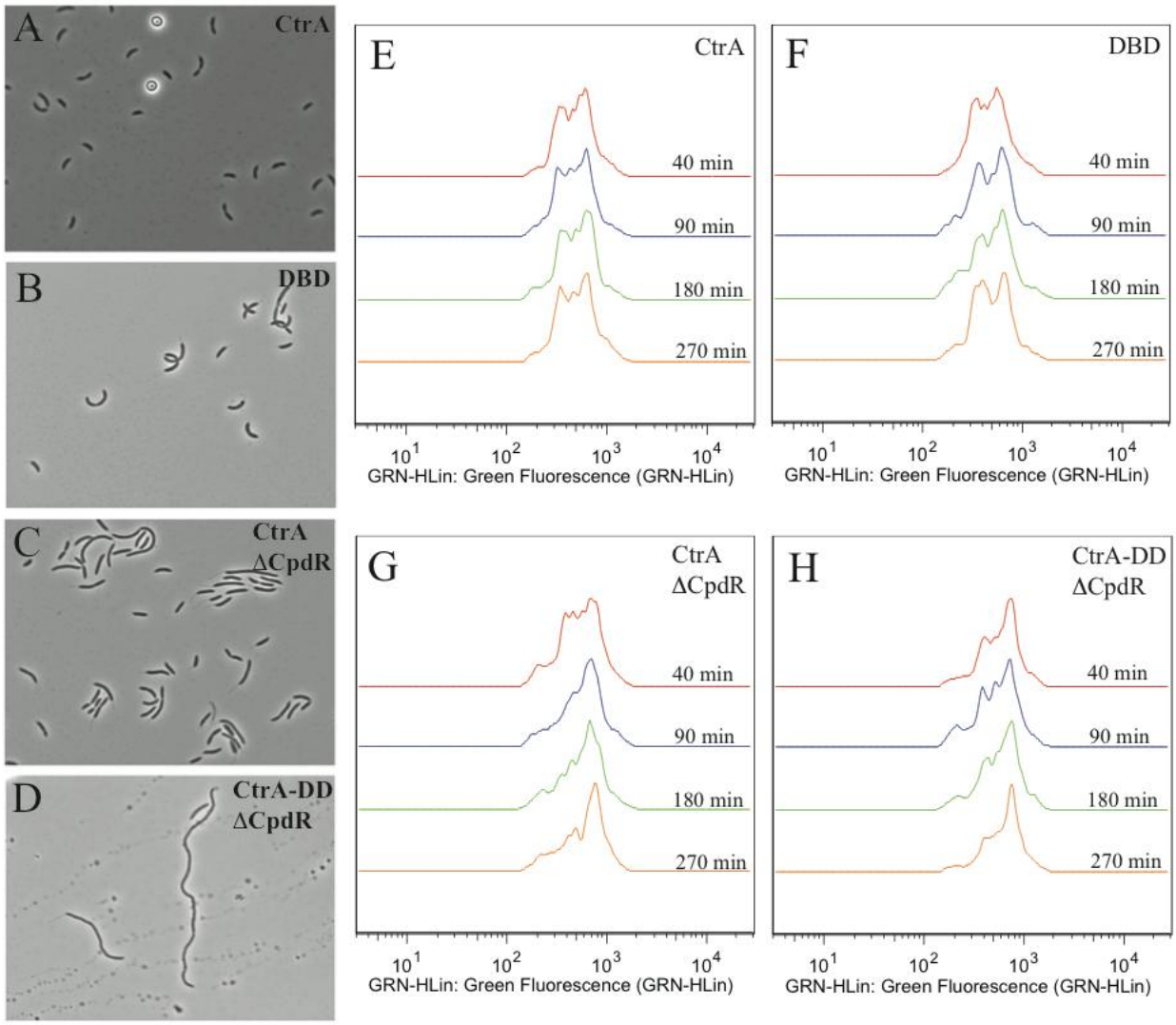


**Figure 11:** Over-expression of CtrA constructs in wild-type and ΔCpdR backgrounds. Asynchronous populations used. All microscopy images taken 270 min post-induction with xylose. **A)** CtrA overexpression in wild-type cells has little effect on cell morphology. **B)** The DNA-binding domain of CtrA shows some filamentation, though not quite to the extent of CtrA-DD [Figure 7]. **C)** ΔCpdR cells over-expressing CtrA show filamentation, similar to that seen for CtrA-DD [Figure 7]. **D)** ΔCpdR cells over-expressing CtrA-DD are extremely filamentous, looking as if they are completely deficient in division. **E)** CtrA over-expression has no effect on the FACS profile; cells still show a fairly equal distribution of G1, S, and G2 cells as expected. **F)** DBD over-expression does not greatly affect the FACS profile of cells. The population is still fairly equally distributed. **G)** ΔCpdR cells expressing CtrA show a distinct G2 arrest, similar to that seen for CtrA-DD [Figure 7]. **H)** Cells over-expressing CtrA-DD show a G2 arrest as well.

