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MECHANISMS OF DIVISION SITE SELECTION AND ROLE OF THE ATP-DEPENDENT PROTEASE CLPXP IN MIND REGULATION AND PROTEOSTASIS IN *E. COLI*

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MECHANISMS OF DIVISION SITE SELECTION AND ROLE OF
THE ATP-DEPENDENT PROTEASE CLPXP IN MIND REGULATION
AND PROTEOSTASIS IN *E. COLI*

BY

CHRISTOPHER JAMES LABRECK

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY DISSERTATION
OF
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ABSTRACT

Cell division in *Escherichia coli* depends on mechanisms to spatially and temporally regulate selection of the division site. In dividing cells, the Z-ring, composed of linear polymers of FtsZ, assembles at the site of septation. For division to yield two identical progeny, the septum must form at the longitudinal cell center, also known as midcell. Septation at locations other than midcell results in uneven division and gives rise to daughter cells with chromosome abnormalities and functional defects, which may arise from chromosome severing or impaired chromosome segregation. Therefore, an organism's overall viability necessitates cellular strategies that ensure correct placement of the Z-ring during the early stages of the division program.

The Min system of *E. coli*, containing the proteins MinC, MinD, and MinE, represents one of the major mechanisms to spatially regulate the assembly of the Z-ring. The Min system oscillates between the poles of cell, preventing polar Z-ring assembly and thereby favoring Z-ring formation at midcell. MinC prevents assembly of the Z-ring by inhibiting FtsZ polymerization through a direct protein-protein interaction. MinD is an ATPase that associates with the membrane upon binding ATP. MinE binds to MinD to stimulate ATP hydrolysis and membrane dissociation, and therefore drives the oscillation of MinD; MinC binds MinD and is a passenger of the oscillation. In Manuscript I we review current models of how MinC disassembles FtsZ polymers and describe the molecular basis for MinDE-dependent oscillation; we further discuss recent insights into the oscillation might be regulated in vivo.

In Manuscript II, we performed a mutagenesis screen to identify specific amino acid substitutions in *minC* conferring cell division defects to *E. coli* cells in vivo. We identified two distinct surface-exposed sites on MinC that are important for direct interactions with FtsZ: one on a cleft in the MinC N-domain and one on the C-domain adjacent to the MinD binding site. MinC mutant proteins that were impaired for the interaction with FtsZ, but not membrane-associated MinD, exhibited slower oscillation in vivo compared to MinC, suggesting that the FtsZ interaction with MinC modulates MinD-dependent oscillation. Furthermore, we showed that both sites on MinC identified in this study were important for the assembly of complexes between FtsZ, MinC, and membrane-associated MinD, and that the importance of each site for complex formation depended on nucleotide binding and hydrolysis by FtsZ. Importantly, we observed that the FtsZ C-terminal end (CTE), which interacts extensively with cell division proteins, was dispensable for complex assembly with dynamic FtsZ polymers formed with GTP, but required for complex assembly with FtsZ-GMPCPP and FtsZ-GDP.

Many cellular processes in *E. coli*, including division, are regulated by degradation of key substrates. ClpXP is an ATP-dependent protease that targets substrates for irreversible proteolysis, including the *E. coli* cell division proteins FtsZ and ZapC; a recent study also suggested that ClpXP degrades FtsA and MinD. In Manuscript III we demonstrate that ClpXP degrades MinD in vitro and prevents copolymer assembly with MinC; furthermore, ClpXP disassembled pre-formed copolymers, which represent a class of ordered aggregates, in vitro. We quantified the rate of MinD degradation and determined that the ClpX Zinc-

binding domain (ZBD) and MinD N-terminal region are both important for recognition and degradation by ClpXP.

ClpXP has been reported to degrade large ordered aggregates in vitro, including FtsZ polymers and the MinCD copolymers described in Manuscript III. In *E. coli*, ClpXP degrades FtsZ polymers in vivo to remodel the Z-ring and accelerate polymer dynamics during constriction. In Manuscript IV, we reported that ClpXP recognizes and degrades aggregated FtsZ and Gfp appended with a ClpX recognition motif (Gfp-ssrA). Furthermore, we demonstrated that ClpX alone is capable of promoting disaggregation of heat-induced aggregates of FtsZ and Gfp, and that ClpX promotes disaggregation of FtsZ following heat shock in vivo.

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PREFACE

This dissertation has been prepared in Manuscript Format according to the guidelines of the University of Rhode Island Graduate School. Manuscript I, “Function and regulation of the *Escherichia coli* Min system”, is formatted as a review article for *Frontiers of Microbiology*. Manuscript II, “MinC N- and C-domain interactions modulate FtsZ assembly, division site selection, and MinD-dependent oscillation in *Escherichia coli*” is published in *Journal of Bacteriology*. Manuscript III, “The AAA+ chaperone protease ClpXP targets the MinD ATPase for proteolysis and modulates MinCD assembly”, is formatted as a research article for *Protein Science*. Manuscript IV, “The Protein Chaperone ClpX Targets Native and Non-native Aggregated Substrates for Remodeling, Disassembly, and Degradation with ClpP,” was published in *Frontiers in Molecular Biosciences* in 2017.

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Manuscript I

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Title: Function and regulation of the *Escherichia coli* Min system

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Abstract

During division in *E. coli*, cell division proteins are spatiotemporally regulated to ensure correct placement of the Z-ring at the cell center and subsequent septation. The Min system of *E. coli*, including the proteins MinC, MinD, and MinE, oscillates between the poles of the cells to inhibit Z-ring formation at non-septal, polar regions. MinC destabilizes dynamic FtsZ polymers to effectively prevent assembly of protofilaments. MinD is an ATPase that associates with the membrane when bound to ATP. MinE binds to membrane-associated MinD to promote stabilization at the membrane or stimulate membrane dissociation by inducing ATP hydrolysis. MinC is a passenger of the oscillation. This review summarizes insights into the function of MinC as a cell division inhibitor and the molecular mechanisms driving and regulating the dynamic patterning of the Min system in vivo.

Introduction

Cell division in *Escherichia coli* is a complex cellular process that requires the concerted effort of a widely conserved set of proteins to separate a single cell into two identical daughter cells (Egan & Vollmer 2013). Cell division is initiated by the assembly of a ring-shaped protein ultrastructure, termed the “Z-ring”, at the longitudinal cell center. The Z-ring contains bundled filaments of the cytoskeletal protein FtsZ that are tethered to the bacterial membrane through protein interactions (Erickson et al. 2010). FtsZ is a

tubulin-like GTPase that polymerizes into linear filaments via the head-to-tail arrangement of FtsZ. FtsZ in the Z-ring functions together with additional cell division proteins to couple membrane scission with remodeling of the bacterial cell wall (Martos et al. 2012).

During the cell cycle, the division machinery is regulated both spatially and temporally to ensure that the Z-ring is positioned at midcell. Mislocalization of the division machinery results in cell filamentation and the production of anucleate minicells (de Boer et al. 1989). In *E. coli*, two key molecular mechanisms constrain Z-ring formation at the cell center: (1) nucleoid occlusion, which prevents FtsZ polymerization over the bacterial chromosome, and (2) the Min system, which inhibits FtsZ assembly at the cell poles. Nucleoid occlusion is mediated by SlmA, which inhibits FtsZ assembly at regions occupied by the nucleoid (Bernhardt & De Boer 2005; Cho et al. 2011). The Min system of *E. coli* comprises the proteins MinC, MinD, and MinE and functions as a potent division inhibitor that rapidly oscillates between the cell poles (Lutkenhaus, et al., 2012). Consequently, the time-averaged concentration of Min proteins is lowest at the cell center (Lutkenhaus 2007). Thus, polar oscillations of the Min system function to restrict Z-ring assembly at the cell center.

Several organisms rely on a Min system to spatially regulate the assembly of the Z-ring; however, the oscillation of Min proteins observed in *E.*

coli is not always preserved in these organisms. Polar gradients of MinC effectively prevent Z-ring assembly at the cell poles in both *E. coli* and *B. subtilis*; however, the polar oscillation of Min proteins observed in *E. coli* is not observed in *B. subtilis*. Instead, DivIVA recruits MinCD complexes to the septal region where MinC prevents the formation of multiple Z-rings at the division septum. Following division, MinC prevents Z-ring assembly at the new cell pole (Gregory, et al., 2008). *Caulobacter crescentus* lacks a Min system entirely and relies on the ATPase MipZ to prevent FtsZ assembly at the cell poles (Rowlett, et al., 2013).

MinC is the component of the Min system that interacts with FtsZ to destabilize polymers. The ATPase MinD associates with the membrane in the ATP-bound dimer conformation, and recruits MinC to the membrane via a direct protein-protein interaction (Raskin and de Boer, 1999b). MinE binds MinD to stimulate ATP hydrolysis and release of MinD from the membrane (Hu & Lutkenhaus 2001; Park et al. 2011). Thus, MinE regulates the ATP-dependent dynamics of MinD with the membrane.

Extensive work conducted over the last two decades has revealed important mechanistic insights into how the Min system functions to antagonize Z-ring formation. Additionally, recent studies have successfully reconstituted Min dynamics in vitro, resulting in new models that describe the molecular basis of Min oscillation in vivo. Here, we discuss how MinC engages

FtsZ to promote polymer destabilization and review recent insights into how MinC, MinD, and MinE assemble into a dynamic polar oscillator.

Binding and destabilization of FtsZ by MinC

FtsZ in the Z-ring is organized into a highly dynamic network of bundled polymers, which contain either FtsZ-GTP or FtsZ-GDP. FtsZ subunits bind and hydrolyze GTP, which stimulates release of FtsZ from the Z-ring. Consequently, cycles of GTP binding and hydrolysis promote the dynamic exchange of subunits between the Z-ring and the cytoplasm. MinC destabilizes FtsZ polymers only when FtsZ undergoes subunit exchange from GTP binding and hydrolysis; accordingly, purified MinC readily disrupts dynamic FtsZ polymers formed with GTP, but does not disrupt static polymers assembled with the non-hydrolyzable GTP analog GMPCPP (Dajkovic et al. 2008; LaBreck et al. 2019). MinC interacts with FtsZ to destabilize polymers via two sites of interaction: one on the N-domain and another at the C-domain (Hu and Lutkenhaus, 2000; Zhou, et al., 2005; Shen and Lutkenhaus, 2009; Dajkovic, et al., 2008; LaBreck, et al., 2019).

The structure of MinC consists of two discrete functional domains that interact with FtsZ and are connected by a flexible linker (Cordell et al. 2001; Hu & Lutkenhaus 2000; LaBreck et al. 2019) (Fig. 1). The MinC N-domain (NTD) fused to MalE inhibits the sedimentation of FtsZ polymers by centrifugation; overproduction of the MinC NTD in *minD* deletion cells prevents

Z-ring assembly and induces widespread cell filamentation (Hu et al. 1999; Hu & Lutkenhaus 2000). The MinC C-domain (CTD) contains a large hydrophobic region that promotes the formation of a stable dimer; a surface-exposed region of the MinC CTD containing a highly conserved 'RSGQ' sequence is important for the interaction with MinD (Szeto et al. 2001; Zhou et al. 2005; Cordell et al. 2001). Overproduction of the MinC CTD also stimulates Z-ring disassembly in vivo and leads to cell filamentation (Shiomi & Margolin 2007). FtsZ polymers in vitro appear shorter and less bundled in the presence of both MinC and the MinC CTD fused to MalE. Furthermore, MalE-MinC and MalE-MinC CTD both reduced the elastic modulus, or stiffness, of FtsZ polymers in vitro, suggesting that the MinC CTD renders FtsZ polymers more susceptible to mechanical stress (Dajkovic, et al., 2008).