*In vivo* degradation of CtrA is much more complicated than its *in vitro* degradation kinetics would suggest. As stated above, many more factors are required for reasons that cannot be adequately shown *in vitro*. I have attempted to further examine some of the factors that may be controlling its turnover *in vivo*. Looking at over-expression of CtrA-DD in an asynchronous population revealed an unexpected arrest in G2 which may be explained by CtrA's varied functions in the cell. In addition to repressing replication, CtrA is also a transcriptional regulator of at least 100 genes in *C. crescentus*. The G2 arrest may occur as a result of misregulation of all the other genes controlled by CtrA. Interestingly, previously published data on another non-degradable mutant of CtrA, CtrAΔ3 [4] showed an arrest in G1 rather than G2 [17]. My results for the CtrA-DD more closely resemble a temperature sensitive CtrA mutant that inactivates CtrA [17]. This could indicate that the CtrA-DD mutant is not active and so is not able to block replication. It could, however, be acting as a negative regulator of the endogenous CtrA, which would deplete the endogenous CtrA as the CtrA-DD is over-expressed. This would cause the misregulation of the many genes that are controlled by CtrA, which could cause the G2 arrest. Another possibility is that the differences between the CtrA-DD and the CtrAΔ3 cause different effects in the cell. CtrA-DD is the full-length CtrA with just the last two alanine residues of the C-terminus mutated to aspartates. The CtrAΔ3 has a deletion of the last 3 residues on the C-terminus, which are then replaced by a GDPIED tag. The differences between these two proteins may be sufficient to explain why the CtrAΔ3 shows a G1 arrest while the CtrA-DD shows a G2 arrest. In the cell, these proteins may have completely different activities even though they are both non-degradable versions of CtrA. The DBD over-expression did not show the G2 arrest. This indicates that the detrimental effects of CtrA-DD may not be related exclusively to its ability to bind DNA. The fact that over-expression of CtrA in a ΔCpdR background led to a phenotype similar to that of CtrA-DD over-expression in wild-type cells suggests that the detrimental effects of CtrA-DD are due to its inability to be degraded. This does not, however, explain why the CtrA-DD in the ΔCpdR background has much more severe effects than the CtrA-DD in

the wild-type background or CtrA in the ΔCpdR background. These results suggest that the CtrA-DD is detrimental to cells due to other reasons than its inability to be degraded. A possibility is that having CtrA-DD and lacking CpdR cause combined effects that are more detrimental to the cell than simply having non-degradable CtrA. CpdR plays a role in the proteolytic regulation of a number of proteins in the cell in addition to CtrA and so the misregulation of these proteins in addition to the inability of CtrA-DD to be degraded may be causing this enhanced phenotype. In addition, CtrA levels may build up more than in the other conditions tested. In the CtrA over-expression in ΔCpdR cells, CtrA doesn't get degraded because CpdR is not able to aid in the localization of ClpXP and CtrA to the same pole in the cell for efficient degradation. However, it is possible that if enough CtrA builds up during the over-expression, ClpXP could engage CtrA for degradation if they came into close enough proximity. This doesn't occur in wild-type cells normally because there is not enough endogenous CtrA to be degraded if it is not localized to the same pole as ClpXP. Endogenous CtrA is also typically bound to DNA, which prevents its degradation, until cells undergo the swarmer to stalk transition. In the CtrA over-expression, a much larger pool of free CtrA is being generated which may occasionally be engaged by ClpXP, and thus some of the CtrA can be degraded. In the CtrA-DD over-expression in ΔCpdR cells, however, CtrA-DD cannot be engaged by ClpXP at all and so during the course of the over-expression there is no way to reduce levels, even if the two proteins come into close contact with each other. In addition, any endogenous CtrA cannot be degraded because ClpXP and CtrA cannot be localized to the same pole due to the absence of CpdR. This may explain why the phenotype of the CtrA-DD over-expression in ΔCpdR is much more severe than CtrA-DD over-expression in wild-type cells or CtrA over-expression in ΔCpdR cells.