FtsZ binding sites on the MinC N- and C-domains

Both the MinC NTD and CTD support an interaction with FtsZ, and extensive work has defined specific sites of interaction on each domain of MinC with FtsZ (Hu and Lutkenhaus, 2000; Zhou et al., 2005; Shen and Lutkenhaus, 2009; Shen and Lutkenhaus 2010; Park et al., 2018; LaBreck et al., 2019). Early genetic studies mapped a mutation to the MinC NTD, *minC(G10D)*, that induced minicell formation in vivo (Labie et al. 1990). Subsequent characterization of this mutation in vitro showed that MinC(G10D) was impaired for destabilization of FtsZ polymers (Hu et al. 1999; LaBreck et al., 2019). Furthermore, overproduction of MinC(G10D) did not result in cell

filamentation or generate minicells in vivo (Hu et al. 1999). Recently, the FtsZ binding site on the MinC NTD has been further defined by mutagenesis studies, which revealed that a cleft in the MinC NTD contains residues that mediate the interaction with FtsZ (Park et al. 2018; LaBreck et al., 2019 (Fig. 1).

Mutagenesis of the MinC CTD aimed to define the MinD-MinC interface. These efforts identified a highly conserved 'RSGQ' amino acid sequence on the surface of the MinC CTD that is required for the interaction with MinD (Zhou et al., 2005). Subsequent work revealed an additional site on the MinC CTD that mediates the interaction with FtsZ (Zhou et al., 2005; LaBreck, et al., 2019). A MinC CTD mutant protein, MinC(R172A), failed to complement the *min* deletion phenotype when co-expressed with MinD and MinE, but remarkably was only mildly impaired for the interaction with MinD (Zhou & Lutkenhaus 2005). Furthermore, MinC Arg 172 is not present in the MinD interface on the MinC CTD. This suggested that the cell division defect resulted from the impaired interaction with FtsZ. Using mutagenesis and biochemical studies, a more recent study identified additional residues on the MinC CTD that are important for division-site selection in vivo and FtsZ polymer disassembly in vitro, but are largely dispensable for the interaction with membrane-bound MinD (LaBreck et al., 2019). These residues map to the surface of the MinC CTD in the vicinity of R172 (Fig. 1). Both the MinC NTD and CTD contain a site that is important for FtsZ polymer destabilization; one

is located at a cavity in the MinC NTD and another site is on the MinC CTD near the MinD interaction site (Fig. 1).

Mechanism of MinC function against FtsZ assembly

MinC is a potent destabilizer of FtsZ assembly (Hu, et al., 1999). Specifically, MinC substoichiometrically inhibits FtsZ polymerization in a concentration-dependent manner that requires GTP binding and hydrolysis (LaBreck et al., 2019); however, MinC does not alter the rate of GTP hydrolysis by FtsZ, (Chen et al. 2013; Hu et al. 1999).

The molecular mechanisms of polymer destabilization by MinC are currently under extensive investigation. Previous genetic work performed in *E. coli* by the Lutkenhaus group has generated a number of insights into how MinC engages FtsZ to promote disassembly. These studies found that mutations in an alpha helix present at the FtsZ subunit-subunit interface, known as helix H10, conferred resistance to overproduction of the MinC NTD in vivo and furthermore that mutations in the FtsZ C-terminal tail abolished binding of MalE-MinC CTD to FtsZ polymers in vitro (Shen & Lutkenhaus 2009; Shen & Lutkenhaus 2010). Based on these observations, it was proposed that the FtsZ C-terminal tail recruits MinC to polymers via the MinC CTD. Recruitment of MinC to FtsZ polymers positions the MinC NTD at the H10 helix within the FtsZ subunit-subunit interface, thereby promoting polymer destabilization (Shen & Lutkenhaus 2009; Shen & Lutkenhaus 2010). GTP

binding and hydrolysis by FtsZ modifies the availability of Helix H10 for MinC binding. In FtsZ polymers, subunits are staggered in a head-to-tail arrangement where the H10 helix is sandwiched between individual subunits, and consequently is not accessible to MinC (Shen and Lutkenhaus, 2010). Therefore a caveat to this model is that polymer breakage by the MinC NTD necessitates the release of the H10 helix following GTP hydrolysis. To address this problem and further define the kinetics of MinC-stimulated disassembly, the Schwille group reconstituted bundled networks of dynamic FtsZ polymers in vitro and examined disassembly by a functional eGfp-MinC fusion protein (Arumugam et al. 2014). They reported that FtsZ subunit turnover induced by GTP hydrolysis occurs stochastically within the filament network and transiently exposes the H10 helix of filament minus ends. MinC can then bind to FtsZ subunits and obstruct incorporation of additional subunits. Furthermore, MinC was found to bind GDP-FtsZ in the polymer and enhance the release of GDP-bound FtsZ from filaments (Arumugam et al. 2014). Thus, MinC decreases the rate of subunit addition to polymers and enhances the GDP-FtsZ detachment rate. Importantly, this work suggests that the interaction between MinC and GDP-FtsZ in polymers is important for destabilization.

GDP-bound FtsZ accounts for more than half of the subunits present in the Z-ring (Chen & Erickson 2009; Arumugam et al. 2014). Several groups have reported direct binding of MinC or MinC-MinD complexes to GDP-FtsZ (Hernandez-Rocamora et al. 2013; Arumugam et al. 2014; LaBreck et al.,

2019). Consistent with a model in which MinC engages GDP-FtsZ, we reported that membrane-associated MinCD complexes bind dynamic FtsZ polymers stronger than static FtsZ polymers assembled with GMPCPP or non-polymerized GDP-FtsZ dimers (LaBreck et al., 2019); unlike static polymers, dynamic FtsZ polymers contain a population of GDP-FtsZ (Arumugam, et al., 2014). Accordingly, the FtsZ C-terminal end (CTE) in dynamic polymers was dispensable for complex formation with MinC and MinD, but necessary for static polymers, suggesting that GDP-FtsZ contained in polymers may be the preferred target of MinC (LaBreck, et al., 2019).

Dynamic localization of Min proteins in vivo

The *E. coli* Min system prevents Z-ring assembly at the cell poles, ensuring that division occurs at midcell. Early models for Min localization envisioned a static system wherein MinC and MinD were restricted to the poles (Zhao et al. 1995; Huang et al. 1996). However, advances in recombinant Gfp-fusion technology in the late 1990's allowed for direct visualization of MinD localization in live cells. Remarkably, Gfp-tagged MinD was observed to oscillate in vivo between the cell poles in a period of ~40 sec (Raskin & de Boer 1999a). The dynamic localization of MinD was observed to depend on MinE, but not on MinC or FtsZ (Raskin & de Boer 1999a). Subsequently, Gfp-MinC was also reported to oscillate between the cell poles in a MinD-dependent manner, suggesting that MinC is a passenger of the oscillation (Raskin & de Boer 1999a; Hu & Lutkenhaus 1999).

In cells lacking a chromosomal copy of *minE*, Gfp-MinD is evenly distributed on the membrane, resulting in global division-site inhibition and cell filamentation (Raskin & de Boer 1999a; Raskin & de Boer 1999b). In contrast, expression of MinE induces rapid polar oscillation of MinD (Raskin & de Boer 1999b). Initial reports of MinE localization in chemically fixed cells revealed that MinE forms a large membrane associated ring-like structure, termed the E-ring, which accumulates near the cell center (Raskin & De Boer 1997); however, in live cells expressing MinE-Gfp the E-ring was observed to be highly dynamic and assemble at the fringes of the MinD polar zone during an oscillation cycle (Hale et al. 2001; Fu et al. 2001). Based on these observations, a model of Min oscillation was proposed where the dynamic localization of the E-ring near the cell pole is important for MinD oscillation; MinE in the E-ring would presumably stimulate the release of MinD from the membrane and subsequent diffusion down a concentration gradient towards the opposite pole.

Molecular basis of MinD oscillation

MinD is a member of the Walker A protein family of cytoskeletal ATPases (WACA), which contain deviant Walker A motifs (K-X₅-GKT, where X is any amino acid) (Löwe & Amos 2009; Michie & Löwe 2006). This motif features two conserved lysine residues that mediate ATP binding, hydrolysis, and oligomerization (Leipe et al. 2002; Lutkenhaus & Sundaramoorthy 2003).

The signature lysine in one protomer contacts ATP bound to the other subunit, thereby mediating dimerization (Lutkenhaus, 2007). WACA ATPases are known to position large protein assemblies and are activated by ATP binding and subsequent dimerization (Lutkenhaus et al. 2012). MinD dimerizes in the ATP-bound form, bringing together the C-terminal membrane targeting sequences of two MinD protomers. Dimerization therefore creates a binding surface with enhanced affinity for the membrane (Szeto et al. 2002; Wu et al. 2011). MinD protomers similarly bind ADP, however, the ADP-bound form of MinD is retained in the cytoplasm as a freely diffusible monomer (Hayashi et al. 2001). Thus, cycles of ATP binding and hydrolysis drive MinD dynamics with the membrane. Importantly, MinE stimulates the ATPase activity of MinD via a direct interaction, and is therefore important for regulating MinD localization (Hu & Lutkenhaus 2001).

Biophysical studies of MinE conformation have contributed greatly to our current understanding of MinDE functional interactions. In solution, MinE exists as a dimer with two distinct functional conformations: (1) an inactive latent form, and (2) an active form that is competent for MinD binding (Fig. 2). The latent form of MinE is a 6 β -stranded structure where the β -sheets and the N-terminal membrane targeting sequences (MTS) of two MinE protomers are packed against the dimer interface (Fig. 2a). The innermost β -sheet contains residues that are important for the interaction with MinD; therefore this conformation precludes MinD binding. In the second, “active” conformation of

MinE, the 6 β -stranded structure undergoes a dramatic structural conversion into a 4 β -stranded structure that is competent for MinD binding. Here, the β 1 strand that was previously occluded in the MinE latent state forms an α -helix that is available for the MinD interaction (Park et al. 2011) (Fig. 2b).

A recent report examining the dynamics of the MinE MTS revealed important mechanistic insights into this remarkable structural reconfiguration. Here, the Lutkenhaus group found that the MTS of MinE each protomer in the latent state is dynamically tethered to the β -strand (Park et al. 2017). This suggests that the MinE MTS becomes transiently exposed in the latent conformation, allowing for membrane association. Due to this intriguing property of the MTS, MinE in the latent state reversibly binds the membrane, functioning as a sensor for membrane-associated MinD (Park et al. 2017). When MinE binds a region of the membrane that lacks MinD, the MinE sensor will revert back to the freely diffusible state. However, when MinE encounters MinD on the membrane, the interaction with MinD induces the conversion of MinE into the 4 β -stranded active conformation. In this state, MinE stimulates ATP hydrolysis by MinD and release from the membrane.

Cell-free reconstitution of Min dynamics in vitro

MinD dynamically interacts with the membrane in a MinE-regulated manner. In recent years, several groups have attempted to recapitulate Min dynamics on supported lipid bilayers (SLB) in vitro in order to explain how this

remarkable patterning occurs in live cells. In a pioneering study by the Schwille group, Min dynamics were observed in vitro using SLBs to mimic the cell membrane. This system consisted of a lipid-coated microscope slide incubated with fluorescently labeled MinD and MinE. In the presence of ATP, MinD formed a dense homogeneous layer on the lipid surface. However, the addition of MinE stimulated the appearance of planar surface waves that propagated at regular velocities (Loose et al. 2008). Importantly, the leading edge of MinE surface waves always followed the trailing edge of MinD wave propagation, consistent with the hypothesis that MinE stimulates the chase and release of MinD from the membrane (Loose et al. 2008). This patterning shared many key similarities with in vivo observations, namely: (1) MinD is evenly distributed on the membrane surface in the absence of MinE, and (2) MinE stimulates regular dynamic patterning of MinD.

Additional groups since have reconstituted MinDE-dependent wave formation in vitro (Ivanov & Mizuuchi 2010; Vecchiarelli et al. 2014), demonstrating that wave propagation on a lipid surface can emerge from the addition of MinD, MinE and ATP in vitro. Although these experiments successfully generate traveling MinD surface waves in vitro, they fail to reconstitute the oscillation observed in vivo. More recently, however, MinD oscillation has been observed in vitro by isolating reactions in rod-shaped micro-compartments or manually tuning the concentrations of MinD and MinE (Zieske & Schwille 2013; Zieske & Schwille 2014; Vecchiarelli et al. 2016). In a

flow cell reaction where the MinD concentration is manually tuned, limiting the MinD levels led to the formation of patterns termed “bursts”, which appeared as radial zones of MinD surrounded by MinE that rapidly expanded and contracted on the lipid surface. During the burst cycle, MinD expansion and contraction on the membrane oscillated at regular intervals, consistent with the patterning observed in live cells (Vecchiarelli et al. 2016). Furthermore, MinE levels were shown to influence MinD binding kinetics during the oscillation cycle in vitro, where lower MinE concentrations paradoxically stabilized MinD binding on the SLB, while higher MinE concentrations stimulated release, challenging the view that the role of MinE is solely to stimulate dissociation of MinD from the membrane (Park et al. 2012; Bonny et al. 2013). Therefore, the MinD:MinE stoichiometry on the membrane, and not just MinE binding, drives MinD dynamics with the membrane. When MinD is in excess on the membrane, MinE forms a stabilizing complex with MinD ($D_2:E_2$), while an excess of MinE promotes the formation of the dissociation complex ($E_2:D_2:E_2$) that results in stimulation of MinD ATP hydrolysis and release from the membrane (Vecchiarelli et al. 2016).

Efforts to reconstruct Min dynamics in vitro have contributed immensely to our understanding of the molecular basis for oscillation in vivo. Based on recent structural and biochemical studies, MinE dimers reversibly bind and scan the membrane for MinD. In the beginning of the oscillation cycle, MinE stabilizes membrane association of MinD at the cell pole; since MinD binds the

membrane faster than MinE, the resulting high MinD:MinE stoichiometry favors the formation of the MinD-association complex ($E_2:D_2$) (Fig. 3A). Membrane expansion of MinD at the cell pole leads to a rapid decline in cytoplasmic MinD levels and a reduced rate of membrane binding, promoting the accumulation of MinE and the MinD-dissociation complex ($E_2:D_2:E_2$) (Fig. 3B). Subsequently, MinE stimulates the release of MinD from the membrane. Lingering MinE prevents rebinding of MinD, thereby promoting MinD accumulation at the opposite pole (Fig. 3C).

Regulation of Min dynamics by the FtsZ interaction

In vivo, MinD and therefore MinC oscillation require MinE, but not FtsZ (Raskin & de Boer 1999a; Raskin & de Boer 1999b). However, although FtsZ is not required to achieve oscillation, FtsZ may modulate the oscillation via the interaction with MinC (Fig. 4i). We recently identified MinC mutant proteins fused to Gfp that oscillate more slowly than MinC (Labreck et al., under review). These mutant proteins, MinC(S16D) and MinC(L194N), are impaired for direct interactions with FtsZ but still bind to membrane-associated MinD in vitro, suggesting that the slow oscillation is related to the impaired interaction with FtsZ. Additionally, we observed transient localization of Gfp-MinC(S16D) to the division septum (LaBreck, et al., 2019). Gfp fusion proteins of the MinC CTD have also been reported to localize to the septum (Johnson et al. 2002; Johnson et al. 2004). Gfp-MinC (S16D), like Gfp-MinC CTD, likely is recruited

to the Z-ring but fails to destabilize FtsZ polymers due to a defective MinC NTD and may be momentarily retained at the septum.

Does MinC alter MinD dynamics with the membrane?

Although MinD and MinE are sufficient to form dynamic patterns on membranes, the Min system also includes MinC, which is actively recruited to membrane-associated MinD. Based on reports examining MinDE-dependent patterning in vitro, it is clear that MinE regulates association of MinD with the membrane. Importantly, MinC and MinE share a similar binding interface on MinD (Wu et al. 2011), but the effect of MinC on MinDE-dependent oscillation remains to be characterized.

MinC and MinD undergo ATP-dependent assembly into large filaments (Ghosal et al. 2014; Conti et al. 2015; Huang et al. 2018). Copolymers readily form under physiological conditions; therefore it is likely that MinCD assembly occurs in vivo. However, copolymer formation is not required for cell viability in vivo, and Gfp-MinD still oscillates in the absence of MinC (Park et al. 2015; Raskin & de Boer 1999a). A recent report speculated that copolymer formation might impact Min oscillation in vivo by adjusting the MinD concentration on the membrane or by sequestering MinD in the cytoplasm (Labreck et al., 2019) (Fig. 4ii). Future studies should address the impact of MinC binding on Min patterning in vitro and in vivo.

Concluding remarks

Considerable progress has been made in recent years to understand how MinC functions with MinD and MinE to restrict division-site selection to midcell, and specifically how MinC engages FtsZ to destabilize polymers. Recent studies reconstituting Min dynamics in vitro have also revealed new mechanistic insights into how Min oscillation might be achieved in vivo. However, many additional questions still remain, including how the oscillation is regulated by protein interactions with MinC, FtsZ and other cell division proteins. Moreover, a study by the Baker group identified MinD as a substrate of the two-component ATP-dependent protease ClpXP, and further demonstrated that MinD turnover was partially controlled in a ClpX-dependent manner (Neher et al. 2006). ClpXP has a prominent role in regulating Z-ring dynamics in *E. coli* via proteolysis of FtsZ, and also degrades the FtsZ bundling protein ZapC (Camberg et al. 2009; Buczek et al. 2016). This begs the question of what role ClpXP may have in modulating the oscillation. Further study should be done to determine whether regulation of MinD levels is important for MinDE-dependent oscillation in vivo.

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Figure 1: Sites on MinC important for FtsZ polymer destabilization

(A) *E. coli* MinC modeled onto *T. maritima* PDB 1HF2 (Cordell, et al., 2001)

Residues identified on the MinC N-domain important for function and C-domain (Park, et al., 2018; LaBreck, et al., 2019) are highlighted as Corey-Pauling-Kolter (CPK) spheres on one protomer of the MinC dimer. The dashed line denotes the MinC dimerization interface.

Fig. 2: Inactive and Active conformations of MinE

(A) Structure of the “inactive” 6 β -stranded conformation of *N. gonorrhoeae* MinE PDB 2KXO (Ghasriani et al. 2010) The MinD-interacting interface is occluded in the dimer structure. (B) Structure of the “Active” 4 β -stranded *E. coli* MinE (I24N) structure in complex with MinD (PDB 3R9J) (Park et al. 2011).

Fig. 3: Model of MinDE-dependent oscillation

(A) MinD associates with the inner face of the cytoplasmic membrane in the ATP-bound state. MinE dimers reversibly bind the membrane, and are stabilized on the membrane by the interaction with MinD. When cytoplasmic MinD levels are high, MinD binds the membrane faster than MinE which results in a high MinD:MinE stoichiometry on the membrane. This stoichiometry favors the formation of the MinD association complex ($D_2:E_2$), which stabilizes MinD propagation on the membrane surface (Vecchiarelli, et al., 2016) (B) As cytoplasmic MinD is depleted through membrane association, the rate of membrane binding slows, shifting the MinD:MinE membrane stoichiometry to higher MinE concentrations. This promotes the formation of the MinD dissociation complex ($E_2:D_2:E_2$) (Vecchiarelli, et al., 2016). (C) MinE stimulates ATP hydrolysis and dissociation of MinD from the membrane. Lingering MinE prevents re-binding of MinD on the membrane, promoting propagation towards the opposite pole.

Fig. 4: Regulation of MinD-dependent oscillation

MinD oscillation is driven by MinE-stimulated membrane dissociation, however recent reports suggest that several mechanisms may function to regulate the oscillation. (i) MinD recruits MinC to the membrane, and forms copolymers with MinC. Copolymerization with MinC may modulate the oscillation by altering the concentration of MinD on the membrane or by sequestering MinD in the cytoplasm away from the membrane. (ii) The FtsZ interaction with MinC modulates the rate of oscillation *in vivo*; MinC mutants impaired for the interaction with FtsZ oscillate slower than wildtype MinC.

Manuscript II

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Title: MinC N- and C-domain interactions modulate FtsZ assembly, division site selection and MinD-dependent oscillation in *Escherichia coli*

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Abstract

The Min system in *Escherichia coli*, consisting of MinC, MinD, and MinE proteins, regulates division site selection by preventing assembly of the FtsZ-ring (Z-ring) and exhibits polar oscillation in vivo. MinC antagonizes FtsZ polymerization and, in vivo, the cellular location of MinC is controlled by a direct association with MinD at the membrane. To further understand the interactions of MinC with FtsZ and MinD, we performed a mutagenesis screen to identify substitutions in *minC* that are associated with defects in cell division. We identified amino acids in both the N- and C-domains of MinC that are important for direct interactions with FtsZ and MinD in vitro, as well as mutations that modify the observed in vivo oscillation of Gfp-MinC. Our results indicate that there are two distinct surface-exposed sites on MinC that are important for direct interactions with FtsZ: one at a cleft on the surface of the N-domain and a second on the C-domain that is adjacent to the MinD interaction site. Mutation of either of these sites leads to slower oscillation of Gfp-MinC in vivo, although the MinC mutant proteins are still capable of a direct interaction with MinD in phospholipid recruitment assays. Furthermore, we demonstrate that interactions between FtsZ and both sites of MinC identified here are important for assembly of FtsZ-MinC-MinD complexes and that the conserved C-terminal end of FtsZ is not required for MinC-MinD complex formation with GTP-dependent FtsZ polymers.

Importance

Bacterial cell division proceeds through the coordinated assembly of the FtsZ-ring, or Z-ring, at the site of division. Assembly of the Z-ring requires polymerization of FtsZ, which is regulated by several proteins in the cell. In *Escherichia coli*, the Min system, which contains MinC, MinD and MinE proteins, exhibits polar oscillation and inhibits the assembly of FtsZ at non-septal locations. Here, we identify regions on the surface of MinC that are important for contacting FtsZ and destabilizing FtsZ polymers.

Keywords

cell division; cytokinesis; patterning; tubulin; divisome

Abbreviations

Amp, ampicillin; Cm, chloramphenicol; Clp, caseinolytic protease; CPK, Corey-Pauling-Kolter; CTD, C-terminal domain; CTE, C-terminal end; FtsA, filamentous temperature sensitive mutant A; FtsZ, filamentous temperature sensitive mutant Z; GDP, guanosine diphosphate; GMPCPP, guanylyl 5'- α,β -methylenediphosphonate; GTP, guanosine triphosphate; Gfp, green fluorescent protein; H10, helix 10; Kan, kanamycin; Min, minicell-forming; NTD, N-terminal domain; Par, partitioning-defective; SImA, synthetically lethal

with a defective Min system protein A; SUV, small unilamellar vesicle; TEM, transmission electron microscopy; ZipA, Z-interacting protein A

Introduction

The cell division pathway in prokaryotes is a widely conserved and highly organized cellular process whereby multiple cell division proteins cooperate to divide a single bacterial cell into two daughter cells [1](#). In *E. coli*, cell division initiates with the assembly of the 'Z-ring', which is composed of the tubulin-like protein FtsZ and several FtsZ-interacting proteins, including FtsA and ZipA, at the site of septation [2](#); [3](#). After Z-ring assembly, additional proteins are sequentially recruited to the division site to insert and remodel the peptidoglycan leading to septation [4](#); [5](#). Normal cell growth and development in bacteria relies on protein-based mechanisms to regulate the location of the division machinery and restrict Z-ring position to the longitudinal center of the cell [6](#). Mislocalization of the division machinery promotes asymmetric cell division, generates daughter cells lacking a copy of the bacterial chromosome, and may lead to chromosomal severing and impaired chromosome segregation [7](#); [8](#); [9](#); [10](#).