# CHAPTER 6

## DISCUSSION AND FUTURE DIRECTIONS

The regulated proteolysis of CtrA *in vivo* has remained a mystery that is none too easy to solve. It is a question I have sought to address through the use of *in vivo* and *in vitro* experiments aimed at identifying its regulation and isolating other factors that may play a role. I have shown that CtrA degradation by ClpXP is inhibited by DNA. This stabilization is caused by directly caused by CtrA binding to DNA. Mutants of CtrA deficient in DNA binding are not stabilized, nor is CtrA in the presence of a mutated CoriC region lacking CtrA binding sites. SciP has been shown to enhance this stability *in vitro* and *in vivo*. SciP does not stabilize CtrA by itself and does not stabilize CtrA in the presence of only 25bp DNA. However, when 50bp DNA is present, such as the *fliF* or *pilA* promoter regions, stabilization is seen. Some mutants of SciP appear to be deficient in this interaction and cannot stabilize CtrA. In addition, CtrA can stabilize SciP in the presence of DNA, preventing SciP degradation by Lon. Mutants of SciP have also been shown to be deficient in the ability to form this CtrA-DNA-SciP complex, as evidenced by their degradation of Lon regardless of the presence of the other components of the complex. These mutants expose residues that are critical for the interaction. This type of complex formation may be a means by which the cell can protect critical cell cycle regulatory components from premature proteolysis. Lon is the main enzyme responsible for SciP degradation, while ClpXP degrades CtrA. The ability of different proteases to recognize and degrade individual parts of the complex suggests a cooperativity in which one protease degrades one component of the complex thereby destabilizing the rest of the complex and opening it up to degradation. This may be a mechanism of controlling CtrA degradation during the swarmer to stalk transition so that the cell cycle can progress properly.

*In vivo* the regulation of CtrA becomes more complicated. Many more factors are needed, many of which are likely currently unknown. I have attempted to elucidate some of the mechanisms by which

this cyclic proteolysis takes place. The results were unexpected. CtrA-DD cells, expected to arrest in G1, actually arrested in G2 instead. The DBD showed some filamentation defects, so perhaps binding to DNA is the reason why CtrA-DD are filamentous. This still does not explain why cells arrest in G2 rather than G1. However, it could be that the mechanism of CtrA coming off the DNA is uncoupled from the actual physical degradation of CtrA. Perhaps the signals present to tell CtrA to come off the DNA can recognize CtrA even if ClpXP cannot degrade it. This would explain why cells can still progress through the cell cycle even though CtrA-DD should still be present in the cell.

To further investigate the regulation of CtrA *in vivo*, I also began a new project that will continue in the future. I used several different strains expressing various pieces of CtrA. One is just the receiver domain, which has been shown to not be degraded; another is the receiver domain+15, which has been shown to be degraded cyclically [18]. I also have the receiver domain attached to an ssrA tag or an ssrA-SS tag. The RR-ssrA is expected to be degraded very rapidly, since ssrA tags are readily degraded by ClpXP. The double serine residues slow down degradation by ClpXP. Both of these are to test if adding another tag to the RR domain will results in cyclic degradation. If the ssrA tagged versions do get degraded in a cyclically regulated fashion, then the RR domain is sufficient for this degradation. I also have GFP+15, RcdA+15, and CpdR+15. These are to test if the 15 residue degradation tag from CtrA is sufficient for cyclic degradation. GFP is a protein that will not be localized in the cell, while RcdA and CpdR should be localized to the old cell pole. If the RcdA+15 and CpdR+15 get degraded cyclically but GFP+15 does not, then localization is also likely playing a part in CtrA's regulated degradation. The RR-ssrA-SS construct has also been put into a ΔsspB background. SspB is a known adaptor of ClpXP, so if this contruct is degraded cyclically then the degradation might be adaptor mediated. Finally, I have RR-ssrA and RR15 in a ΔCpdR background. CpdR is known to be required for *in vivo* CtrA degradation. It could be required as an adaptor for the cyclic degradation or as a means of helping localize ClpXP and CtrA to the

same area. I have not gotten results yet for these constructs, so this is a project that will need to be continued in the future but could yield important information on the regulation of CtrA.