Division site selection in *E. coli* predominantly depends on two mechanisms: (1) nucleoid occlusion mediated by SlmA, which blocks Z-ring assembly over the bacterial chromosome by binding to DNA and FtsZ, and (2) the Min system, which prevents Z-ring formation at the cell poles [3](#); [8](#); [11](#); [12](#); [13](#); [14](#); [15](#); [16](#); [17](#); [18](#). *E. coli* lacking both the Min system and nucleoid occlusion are not

viable in rich media, and *min* mutants of *Neisseria gonorrhoeae* and *Escherichia coli* O157:H7 are less virulent [8](#); [19](#); [20](#). *E. coli min* mutants display filamentation in liquid culture and produce anucleate minicells that arise from polar divisions [7](#). *N. gonorrhoeae min* mutants are enlarged compared to wild type cells and undergo cell lysis [21](#); [22](#).

The Min system of *E. coli* is composed of three proteins, MinC, MinD, and MinE, that exhibit coordinated polar oscillation in vivo. MinC destabilizes GTP-dependent FtsZ polymers in vitro [23](#); [24](#); [25](#). In the current model, the MinC N-domain binds to the FtsZ-FtsZ subunit interface, preventing the head to tail assembly of FtsZ protomers [26](#); [27](#). Overexpression of the MinC C-domain, which is also the dimerization domain, inhibits cell division in vivo and has been suggested to inhibit lateral associations between FtsZ filaments, and the C-domain also contains the MinD binding site [25](#); [28](#); [29](#); [30](#). An amino acid on the MinC N-domain, G10, was previously reported to be important for FtsZ disassembly [23](#). In a recent study by the Lutkenhaus group, several additional N-domain residues (K9, F42, K35, A39) on MinC were implicated in the FtsZ interaction [31](#).

MinD is a member of the Walker A Cytoskeletal ATPases (WACA) protein family and contains a deviant Walker A motif [32](#); [33](#). MinD controls the cellular distribution of MinC through a direct interaction [34](#). MinD associates with the inner face of the cytoplasmic membrane in its ATP-bound dimer conformation [35](#). MinE promotes the dissociation of MinD from the membrane by stimulating ATP

hydrolysis and release from the membrane surface [36](#); [37](#). Following MinD membrane displacement, MinE has been observed to remain at the membrane via an N-terminal membrane targeting helix [38](#); [39](#), possibly serving to prevent re-binding of MinD at the same position. Indeed, reconstitution of MinCDE patterning in vitro reveals that MinD propagates at the leading edge of MinE waves [40](#).

It was previously demonstrated by our group and the Lowe group that MinC and MinD form copolymers in vitro in the presence of ATP [41](#); [42](#), likely containing alternating MinC and MinD dimers as observed in a crystal structure of *Aquifex aeolicus* MinD in complex with the MinC dimerization domain [41](#). In addition to *E. coli* and *A. aeolicus*, MinCD copolymerization has been detected for *Pseudomonas aeruginosa* [43](#). After ATP-dependent assembly, MinCD copolymers remain stable, but rapidly disassemble in the presence of MinE, suggesting that nucleotide hydrolysis and/or displacement of MinC with MinE mediates disassembly [41](#); [42](#). Although copolymers assemble robustly in vitro, oscillation of MinD in vivo is dependent on MinE and does not require MinC [34](#); [44](#). Moreover, cells expressing MinD heterodimers that bind to MinC but fail to form copolymers display wild type morphology [45](#). Therefore, MinCD copolymer formation is not required for polar oscillation of MinD in vivo and it is unclear if MinCD copolymers assemble in vivo.

To gain further mechanistic insight into the functional interactions of MinC, FtsZ, and MinD, we performed random mutagenesis on *minC* and constructed a

library of strains expressing the randomly mutagenized genes from the chromosomal *minC* locus. By screening recombinants for morphological defects in vivo and testing purified MinC mutant proteins for protein-protein interactions with FtsZ and MinD in vitro, we mapped two distinct FtsZ binding sites on the surface of MinC: one at the cleft in the MinC N-domain and an additional region on the surface of the MinC C-domain. We show that FtsZ directly interacts with complexes of MinC and MinD in the absence and presence of GTP, the condition that stimulates FtsZ polymerization. Finally, we monitored the rate of MinC oscillation in vivo using Gfp-MinC fusion proteins and observed impaired oscillation for both N- and C-domain mutant proteins. These results show that site-specific determinants on the MinC N- and C-domains contribute to FtsZ-MinC-MinD complex formation in the presence and absence of GTP, and that complex formation may modulate Min oscillation in vivo.

Results

Identification and mapping of MinC regions important for cell division in vivo

Cells deleted for *minC* fail to effectively regulate division site selection, leading to aberrant Z-ring placement and the production of minicells and short filamentous cells [7](#); [12](#); [46](#); [47](#). To identify residues on MinC important for regulating Z-ring placement in vivo, we generated a library of strains

containing substitutions in the chromosomal copy of *minC* by PCR-based random mutagenesis. Mutagenized *minC* gene products were then inserted into a *minC* deletion strain (CL0030) containing a kanamycin resistance gene adjacent to *parE*, which encodes a DNA gyrase inhibitor and is under the control of a rhamnose-inducible promoter (Table 1); in this strain, the *kan-P_{rha}-parE* functional cassette is located at the *minC* chromosomal locus. When expressed, ParE prevents cell growth, thus providing selection for recombinants. Mutagenized *minC* from the library was inserted at the chromosomal *minC* locus. Successful recombinants were selected by growth on rhamnose and confirmed by sequencing.

During exponential growth in liquid culture, cells deleted for *minC* are filamentous, with a mean length of $5.28 \pm 0.2 \mu\text{m}$ ($n = 250$) and produce minicells (23.7%) (Fig. 1a and Table 2). In contrast, wild type cells containing functional *minC* have a mean length of $2.47 \pm 0.05 \mu\text{m}$ ($n = 250$) and do not generate minicells (Fig. 1a and Table 2). We screened the library of *minC* mutants by microscopy for cell length defects and minicells and sequenced chromosomal *minC* from selected recombinants to identify amino acid substitutions that confer a functional defect. To confirm that this system can be used to successfully restore wild type morphology to a *minC* deletion strain, we replaced the *kan-Prha-parE* cassette at the *minC* locus with a wild type *minC* gene ($\Delta \textit{minC}::\textit{minC}^+$) and measured average cell length and expression of MinC by immunoblotting (Fig. 1a-c). Overall cell morphology,

cell length distribution, and MinC expression levels were similar between the wild type MG1655 strain (*minC*⁺) and the restored *minC* recombinant strain (Δ *minC*::*minC*⁺), whereas the strain deleted for *minC* (Δ *minC*::*kan-P_{rha}-parE*) contained many longer cells, minicells and no detectable MinC (Fig. 1a-c and Table 2).

Using the mutant library, we identified amino acid substitutions associated with defects in MinC function (Table S1). Nearby surface exposed amino acid residues were also targeted by site-directed mutagenesis. In addition, we also constructed two mutations reported in the literature, G10D and R172A [23](#); [30](#); [48](#) (Table S1). Altogether, we characterized nine single amino acid substitutions in *minC* associated with cell division defects as indicated by length and/or minicell production in vivo (G10D, S12D, S16D, R172A, V18D, S134F, E193K, L194N, and Y201A (Fig. 2a and 2b) (Table 2); MinC mutant proteins characterized were reactive to MinC antisera (Fig. 2c). Several targeted mutations, including S11D, M93K, E156A, L203N, L174N, and G199D did not produce a cell division defect and were not examined further (Fig. S1 and Table S1).

Residues on the MinC N- and C-domains mediate direct binding to FtsZ

Residues identified in the screen map to both the MinC N- and C-terminal domains (Fig. 3a), and are depicted as Corey-Pauling-Koltun (CPK)

spheres, based on the structural model of *E. coli* MinC generated by modeling the amino acid sequence onto the crystal structure available for *Thermotoga maritima* MinC [49](#); [50](#); [51](#). This is consistent with previous findings that the MinC N-domain prevents the sedimentation of FtsZ polymers, while the MinC C-domain abrogates lateral interactions between polymers [25](#); [29](#). Overexpression of either the MinC N-terminal domain (NTD) or the C-terminal dimerization domain (CTD) in vivo blocks cell division [24](#); [29](#).

To determine if the *minC* mutations identified in the screen disrupt the interaction with FtsZ, we purified and assayed each MinC mutant protein for FtsZ destabilization in vitro. Purified FtsZ (6 μ M) was polymerized with GTP (2 mM) under steady state conditions in the presence of a GTP-regenerating system, and then increasing amounts of MinC (0 to 4 μ M) were added. FtsZ polymers were collected by ultracentrifugation and the amount of FtsZ in the pellet was quantified. MinC is a potent destabilizer of FtsZ polymers, and we detected that substoichiometric amounts of purified MinC (0.5 to 4 μ M dimer) were sufficient to inhibit sedimentation of FtsZ (6 μ M) polymers by 87% (Fig. 3b, 3c and Fig. S2). Next, we tested MinC mutant proteins for destabilization of FtsZ polymers in sedimentation assays. All mutant proteins purified similarly to wild type MinC and eluted as a dimer by size exclusion chromatography, except for MinC(V18D), which was insoluble (data not shown). Our results show that all three MinC N-domain mutant proteins tested (G10D, S12D, and S16D) were defective for preventing sedimentation of dynamic FtsZ polymers and resulted in less than 5%

inhibition at 1 μ M (Fig. 3b).

Next, we tested if MinC proteins containing C-domain mutations prevented FtsZ polymer sedimentation. Compared to wild type MinC, MinC(L194N) and MinC(R172A) were poor inhibitors of FtsZ sedimentation, resulting in 90% and 80% loss of function at 1 μ M, respectively (Fig. 3c and Fig. S2), similar to MinC N-domain mutants (Fig. 3b and Fig. S2). MinC(Y201A) and MinC(E193K) were partially defective compared to wild type MinC (55% and 35% loss of function, respectively) (Fig. 3c and Fig S2). MinC(S134F), which contains a mutation in the known MinD binding site (S134) ³⁰, was a potent inhibitor of FtsZ polymerization, similar to wild type MinC (Fig. 3c and Fig. S2). Together, these results indicate that the two regions of MinC mapped in this study are largely responsible for destabilizing FtsZ dynamic polymers; one is located as a cleft on the MinC N-domain and a second maps to the surface of the C-terminal dimerization domain outside of the MinD interaction site (Fig. 3a, Fig. S3a-S3c).