CHAPTER 7

MATERIALS AND METHODS

*List of Strains*

*E. coli* strains

| Database Number | Cell Type | Vector | Media |
|---|---|---|---|
| EPC 163 | BL21DE3 | 375 GFP-RR15 | LB+Amp |
| EPC 164 | BL21DE3 | 375 GFP-CtrA | LB+Amp |
| EPC 453 | Top10 | 152 CtrA | LB+Kanamycin |
| EPC 455 | Top10 | 152 R198A | LB+Kan |
| EPC 456 | Top10 | 152 T213A | LB+Kan |
| EPC 457 | BL21DE3 | His-SciP-M2 | LB+Ampicillin |
| EPC 458 | Top10 | pBAD Lon | LB+Chloramphenicol |
| EPC 459 | ΔLon | none | LB |
| EPC 460 | ΔLon | pBAD Lon | LB+Chlor |
| EPC 461 | Top10 | 375 GFP-DBD | LB+Amp |
| EPC 464 | Top10 | His-SciP wt | LB+Amp |
| EPC 465 | Top10 | His-SciP R35A | LB+Amp |
| EPC 466 | Top10 | His-SciP R40A | LB+Amp |
| EPC 467 | Top10 | His-SciP E57A | LB+Amp |
| EPC 468 | DH5α | pRX GFP15 | LB+Kan |

*C. crescentus* strains

| Database Number | Cell Type | Vector | Media |
|---|---|---|---|
| CauloPC 105 | CB15N | 152 CtrA | PYE+Kan |
| CauloPC 106 | CB15N | 152 CtrA-DD | PYE+Kan |
| CauloPC 108 | CB15N | 152 T213A | PYE+Kan |
| CauloPC109 | CB15N | 152 R198A | PYE+Kan |
| CauloPC 116 | CB15N | 498 CtrA | PYE+Tetracycline |
| CauloPC 117 | CB15N | 498 CtrA DBD | PYE+Tet |
| CauloPC 118 | ΔCpdR | 498 CtrA | PYE+Tet |
| CauloPC 119 | ΔCpdR | 498 CtrA-DD | PYE+Tet |

*Oligo Preparation, Plasmid Isolation, and Transformation*

25bp and 50bp sections of DNA were made by mixing forward and reverse oligos for each piece of DNA in a 1:1 ratio along with 100mM KCl. Oligos were then heated at 95$^{o}$C for 10 minutes and cooled slowly in the heat block to allow for proper annealing. Oligo sequences can be found in the table below.

| | ***flif* sequences** |
|---|---|
| **Forward** | ggaaagcctgacgatcggcagataaacgcctcgtttaccttgtactgg |
| **Reverse** | ccagtacaaggtaaacgaggcgtttatatctgccgatcgtcaggcctttcc |

| | ***pilA* sequence** |
|---|---|
| **Forward** | ttggcagcgatcgcggagtgcatggttaagaacaaataacggtaaataca |
| **Reverse** | tgtatttaccgttatttgttcttaaccatgcactccgcgatcgctgccaa |
| | ***CoriC* sequence** |
| **Foward** | tggggTTAAcgctctgTTAAtcatg |
| **Reverse** | catgaTTAAcagagcgTTAAcccca |

Strains harboring plasmids for SciP and its mutants were obtained from the Laub lab. Plasmids were miniprepped using the Bio Basic EZ-10 spin column plasmid isolation kit and following the included for high copy number plasmids. Purified plasmids were then transformed into BL21 cells. Cells were thawed on ice before 5uL of isolated plasmid was added to each 100uL aliquot. Tubes were flicked gently to mix before being transferred to an electroporation cuvette that had been sitting on ice. Cells were then electroporated using an Eppendorf Electroporator 2510 before 500uL of LB was added. Cells were transferred to a new 1.5mL Eppendorf tube and recovered 1 hour in a 37$^{o}$C shaker before being plated on LB plates containing ampicillin (100ug/mL). Plates were incubated overnight at 37$^{o}$C (without shaking).