Mutations in the MinC CTD differentially impair recruitment to MinD-containing vesicles and copolymer formation with MinD

MinD recruits MinC to small unilamellar vesicles (SUVs) in the presence of ATP in vitro ^{52; 53}. To investigate if MinC mutant proteins are capable of binding to MinD, we tested them for recruitment to MinD-associated SUVs. MinC and MinC mutant proteins (4 μ M) were added to reactions containing MinD (4 μ M), SUVs

(0.25 mg ml⁻¹) and ATP (4 mM). The reactions were centrifuged at low speed to collect membrane-associated complexes, and the amount of MinC, wild type or mutant protein, fractionating with the SUV pellet was quantified. Mutations in the MinC N-domain only modestly impaired binding of purified MinC to SUV-associated MinD (Fig. 3d). This is expected since binding of MinC to MinD is mediated by the MinC C-terminal domain outside of the dimerization interface and includes a conserved motif (133-RSGQ-136) [30](#). Accordingly, MinC(S134F) failed to fractionate with MinD (Fig. 3d) since S134 is present at the MinD interaction site [30; 41](#). MinC(E193K), and MinC(R172A), are located outside of the MinD interaction site, based on the structural model [30; 41](#) (Fig. S3a and S3b); however, surprisingly they are defective for recruitment to SUVs via MinD by 97% and 63%, respectively (Fig. 3d). In a previous study, a MalE-MinC C-terminal domain fusion protein containing the R172A substitution also showed reduced phospholipid association with MinD, which is consistent with these observations [30](#). MinC(L194N) and MinC(Y201A) were only partially defective for recruitment to SUV-associated MinD (Fig. 3d). Together, our results confirm that mutations in the reported MinD interaction site, which includes S134, prevent recruitment of MinC to SUV-associated MinD and mutations in the MinC N-domain do not impair binding to MinD in the presence of SUVs. Furthermore, a large functional defect was observed for MinC(E193K), which failed to be recruited to MinD-SUV complexes but is outside of the putative MinD interaction site [30; 41](#) (Fig. 3d, Fig. S3a and S3b).

Recently, our group and the Lowe group demonstrated that MinC and MinD form copolymers in the presence of ATP [41; 42](#). The crystal structure of an *A. aeolicus* copolymer containing alternating dimers of the MinC C-terminal domain and MinD has been reported, but the MinC N-domain is not present in the structure (Fig. S3a and S3b) [41](#). Therefore we tested if MinC mutant proteins are capable of copolymer formation with MinD in the presence of ATP by negative staining and transmission electron microscopy (TEM), 90° angle light scatter, and sedimentation assays. MinC wild type and mutant proteins (4 μM) were incubated with MinD (8 μM) and ATP (4 mM) and then reactions were applied to copper grids and analyzed by negative staining TEM. We detected robust copolymer formation by wild type MinC, MinD, and ATP, and detected no copolymers when MinC was omitted (Fig. 3e). As expected, copolymers with MinD were observed for MinC(G10D), MinC(S12D), and MinC(S16D), which map to the MinC NTD (Fig. 3a and 3e). However, we did not detect copolymers by any of the MinC C-domain mutant proteins tested by TEM (Fig. S4a). MinC S134 is present at the MinC-MinD copolymer interface, based on the *A. aeolicus* crystal structure [41](#). Accordingly, we did not detect copolymers in reactions containing MinC(S134F) and MinD with ATP (Fig. S4a). Surprisingly, MinC C-terminal domain mutations, including R172A, E193K, L194N and Y201A, which are located on the surface but are not present at the putative MinC-MinD copolymer interface or the dimerization interface, did not exhibit copolymers by TEM (Fig. 3a, S3a, S3b and S4a) [41](#).

To confirm that MinC CTD mutant proteins do not copolymerize with MinD, we monitored 90° angle light scatter associated with reactions containing MinC mutant proteins, MinD, and ATP. The addition of ATP stimulated light scatter in reactions containing wild type MinC and MinC NTD mutant proteins, but very little scatter was detected for MinC CTD mutant proteins (Fig. S4b). Next, we monitored copolymer formation using sedimentation assays. Copolymer assembly was stimulated by addition of ATP, and polymers were collected by ultracentrifugation. The amount of MinD present in copolymers with MinC wild type or mutant protein was determined by SDS-PAGE and densitometry. As expected, we observed that the amount of MinD in pellet fractions was reduced by 68-86% in reactions containing MinC CTD mutant proteins (Fig. S4c). All MinC proteins tested here form stable dimers by size exclusion chromatography indicating that the CTD is properly folded (data not shown). Finally, we also observed changes in the amounts of copolymers stimulated to form by addition of ATP in reactions containing MinD and MinC NTD mutant proteins. In light scattering assays, MinC(G10D) and MinC(S12D) showed a larger amplitude increase by light scattering than wild type MinC, suggesting that the mutant proteins may be slightly more efficient at copolymer assembly with MinD than wild type MinC (Fig. S4b). In contrast, MinC(S16D) showed a smaller amplitude increase with ATP and MinD by light scattering suggesting that it is less efficient than wild type MinC for copolymer formation; however, we observed copolymers of MinD and MinC(S16D) by TEM (Fig. 3e and S4b). All three MinC NTD mutant proteins were partially defective for assembly of large complexes collected by

ultracentrifugation, although to a lesser extent than all of the MinC CTD mutant proteins. However, both light scattering and ultracentrifugation assays may underreport small polymers. Together, these results suggest that there are additional residues in the MinC CTD, which are located outside of the putative MinD copolymer interface, that are important for assembly of MinC-MinD copolymers from *E. coli*.

The MinC-FtsZ interaction modulates MinC oscillation in vivo

MinC, fused to Gfp (Gfp-MinC), exhibits rapid pole-to-pole oscillation regulated by MinD and MinE [34](#); [54](#). To determine if MinC mutant proteins are capable of polar oscillation in vivo, we constructed *gfp-minC* and performed site-directed mutagenesis to incorporate two substitution mutations identified in the screen, *gfp-minC(S16D)* and *gfp-minC(L194N)*. Then *gfp-minC* wild type and mutant genes were reinserted into the $\Delta minC::kan-P_{rha}-parE$ deletion strain at the native locus by lambda Red recombination. Cells were grown to early log phase and Gfp-MinC localization was examined by confocal fluorescence microscopy.

To detect Gfp-MinC oscillation, we monitored fluorescence in live, dividing cells at 5 second intervals and measured the fluorescence amplitude across the long axis of the cell for each time series (Fig. 4a). We observed that Gfp-MinC accumulates at one cell pole, redistributes to the opposing pole, and

then returns, completing one oscillation cycle, in an average of 42.0 ± 8 seconds ($n=20$) (Fig. 4b), with a minimum observed time of 35 seconds and a maximum observed time of 55 seconds, which is consistent with previous reports [34](#); [54](#). We plotted the amplitude of fluorescence as a function of cell length (nm) at each interval and calculated the rate for directional movement of fluorescence foci along the longitudinal axis of the cell. In cells expressing Gfp-MinC, this rate is $169.5 \pm 5.4 \text{ nm sec}^{-1}$ (Fig. 4c).

It was previously reported that a chimera containing Gfp and the MinC C-domain oscillated similarly to full-length MinC, suggesting that the MinD interaction only requires the C-domain of MinC [55](#); however, this fusion protein also transiently associated with the division septum, which was not observed by wild type Gfp-MinC. Next we tested if Gfp-MinC(S16D), which is competent for interacting with SUV-associated MinD but defective for destabilizing FtsZ polymers in vitro, oscillates in vivo. The rate of oscillation of Gfp-MinC(S16D) is 26.6% slower ($124.4 \pm 9.6 \text{ nm min}^{-1}$) than Gfp-MinC (Fig. 4c). We also observed septal localization, or pausing, of Gfp-MinC(S16D) in approximately 10% of cells (Fig. 4d) ($n=20$ cells exhibiting oscillation).

Next, we tested if Gfp-MinC(L194N) also exhibits impaired oscillation in vivo. In vitro, MinC(L194N) is also defective for destabilizing FtsZ polymers (Fig. 3c). Interestingly, we observed that 68% of cells expressing Gfp-MinC(L194N) contained diffuse cytoplasmic fluorescence and failed to oscillate (data not

shown), in contrast to Gfp-MinC and Gfp-MinC(S16D), where none or 12% failed to oscillate, respectively. In the cells exhibiting Gfp-MinC(L194N) oscillation, the rate was 37.7% slower ($105.6 \pm 7.4 \text{ nm min}^{-1}$) than wild type Gfp-MinC (Fig. 4b and 4c). Interestingly, MinC(L194N) is recruited to SUVs by MinD although slightly less than wild type MinC; however, it is defective for copolymer formation with MinD (Fig. 3d and Fig. S4a-S4c). Together, these results show that mutations in the MinC N- and C-domains known to disrupt the FtsZ interaction above also impair oscillation in vivo.

Recruitment of FtsZ to phospholipid vesicles by MinC and MinD

Finally, we tested if FtsZ can be recruited to SUV-associated complexes of MinC and MinD using purified proteins in vitro. To detect complex formation between MinC, MinD, and FtsZ with SUVs, we used active, fluorescent FtsZ, labeled with Alexa Fluor 488 (FL-FtsZ). The current model suggests that FtsZ polymers first bind to the MinC C-domain via the FtsZ C-terminal end (CTE) [26; 27; 56](#). The Z-ring contains dynamic FtsZ polymers [57; 58](#), therefore we monitored dynamic FtsZ polymers assembled with GTP, which we know are sensitive to disassembly by MinC [25; 26](#), for recruitment to SUV-associated complexes of MinC and MinD. Increasing amounts of FtsZ (0 to 250 pmol), polymerized with GTP and including a GTP regenerating system, were added to reactions containing preassembled complexes of MinC (4 μM), MinD (4 μM), SUVs (0.25 mg ml^{-1}), and ATP (4 mM). Complexes were collected by low speed centrifugation and the

amount of FtsZ recruited was quantified by fluorescence intensity. Without MinC or MinD, few FtsZ polymers were detected in the pellet (Fig. 5a). We observed that MinC-MinD-SUV complexes recruit FtsZ in the presence of GTP, and that the recruitment was dependent on FtsZ concentration, with approximately 70 pmol, or 28% of the total FtsZ in the reaction, recruited to SUVs (Fig. 5a). Next, we monitored SUV-recruitment of stable FtsZ polymers in the presence of GMPCPP. FtsZ-GMPCPP was not detected in pellets without MinC or MinD (Fig. 5b). As expected, we also observed concentration-dependent association of stable FtsZ polymers to Min complexes (Fig. 5b); however, the amount of FtsZ recruited to SUVs in the presence of GMPCPP was reduced by 70%, compared to in the presence of GTP (Fig. 5a and 5b). Previous reports suggested that the MinC N-domain may interact with an open FtsZ protofilament interface, and therefore this region should be also accessible in FtsZ monomers and dimers [27](#). To determine if MinC recruits FtsZ to SUV-associated complexes containing MinD via this site, we repeated the SUV recruitment assay in the presence of GDP to promote FtsZ dimer formation and also detected a concentration-dependent increase in the amount of FtsZ localizing to SUV-associated complexes containing MinD and wild type MinC (Fig. 5c). Our results indicate that complexes of FtsZ-MinC-MinD can be assembled with both polymerized and non-polymerized FtsZ, in contrast to previous reports [25; 26](#). A subsequent study also showed that FtsZ-GDP binds to MinC [59](#). To confirm that SUV-recruitment is dependent on the MinC-MinD interaction, and does not result from non-specific trapping in SUVs, we repeated the SUV-recruitment assay with Gfp in the

presence of MinC (4 μ M), MinD (4 μ M), ATP (4 mM), and, where indicated, GDP (2 mM), GMPCPP (0.2 mM), or GTP (1 mM) and a GTP regenerating system, where indicated. As expected, we detected no SUV-associated Gfp in the pellet (Fig. S5). A recent report also indicated that MinD binds to FtsZ directly; however, we detected no recruitment of FtsZ to MinD-SUV complexes without MinC (data not shown) indicating that under these conditions, MinC is required for interaction with FtsZ⁶⁰.