*Protein Purification*

For purification of SciP variants, overnight cultures were diluted into LB with ampicillin (100 ug/ml) and induced with 0.4 mM IPTG following 2-3 hours of growth at 37$^{o}$C. After 3 hours of induction, cells were spun down at 5000rpm 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$^{o}$C. The day of purification, cells were thawed on ice before being lysed by high pressure disruption using a Microfluidizer (Microfluidics, Newton, MA). Following clarification by centrifugation at 15,000 x g, lysate was applied to a 1mL Ni-NTA column pre-equilibrated with lysis buffer at room temperature. 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, concentratied, and aliquoted before being frozen at -20$^{o}$C for future use. CtrA and GFP-CtrA were purified similarly. ClpX and ClpP were purified as previously described [3, 12]. Lon was purified based on previously published work [7], except cells were grown in LB with chloramphenicol (30ug/mL). 30kDa concentrators were used to concentrate Lon fractions, and fractions were typically concentrated further than the final 5mL concentration described in the protocol to yield stock concentrations around 1.5uM.

*Degradation Assays*

Protein degradation was monitored using SDS-PAGE. For reactions monitoring CtrA degradation, the concentrations were as follows unless otherwise noted: 0.4uM ClpX, 0.8uM ClpP, 4mM ATP, 75ug/mL creatine kinase, 5mM creatine phosphate, 5uM CtrA, 5uM DNA, and 5uM SciP. H-buffer

(25mM HEPES-KOH pH=7.4, 100mM KCl, 10% glycerol, 10mM beta-mercaptoethanol) was added to bring volumes up to desired reaction volume. For assays where SciP degradation was monitored, the concentrations of the components was the same as listed above, except 0.5uM Lon was used as the protease and Lon degradation buffer (25mM Tris pH=8.0, 100mM KCl, 10mM $MgCl_2$, 1mM DTT) was used instead of H-buffer. Reactions were set up with the necessary components and initiated with ATP. Aliquots were removed at the specified times and mixed with SDS loading dye to quench the reaction before being flash frozen in dry ice. Samples were then heated at 65 for 5min and proteins were separated by SDS-PAGE. Gels were stained using Coomassie Blue G-250, scanned using a flatbed scanner and quantified using ImageJ. Protein degradation was also monitored by fluorescence. A GFP-tagged version of CtrA was used in which degradation was monitored via loss of fluorescence over time. Final concentrations in these assays unless otherwise stated were as follows: 0.4uM ClpX, 0.8uM ClpP, 4mM ATP, 75ug/mL creatine kinase, 5mM creatine phosphate, 50nM GFP-CtrA, 5uM DNA, and 1uM SciP. 20uL reactions were set up in a corning 384 well flat-bottom, non-binding plate and incubated at 30oC for 10 minutes in a Spectramax M5 (Molecular Devices) plate reader. Reactions were initiated with ATP and then read by the plate reader at the following settings: 440nm excitation, 510nm emmision, 495nm cutoff. Fluorescence was read at 15 second intervals for 2 hours.

*In Vivo Assays*

Asynchronous cell cultures were grown in PYE+appropriate antiobiotic overnight at 30$^{o}$C. Cultures were back-diluted to an OD of 0.1-0.2 and allowed to grow to OD 0.3-0.4 (approximately 1 doubling). Cultures were then induced with xylose for the specified time and time points were taken. For synchronized cultures, cells were grown in the same way, induced for an hour with xylose then pelleted. Pellets were resuspended in a mixture of cold 1x M2 salts and percoll (1:1 ratio) and subjected to density centrifugation (15,000 x g, 20 min). Two bands resulted, an upper band of stalked and predivisional cells and a lower band of swarmer cells. The swarmer cells were collected and washed with cold 1x M2 salts. The final swarmer cell population was resuspended in 100uL of PYE and released into pre-warmed PYE+antibiotic at 30$^{o}$C. Samples were taken at the indicated times for flow cytometry analysis. 300uL samples were taken for each time point and mixed with 700uL ethanol to fix the cells. Cells were stored at 4$^{o}$C until ready to use (at least 4 hours). Cells were then prepared for analysis. Samples were spun at 6,000 x g for 4 minutes and the supernatant was carefully removed from the pellets. Cells were then resuspended in 250uL FACS buffer (10mM Tris HCl unpHed, 1mM EDTA from 0.5M stock at pH=8.5, 50mM NaCitrate unpHed, 0.01% Triton X-100) and spun again. The pellets were resuspended in the same volume of FACS buffer, but this time were resuspended using an insulin syringe. 1uL of 20mg/mL RNase was then added to each sample and samples were incubated for 30 minutes at 37oC. After incubation another 250uL FACS buffer was added to each sample. 0.3uL of these prepared samples were then added to 200uL FACS buffer + 1uM sytox green and mixed in a Falcon 35-3077 96-well Microtest U-Bottom Tissue Culture Treated plate.

REFERENCES

1.) Bowers ML, Shapland EB, and Ryan KR. *Who's in charge here? Regulating cell cycle regulators*. Curr Op Microbiol, 2008. **11**(6): 547-552.

2.) Brun YV and Curtis PD. *Getting in the Loop: Regulation of Development in Caulobacter crescentus.* Microbiol Mol Biol Rev, 2010. **74**(1): 13-41.

3.) Chien P, Perchuk BS, Laub MT, Sauer RT, and Baker TA. *Direct and adaptor-mediated substrate recognition by an essential AAA+ protease.* PNAS, 2007. **104**(16): 6590-6595.

4.) Domian IJ, Quon KC, and Shapiro L. *Cell Type-Specific Phosphorylation and Proteolysis of a Transcriptional Regulator Controls the G1-to-S Transition in a Bacterial Cell Cycle.* Cell, 1997. **90**(3): 415-424.

5.) Duerig A, Abel S, Folcher M, Nicollier M, Schwede T, Amiot N, Giese B, and Jenal U. *Second messenger-mediated spatiotemporal control of protein degradation regulates bacterial cell cycle progression*. Genes & Dev, 2009. **23**: 93-104.

6.) Gora KG, Tsokos CG, Chen YE, Srinivasan BS, Perchuk BS, and Laub MT. *A cell-type-specific protein-protein interaction modulates transcriptional activity of a master regulator in Caulobacter crescentus.* Mol cell, 2010. **39**(3): 455-467.

7.) Gur E and Sauer RT. *Recognition of misfolded proteins by Lon, a AAA+ protease*. Genes and Dev. 2008. 22: 2267-2277.

8.) Hughes V, Jiang C, and Brun Y. *Caulobacter crescentus.* Curr Biol, 2012. **22**(13): R507-R509.

9.) Iniesta AA, McGrath PT, Reisenaur A, McAdams HH, and Shapiro L. *A phopho-signaling pathway controls the localization and activity of a protease complex critical for bacterial cell cycle progression*. PNAS, 2006. **103**(29): 10935-10940.

10.) Jenal U. *The role of proteolysis in the Caulobacter crescentus cell cycle and development*. Research in Microbiol, 2009. **160**(9): 687-695.

11.) Kirkpatrick CL and Viollier PH. *Decoding Caulobacter development*. FEMS Microbiol Rev, 2012. **36**(1): 193-205.

12.) Levchenko I, Seidel M, Sauer RT, and Baker TA. *A specificity-enhancing factor for the ClpXP degradation machine*. Science, 2000. **289**(5488): 2354-2356

13.) Martinez-Hackert E and Stock AM. *The DNA-binding domain of OmpR: crystal structure of a winged helix transcription factor*. Structure, 1997. **5**(1): 109-124.

14.) Narayanan A, Paul LN, Tomar S, Patil DN, Kumar P, Yernool DA. *Structure-Function Studies of DNA Binding Domain of Response Regulator KdpE Reveals Equal Affinity Interactions at DNA Half-Sites.* Plos One, 2012. **7**(1):e30102. Epub 2012 Jan 23.

15.) Okamura H, Hanaoka S, Nagadoi A, Makino K, and Nishimura Y. *Structural comparison of the PhoB and OmpR DNA-binding/transactivation domains and the arrangement of PhoB molecules on the phosphate box*. J Mol Biol, 2000. **295**(5): 1225-1236.

16.) Pruteanu M, Neher SB, Baker TA. *Ligand-controlled proteolysis of the Escherichia coli transcriptional regulator ZntR*. J Bacteriol, 2007. **189**(8): 3017-3025.

17.) Quon KC, Yang B, Domian IJ, Shapiro L, and Marczynski GT. *Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin.* Biochemistry, 1998. **95**: 120-125.

18.) Ryan KR, Judd EM, and Shapiro L. *The CtrA Response Regulator Essential for Caulobacter crescentus Cell-cycle Progression Requires a Bipartite Degradation Signal for Temporally Controlled Proteolysis.* J Mol Biol, 2002. **324**(3): 443-455.

19.) Taylor JA, Wilbur JD, Smith SC, and Ryan KR. *Mutations that alter RcdA surface residues decouple protein localization and CtrA proteolysis in Caulobacter crescentus*. J Mol Biol, 2009. **394**(1): 46-60.