To determine if the sites that we identified on the MinC N- and C-domains are important for recruitment of FtsZ under the conditions tested, we compared MinC(S16D) and MinC(L194N) to wild type MinC in SUV sedimentation assays. In the presence of GTP and a regenerating system, we detected 40% less FtsZ associated with SUVs and MinD complexes with MinC(S16D) than with wild type MinC, and a modest but insignificant reduction with MinC(L194N) (Fig. 5d). In the presence of GMPCPP, we detected 82% less FtsZ in SUV-associated complexes containing MinD and MinC(L194N) (Fig. 5e) compared to wild type MinC, but a small reduction associated with MinC(S16D)-containing complexes that was not significant (Fig. 5e). Next, we tested if MinC(S16D) and MinC(L194N) recruit non-polymerized FtsZ to SUV-associated complexes with MinD. We detected 68% less FtsZ present in complexes with MinC(L194N), and 94% less FtsZ in complexes with MinC(S16D) compared to wild type MinC (Fig. 5f). Interestingly, these results show that in the presence of GDP, FtsZ interacts with both domains of MinC bound to MinD and SUVs. To confirm that MinC secondary structure

was not perturbed by either of these substitutions, circular dichroism spectra of MinC(S16D) and MinC(L194N) were measured and shown to be similar to wild type MinC (Fig. S6). Together, our results suggest that while stable FtsZ polymers require only the FtsZ-MinC CTD interaction to form a complex with MinC and MinD, non-polymerized FtsZ requires contacts with each domain of MinC for complex assembly. Additionally, dynamic FtsZ polymers assembled with GTP are recruited to MinC much more efficiently than either FtsZ-GMPCPP or FtsZ-GDP, and mutations in either the MinC NTD or CTD only modestly impair recruitment of dynamic FtsZ polymers assembled with GTP. Notably, although MinC(S16D) and MinC(L194N) are able to efficiently recruit dynamic FtsZ polymers to MinD-associated SUVs, they are defective for disassembly of FtsZ polymers in vitro (Fig. 3b-c and 5d).

The FtsZ C-terminal end is required for FtsZ-MinC-MinD complex formation with phospholipids

The FtsZ C-terminal end (CTE), consisting of residues 370 through 383, directly interacts with FtsA, ZipA, MinC, SlmA, ClpXP, and ZapD [18](#); [27](#); [58](#); [61](#); [62](#); [63](#); [64](#); [65](#); [66](#). Several reports implicate the FtsZ CTE in mediating interactions with MinC [26](#); [64](#). In vivo, inhibition of cell division by the MinC CTD requires the FtsZ CTE [26](#), suggesting that FtsZ engages the MinC C-domain. Additionally, a Gfp-MinC C-domain fusion protein is recruited to the Z-ring in vivo and recruitment

requires isoleucine 374 of the FtsZ CTE and R172 of MinC [26; 29; 30; 55; 67](#). Based on these observations, we investigated if recruitment of FtsZ to SUV-associated complexes containing MinC and MinD requires the FtsZ CTE in reactions containing fluorescent, truncated FtsZ [FL-FtsZ(Δ C18)], FtsZ [FL-FtsZ(Δ C9)], and FtsZ [FL-FtsZ(Δ C4)]. Surprisingly, all FtsZ C-terminal mutant proteins were efficiently recruited to SUV complexes containing MinC and MinD in the presence of GTP and a regenerating system, including FL-FtsZ(Δ C18) (Fig. 5g). However, FL-FtsZ(Δ C18) was not recruited to SUV complexes containing MinC and MinD in the presence of GMPCPP or GDP (Fig. 5h and 5i). Additionally, recruitment of both FtsZ(Δ C9) and FtsZ(Δ C4) mutant proteins in the presence of GTP, GMPCPP, or GDP was similar to FtsZ (Fig. 5g-i). Since residue I374 is present in both the FtsZ(Δ C9) and FtsZ(Δ C4) truncations, but not in the FtsZ(Δ C18) mutant, we purified and tested FtsZ(I374V) for the interaction with MinCD. Interestingly, the I374V mutation alone did not impair recruitment of FtsZ to MinC-MinD-SUV complexes in the presence of GDP or GMPCPP in vitro, and we observed only a minor reduction with GTP (Fig. 5g-i). These results suggest that amino acids 366 through 374 of FtsZ are important for binding of MinC-MinD-SUV complexes to FtsZ dimers and GMPCPP-stabilized polymers, but not essential to FtsZ polymers assembled with GTP.

Discussion

Mapping the FtsZ interaction sites on MinC

We mapped two regions on the surface of MinC important for controlling septal localization using mutagenesis and site-specific recombination, followed by functional analyses in vivo and in vitro. Several purified MinC mutant proteins, with mutations in both the N-domain and the C-domain of MinC, failed to effectively prevent the GTP-dependent sedimentation of FtsZ in vitro but were recruited to SUVs by MinD (Fig. 3a-d), suggesting that cell division defects associated with chromosomal *minC* mutants are likely due to an impaired FtsZ interaction. Moreover, MinC mutant proteins with substitutions in the N-domain (S16D) or the C-domain (L194N) bind to dynamic FtsZ polymers but are defective for promoting polymer disassembly, in contrast to wild type MinC. MinC is a potent inhibitor of FtsZ polymerization; disassembly of dynamic FtsZ polymers, assembled with GTP and a regenerating system, occurred at substoichiometric (1:12) amounts of MinC (0.5 μ M MinC dimer and 6 μ M FtsZ) (Fig. 3b, 3c and Fig. S2). This ratio is inconsistent with MinC promoting polymer disassembly via a FtsZ sequestration mechanism. Together, there are two distinct sites of FtsZ interaction on the surface of a single MinC protomer, with four total sites accessible on a dimer (Fig. 6a). Both interaction sites are important for destabilizing FtsZ polymers: one site is at an N-domain cleft (G10, S12, S16) of a protomer and another site is on the outer surface of the C-terminal dimerization domain (R172, E193, L194N, Y201), as modeled here (Fig. 3a) and identified on a recent structure of the CTD of *E. coli* MinC [68](#). Importantly, MinC accommodates interactions simultaneously with MinD and FtsZ and the regions do not appear to

overlap based on FtsZ recruitment assays and structural modeling of the MinC-MinD interface from *A. aeolicus* (Fig. 5 and Fig. S3a-S3c) ⁴¹. Our results are in agreement with a recent study of the MinC N-domain, which implicated nearby residues K9, F42, K35 and A39 in contacting FtsZ and are adjacent to the N-domain cleft described here ³¹. The phenotypic defects associated with MinC N- and C-domain mutations in this study do not result from overexpression, since MinC mutant proteins were expressed to near wild type levels from the chromosome.

Conservation of the MinC-MinD interface

MinC and MinD assemble into copolymers in the presence of ATP in vitro ^{41; 42}. The Lowe group reported a crystal structure for the *A. aeolicus* MinC CTD-MinD complex as alternating dimers arranged as a copolymer, revealing two MinC-MinD interaction sites ⁴¹. The first site contains a conserved 'RSGQ' motif in MinC and helix 7 of MinD, while a secondary site is mediated by contacts between MinD helix 8 and MinC helix 3, although this helix does not appear to be present in *E. coli* MinC ⁴¹. To determine if the mutations identified here in MinC also impair copolymerization with MinD, we purified MinC mutant proteins and measured copolymer formation by EM, light scattering and in sedimentation assays. MinC(S134F) contains a mutation in the first predicted MinC-MinD interaction site (i.e., RSGQ) and is severely impaired for copolymer formation with MinD (Fig. S4a-S4c). We mapped MinC R172, E193, L194, and Y201 onto a

structural model of the *E. coli* MinC C-domain and observed that they are absent from both predicted MinC-MinD interfaces (Fig. S3a and S3b). Surprisingly, the purified C-domain mutant proteins were all impaired for copolymer formation, however they form dimers (data not shown) suggesting that the C-domains are folded correctly and they are recruited to SUVs by MinD with the exception of MinC(E193K) (Fig. 3d and S4a-S4c). This suggests that the interaction between MinC and MinD in the copolymer is different from the interaction with MinD at the membrane. The role of the copolymer during oscillation of the Min system or cell division is not yet understood, and a recent report suggests that copolymer formation is not required for division [45](#).

Slow oscillation by Gfp-MinC mutant proteins

Oscillation of Gfp-MinD in vivo and, by extension Gfp-MinC, requires MinE to promote ATP hydrolysis by MinD, but does not require FtsZ [34](#); [44](#). Remarkably, we found that Gfp-MinC mutant proteins impaired for disassembly of dynamic FtsZ polymers (S16D and L194N) oscillate 27% and 38% slower, respectively, in vivo compared to Gfp-MinC (Fig. 3b, 3c and 4c). This is likely not due to a perturbed interaction with membrane-associated MinD, since purified MinC(S16D) and MinC(L194N) were recruited to MinD-bound SUVs (Fig. 3d). Both of these mutants are impaired for destabilizing dynamic FtsZ polymers, yet both recruit FtsZ polymers assembled with GTP to MinC-MinD-SUV complexes (Fig. 3b, 3c and 5d). In vivo, how could FtsZ binding modulate MinC oscillations?

Pausing at the division septum could account for the slow oscillation of Gfp-MinC(S16D). Consistent with this, we observed transient localization of Gfp-MinC(S16D) at the Z-ring in 10% of cells (Fig. 4d), suggesting productive recruitment to dynamic FtsZ polymers in vivo. Failure to destabilize polymers at the Z-ring may lead to the prolonged localization observed. Accordingly, the MinC C-domain tagged with Gfp also pauses at the septum, consistent with failure to disassemble the ring^{55; 64}. MinC(L194N) is also able to recruit FtsZ polymers assembled with GTP to MinD-associated SUVs (Fig. 5d), raising the possibility that MinC(L194N) may also be recruited to FtsZ polymers in vivo, although we did not detect septal localization or pausing of Gfp-MinC(L194N).

A recent report showed that the stoichiometry of MinE and MinD on the membrane may modulate MinE-stimulated stabilization and release of MinD ⁶⁹. MinD oscillation in vivo and wave propagation in vitro occurs in the total absence of MinC ^{34; 38; 40; 44; 69}. The Lutkenhaus group showed that expression of MinD heterodimers impaired for MinC copolymerization, but not MinC binding, restore wild type morphology to a *min* deletion strain ⁴⁵, suggesting that copolymer formation is not required to effectively regulate Z-ring formation in vivo under the conditions tested. However, engagement of MinD by MinC could serve to constrain the accessible population of MinD, either through sequestration on the membrane surface or in the cytoplasmic pool, and therefore modulate the propagation of MinD across the phospholipid surface. If copolymers modulate the availability and membrane concentration of MinD, then the slow oscillation

observed for MinC(L194N) could alternatively result from impaired copolymer formation with MinD (Fig. 4b, 4c and Fig. S4a-S4c).

Destabilization of FtsZ polymers by MinC

FtsZ assembles into dynamic polymers in the presence of GTP, with more than half of the FtsZ within filaments suggested to exist in a GDP-bound state [70](#); [71](#). MinC substoichiometrically destabilizes FtsZ polymers, without MinD, in a concentration-dependent manner and destabilization requires active GTP hydrolysis (Fig. 3b and 3c). MinC does not slow the rate of GTP hydrolysis by FtsZ²³, which is consistent with MinC rapidly releasing FtsZ. In a model for how MinC destabilizes FtsZ polymers, the Lutkenhaus group proposed that the FtsZ CTEs in FtsZ filaments recruit the MinC CTD, thus positioning the MinC NTD at the FtsZ H10 helix within the FtsZ subunit-subunit interface and promoting the breakage of polymers [27](#). More recently, it was suggested that potent disassembly activity by MinC in the presence of MinD may occur through a severing mechanism³¹. Our results extend this model by revealing that nucleotide occupancy of FtsZ differentially affects engagement by MinC at each site mapped in this study. We further demonstrate that recruitment of FtsZ assembled with GTP to MinC does not require the CTE of FtsZ. Pausing of Gfp-MinC(S16D) at the septum is consistent with a model in which a MinC NTD interaction with FtsZ in a filament promotes release of the FtsZ subunit from the filament by a severing

mechanism. Recently, the Schwille group also demonstrated that MinC enhances the detachment rate of GDP-bound FtsZ from polymers [70](#).

FtsZ subunit turnover due to GTP hydrolysis occurs stochastically within filaments, resulting in exposed minus ends containing the H10 helix [70](#). Since the FtsZ H10 helix would be exposed in GDP-bound FtsZ, this suggests that MinC should interact with GDP-FtsZ. A previous study from the Rivas group reported that MinC strongly binds GDP-FtsZ [59](#). Consistent with this, we detected complex formation between free GDP-FtsZ and SUV-associated MinC-MinD, which required both MinC NTD and CTD sites (Fig. 5c and 5f), but recruitment of GDP-FtsZ was much less efficient than recruitment of dynamic FtsZ polymers, consistent with a weaker interaction. Together, this suggests that a GDP-bound FtsZ subunit may be conformationally distinct in the polymer compared to the dimer.

In the model of MinC engaging FtsZ, the MinC-FtsZ interaction is promoted by FtsZ GTP binding and hydrolysis and, although the interaction between the FtsZ CTE and MinC is not essential for recruitment of dynamic FtsZ polymers to MinC-MinD-SUV complexes, the CTE is important for binding of MinC to GMPCPP-stabilized FtsZ polymers and FtsZ-GDP dimers (Fig. 5g-i and 6a-d). In the model, MinC residues R172, E193, L194, and Y201, which are clustered on the MinC C-domain, mediate an interaction with residues 366-374 of FtsZ. The cleft at the MinC N-domain, containing residues G10, S12 and S16, is

important for binding to the FtsZ polymerization domain, likely at the protofilament interface (Fig. 6a). Both MinC NTD and CTD sites mapped in this study are important for promoting efficient FtsZ polymer disassembly or severing (Fig. 6d). Spatially, the distance between two MinC N-domain clefts in a dimer is approximately 80% larger than the distance between adjacent subunit interfaces in an FtsZ protofilament (76 Å and 42 Å, respectively) (Fig. 6a). Although the MinC N-domain is likely considerably flexible in position relative to the C-domain due to a long tethering region between them, given this distance, it may be more likely that a MinC dimer would interact with, and subsequently destabilize, two separate FtsZ protofilaments, which is consistent with MinC acting as a potent inhibitor of FtsZ polymerization

Material and Methods

Bacterial strains, plasmids, and growth conditions

Lennox media (LB) was inoculated with stationary phase cultures of wild type *E. coli* MG1655 or mutant strains, and cultures were grown at 30°C to an OD₆₀₀ of approximately 0.5. Strains containing chromosomal *gfp-minC* were grown in the presence of L-arabinose (100 μM) to induce expression of *gfp-minC* [72](#).

Mutagenesis and screening

Plasmids containing *minC* were mutagenized by amplification with the Mutazyme II error-prone polymerase (Agilent) and by site-directed mutagenesis (Agilent). Mutagenized *minC* gene products were introduced into the chromosomal copy of *minC* using λ -red recombination ⁷³ (J. Teramoto, K. A. Datsenko, and B. L. Wanner, unpublished results). MG1655 $\Delta minC::kan-Prha-parE$ containing pKD46 was grown as described in *Bacterial strains, plasmids, and growth conditions* to an OD₆₀₀ of approximately 0.4 in the presence of ampicillin (100 μ g ml⁻¹) and L-arabinose (20 mM). Cells were electroporated with mutagenized *minC* gene products and recombinants containing chromosomal *minC* mutants were selected by growth on M9 minimal media agar plates with 1% L-Rhamnose. Subsequently, all recombinants were confirmed by sequencing. Recombinant strains containing mutagenized *minC* (1020 strains) were isolated and screened for cell length by microscopy. Of the 240 isolates exhibiting a filamentous phenotype, 190 contained premature stop codons or missense mutations, 8 had single nucleotide substitutions, and 42 contained two or three nucleotide substitutions, which were reconstructed as single substitutions.

Microscopy

Wild type and mutant cells were grown as described in *Bacterial strains, plasmids, and growth conditions*. Cells were collected by centrifugation at

5,000 x *g*, washed in Tris Buffered Saline with EDTA (1 mM), and were fixed to glass slides with poly-L-lysine coated coverslips. Cells were imaged by differential interference contrast (DIC) microscopy with a Zeiss AxioCam HRc high-resolution camera. Images were prepared using Adobe Photoshop CS3 and cell lengths were measured using NIH ImageJ software. To monitor the oscillation of Gfp-MinC mutant proteins, strains containing *gfp-minC* were grown as described and applied to glass slides with agarose gel pads (5% w/v) containing M9 minimal media supplemented with 0.2% glucose. Live cells were visualized by confocal fluorescence microscopy with a Zeiss AxioImager M2 imaging system equipped with a LSM 700 confocal module with excitation and emission set to 488 and 525, respectively. To determine the rate of fluorescence movement across the longitudinal cell axis (nm min^{-1}), images of live cells were captured in 5-second intervals for 20 frames. The peak fluorescence was determined for each image using NIH ImageJ software and plotted as a function of distance traveled from the originating pole.

Protein extraction and Immunoblotting

Wild type and mutant strains were grown as described in *Bacterial strains, plasmids, and growth conditions*. Cells were collected by centrifugation at 5,000 x *g* and resuspended into 1 ml of LB Lennox medium. Protein was extracted by the addition of 15% TCA (*v/v*) and protein concentration was determined by the Bicinchoninic acid assay (BCA). Protein extracts (75 μg)

were analyzed by SDS-PAGE and immunoblotted with antibodies directed against MinC. Anti-MinC antisera was generated in rabbits using purified wild type MinC (Noble Life Sciences, Inc.)

Expression and purification of proteins

Native MinC, MinD and FtsZ wild type and mutant proteins were overexpressed in *E. coli* BL21 (λ DE3) and purified as described [42](#); [61](#); [74](#). Size exclusion chromatography performed as part of the purification confirmed dimerization of MinC and MinC mutant proteins. Protein concentrations are reported as FtsZ monomers, MinC dimers, and MinD dimers. Where indicated, circular dichroism spectra were obtained for reactions containing MinC (1 μ M) or MinC mutant proteins (1 μ M), where indicated, in assembly buffer on a Jasco J-1100 CD spectrophotometer at room temperature using a 0.1 mm quartz cuvette. Data were collected from 200-250 nm, averaged over 5 scans.

FtsZ Polymerization Assays with MinC

Purified FtsZ was incubated with GTP (2 mM) and a GTP-regenerating system containing acetyl phosphate (15 mM) and acetate kinase (25 μ g ml⁻¹) in the presence of increasing MinC or MinC mutant protein concentration (0 to 4 μ M). Reactions were performed in assembly buffer containing 2-(N-morpholino)ethanesulfonic acid (MES) (50 mM, pH 6.5), KCl (100 mM), MgCl₂

(10 mM), and polymers were collected by centrifugation at 129,000 x *g* for 30 min at 23°C in a Beckman TLA 120.1 rotor. Pellets containing polymers were resuspended in boiling lithium dodecyl sulfate (LDS) loading buffer and analyzed by SDS-PAGE and densitometry using NIH ImageJ.

Phospholipid recruitment assays with MinD and MinC

MinD (4 μ M) was added to reactions containing SUVs (0.25 mg ml⁻¹), MinC (4 μ M) or MinC mutants (4 μ M), where indicated, and ATP (4 mM) in assembly buffer. Small unilamellar vesicles from *E. coli* extracts (Avanti Polar lipids) were prepared as described in Conti, et al., 2018 [42](#). Reactions were incubated at 30°C for 10 min and phospholipids were collected by centrifugation at 14,000 x *g* for 15 min at 23°C. SUV-containing pellets were resuspended in LDS loading buffer and analyzed by SDS-PAGE and densitometry.

Where indicated, purified FtsZ or FtsZ mutant proteins labeled with Alexa Fluor-488, or Gfp, were pre-mixed with GMPCPP (0.2 mM), GDP (2 mM), or GTP (1 mM) and a GTP regenerating system containing acetyl phosphate (15 mM) and acetate kinase (25 μ g ml⁻¹) where indicated, and increasing amounts of FtsZ or Gfp with nucleotide was added to reactions containing MinCD complexes (4 μ M each) pre-assembled onto SUVs as described. Active, fluorescent FtsZ wild type and mutant proteins were

generated by labeling with Alexa Fluor-488 succinimidyl ester and cycling through GTP-dependent polymerization as described [61](#). Reactions containing GMPCPP or GTP were incubated for 5 min at 23°C and SUVs were collected by centrifugation at 10,000 x *g* for 2 min 23 °C. Reactions containing GDP were incubated for 10 min at 30°C and SUVs were collected by centrifugation at 14,000 x *g* for 15 min at 23 °C. SUV-containing pellets were resuspended in assembly buffer and pellet-associated fluorescence was measured with excitation and emission wavelengths set for 490 nm and 525 nm, respectively (Alexa Fluor 488), or 395 nm and 510 nm, respectively (Gfp).

Copolymer assays with MinC and MinD

MinD (8 μ M) was added to reactions containing MinC (4 μ M) or MinC mutant proteins (4 μ M), where indicated, in assembly buffer. ATP (4 mM) was added to initiate copolymer formation, and reactions were incubated for 5 min at 23°C. Polymers were collected by centrifugation at 129,000 x *g* for 30 min at 23 °C in a Beckman TLA 120.1 rotor. Pellets were resuspended in LDS loading buffer (1X), and the amount of pellet-associated MinD was determined by SDS-PAGE and densitometry.

To monitor MinCD copolymer formation by light scatter, MinC (4 μ M) or MinC mutant proteins (4 μ M), where indicated, were added to reactions containing MinD (8 μ M) in assembly buffer. 90° angle light scatter was

monitored with excitation and emission wavelengths set to 450nm. After 5 min, ATP (4 mM) was added and light scatter was monitored for an additional 30 min.

Electron Microscopy

Reactions containing MinD (8 μ M), MinC (4 μ M) and MinC mutant proteins (4 μ M), where indicated, and ATP (4 mM), in assembly buffer were applied to 300-mesh carbon/formvar-coated grids, fixed with glutaraldehyde (1%), and stained with uranyl acetate (2%). Samples were imaged by transmission electron microscopy using a FEI Tecnai G2 Spirit BioTWIN 80Kv instrument equipped with a SIS Morada 11 Megapixel camera.

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Table 1. *E. coli* strains and plasmids.

Strain or Plasmid	Genotype	Source, reference or Construction
Strains		
MG1655	<i>LAM-rph-1</i>	76
BL21 (ΔDE3)	<i>F⁻ ompT gal dcm lon hsdSB(rB⁻ mB⁻) λ(DE3)[lacI lacUV5-T7 gene 1 ind1 sam7 nin5]</i>	EMD Millipore, USA
CL0030	MG1655 Δ <i>minC</i> :: <i>kan-Prha-parE</i>	pKD267; λred
CL0048	MG1655 Δ <i>minC</i> :: <i>minC</i> ⁺	CL0030; λred
CL0062	MG1655 <i>minC</i> (G10D)	CL0030; λred
CL0064	MG1655 <i>minC</i> (S12A)	CL0030; λred
CL0068	MG1655 <i>minC</i> (S16D)	CL0030; λred
CL0070	MG1655 <i>minC</i> (S12D)	CL0030; λred
CL0085	MG1655 <i>minC</i> (S11A)	CL0030; λred
CL0093	MG1655 <i>minC</i> (L194P)	CL0030; λred
CL0100	MG1655 <i>minC</i> (S11D)	CL0030; λred
CL0156	MG1655 <i>minC</i> (S134F)	CL0030; λred
CL0272	MG1655 <i>minC</i> (L218Q)	CL0030; λred
CL0343	MG1655 <i>minC</i> (I197T)	CL0030; λred
CL0369	MG1655 <i>minC</i> (V147D)	CL0030; λred
CL0386	MG1655 <i>minC</i> (A70P)	CL0030; λred
CL0389	MG1655 <i>minC</i> (A155P)	CL0030; λred
CL0396	MG1655 <i>minC</i> (V18D)	CL0030; λred
CL0397	MG1655 <i>minC</i> (S69P)	CL0030; λred
CL0401	MG1655 <i>minC</i> (E193V)	CL0030; λred
CL0403	MG1655 <i>minC</i> (L203N)	CL0030; λred
CL0405	MG1655 <i>minC</i> (A84T)	CL0030; λred
CL0406	MG1655 <i>minC</i> (R172A)	CL0030; λred
CL0408	MG1655 <i>minC</i> (Y201A)	CL0030; λred
CL0410	MG1655 <i>minC</i> (M93K)	CL0030; λred
CL0413	MG1655 <i>minC</i> (E193K)	CL0030; λred
CL0414	MG1655 <i>minC</i> (E156A)	CL0030; λred
CL0416	MG1655 <i>minC</i> (L174N)	CL0030; λred
CL0418	MG1655 <i>minC</i> (G199D)	CL0030; λred
CL0428	MG1655 <i>pBAD-gfp-minC</i>	CL0030; λred
CL0450	MG1655 <i>pBAD-gfp-minC</i> (S16D)	CL0030; λred
CL0468	MG1655 <i>pBAD-gfp-minC</i> (L194N)	CL0030; λred
Plasmids		
pET-MinC	<i>kan</i>	42
pET-MinD	<i>kan</i>	42
pET-MinC(G10D)	<i>kan</i>	(This study)
pET-MinC(S11A)	<i>kan</i>	(This study)
pET-MinC(S11D)	<i>kan</i>	(This study)
pET-MinC(S12D)	<i>kan</i>	(This study)
pET-MinC(S16D)	<i>kan</i>	(This study)
pET-MinC(V18D)	<i>kan</i>	(This study)
pET-MinC(S69P)	<i>kan</i>	(This study)
pET-MinC(S134F)	<i>kan</i>	(This study)
pET-MinC(A155P)	<i>kan</i>	(This study)
pET-MinC(V165D)	<i>kan</i>	(This study)
pET-MinC(R172A)	<i>kan</i>	(This study)
pET-MinC(E193K)	<i>kan</i>	(This study)
pET-MinC(L194N)	<i>kan</i>	(This study)
pET-MinC(L194P)	<i>kan</i>	(This study)
pET-MinC(I197T)	<i>kan</i>	(This study)
pET-MinC(Y201A)	<i>kan</i>	(This study)
pKD46	<i>amp</i>	73
pKD267	<i>kan</i>	B. Wanner ^a
pBAD33- <i>gfp</i>	<i>cat</i>	(This study)
pBAD33- <i>gfp-minC</i>	<i>cat</i>	(This study)
pBAD33- <i>gfp-minC</i> (S16D)	<i>cat</i>	(This study)
pBAD33- <i>gfp-minC</i> (L194N)	<i>cat</i>	(This study)

^a J. Teramoto, K. A. Datsenko, and B. L. Wanner (unpublished results)

Table 2. Minicell percentages and cell lengths of *E. coli* strains

Strain	Minicell Percentage (%) ^a	Mean cell length (μm) ^a	<i>p</i> -value ^b
MG1655 (<i>minC</i> ⁺)	0.0	2.47 ± 0.05	
MG1655 Δ <i>minC::minC</i> ⁺	0.0	2.78 ± 0.05	
Δ <i>minC::gfp-minC</i>	<0.5	2.41 ± 0.04	
Δ <i>minC::kan-Prha-parE</i>	23.7	5.28 ± 0.20	<.01
MG1655 <i>minC</i> (G10D)	10.8	5.85 ± 0.18	<.01
MG1655 <i>minC</i> (S12D)	3.6	3.42 ± 0.11	<.01
MG1655 <i>minC</i> (S16D)	8.6	5.59 ± 0.20	<.01
MG1655 <i>minC</i> (V18D)	23.5	3.05 ± 0.12	<.01
MG1655 <i>minC</i> (S134F)	12.7	3.15 ± 0.10	<.01
MG1655 <i>minC</i> (R172A)	11.0	3.73 ± 0.16	<.01
MG1655 <i>minC</i> (E193K)	8.0	2.73 ± 0.12	≤.02
MG1655 <i>minC</i> (L194N)	3.9	2.76 ± 0.17	≤.05
MG1655 <i>minC</i> (Y201A)	4.8	2.51 ± 0.09	n.s.

^a Minicell percentage generated by analyzing images containing at least 215 cells for minicells. Mean cell length was calculated by measuring at least 215 cells: minicells were omitted from length measurements. Error values are reported as SEM.

^b *p* values for each data set are compared to the wild type strain and were calculated by Welch's *t* test. (n.s., not significant)

Fig. 1. Cell morphology of strains deleted for *minC* and restored with *minC* PCR products. (a) Log phase cultures of MG1655 (*minC*⁺), $\Delta minC::kan-P_{rha}-parE$, and $\Delta minC::minC^+$ were added to poly-L-lysine coated coverslips and examined by DIC microscopy. Size bar is 2 μ m. Arrowheads denote minicells. (b) Box and whiskers plot of cell lengths (μ m) for wild type MG1655 (*minC*⁺), $\Delta minC::kan-P_{rha}-parE$, and $\Delta minC::minC^+$ strains (each n = 250 cells). The box represents the interquartile range. The line in the middle of the box is plotted at the median (***) is p < .0001). (c) Protein extracts (75 μ g) from MG1655 (*minC*⁺), $\Delta minC::kan-P_{rha}-parE$, $\Delta minC::minC^+$ and purified MinC (10 ng) were immunoblotted with anti-MinC antisera as described in *Materials and Methods*.

Fig 2. Cell morphology and cell length distribution of strains expressing chromosomal *minC* mutants. (a) MG1655 (*minC*⁺), $\Delta minC::kan-P_{rha}-parE$, and strains containing chromosomal *minC* mutant genes were grown as described in *Materials and Methods* and examined by DIC microscopy. Size bar is 2 μ m. (b) Box and whiskers plot of cell lengths (μ m) were plotted for wild type and *minC* mutant strains. At least 215 cells were measured for each strain. The box represents the interquartile range. The line in the middle of the box is plotted at the median; all measured values were plotted. (c) Protein extracts (75 μ g) from MG1655 (*minC*⁺), $\Delta minC::kan-P_{rha}-parE$, and strains containing chromosomal *minC* mutant genes were immunoblotted with anti-MinC antisera as described in *Materials and Methods*.

Fig 3. MinC mutant proteins are impaired for destabilizing FtsZ polymers, MinC-MinD copolymer formation, and recruitment to SUVs by MinD. (a) *E. coli* MinC modeled onto *T. maritima* MinC PDB 1HF2 ⁴⁹; the dashed line represents the dimerization interface. MinC residues that were mutagenized are indicated as CPK spheres on one protomer: N- and C-terminal domains are labeled on the opposing protomer as NTD and CTD, respectively. (b-c) Inhibition of FtsZ polymerization by MinC and MinC mutant proteins was monitored by sedimentation of FtsZ polymers. FtsZ polymerization assays were performed as described in *Material and Methods* in reactions containing FtsZ (6 μ M), MinC or MinC mutant (0 to 4 μ M), where indicated, and GTP (1 mM) with a GTP regenerating system. Pellet-associated FtsZ was quantified by SDS-PAGE and densitometry. (d) MinC recruitment to SUVs by MinD was monitored by sedimentation assays as described in *Materials and Methods* in reactions containing MinD (4 μ M), MinC (4 μ M) or MinC mutant protein (4 μ M), where indicated, SUVs (0.25 mg ml⁻¹), and ATP (4 mM). SUV-associated MinC was quantified by SDS-PAGE and densitometry. Data from at least three replicates are shown as mean $\pm$ SEM (* is $p < .01$; ** is $p < .001$; *** is $p < .0001$). (e) MinC-MinD copolymer formation was visualized by transmission electron microscopy. Reactions containing MinD (8 μ M), ATP (4 mM), and MinC (4 μ M) or MinC mutant proteins (4 μ M), where indicated, were applied to carbon/formvar-coated grids, stained with uranyl acetate and visualized by EM as described.

Fig 4. Oscillation of Gfp-MinC and Gfp-MinC mutant proteins. (a) Live cells expressing Gfp-MinC from the chromosome were grown to log phase and imaged by confocal fluorescence microscopy as described in *Materials and Methods*. Frames were captured in 5 sec increments, and the fluorescence intensity across the long axis of the cell for each frame was plotted for a representative cell. The cell image used for quantification is shown within each inset. Scale bar is 1 μm . (b) Live cells containing chromosomal *gfp-minC* (top), *gfp-minC*(S16D) (middle), and *gfp-minC*(L194N) (bottom) were grown and imaged by confocal fluorescence microscopy as described in *Materials and Methods*. Panels showing one complete oscillation of Gfp fluorescence are shown adjacent to a DIC image of each cell. Scale bar is 1 μm . (c) Box and whiskers plot showing the rate of fluorescent foci tracking (nm sec^{-1}) in live cells expressing chromosomal *gfp-minC* mutations. Live cells expressing chromosomal *gfp-minC*, *gfp-minC*(S16D), and *gfp-minC*(L194N) were grown and imaged by confocal fluorescence microscopy, and the rate of Gfp movement (nm sec^{-1}) was determined as described in *Materials and Methods*. The box represents the interquartile range. The median rate of Gfp movement is represented by a line within each box, and the mean is represented by a “+” symbol. Twenty replicate cells that exhibited oscillation were analyzed for each strain (** is $p < .001$; *** is $p < .0001$). (d) Live cells expressing chromosomal *gfp-minC*(S16D) were grown and imaged by confocal fluorescence microscopy as described in *Materials and Methods*. Gfp-MinC(S16D) fluorescence

localized at the septum is indicated by a white arrow in the fluorescence panel.

Scale bar is 1 μm .

Fig 5. FtsZ complex formation with MinC, MinD and SUVs. (a-c) FtsZ-MinC-MinD SUV-sedimentation assays were assembled in reactions containing FtsZ (0 to 10 μM), SUVs (0.25 mg ml^{-1}), MinC (4 μM) and MinD (4 μM), where indicated, GTP (1 mM) and a GTP regenerating system, GMPCPP (0.2 mM), or GDP (2 mM), where indicated, and ATP (4 mM) as described in *Materials and Methods*. SUV-associated FtsZ (pmol) in the presence (black squares) or absence (black triangles) of MinC-MinD was collected by centrifugation as described in *Materials and Methods* and quantified by measuring the fluorescence associated with pellet fractions. (d-f) FtsZ-MinC-MinD SUV-sedimentation assays were performed as described in *Materials and Methods* in reactions containing FtsZ (10 μM), SUVs (0.25 mg ml^{-1}), MinD (4 μM), MinC (4 μM), MinC(S16D) (4 μM), or MinC(L194N) (4 μM), where indicated, GTP (1 mM) and a GTP regenerating system, GMPCPP (0.2 mM), GDP (2 mM), where indicated, and ATP (4 mM). SUV-associated FtsZ (pmol) was collected by centrifugation as described in *Materials and Methods* and quantified by measuring the fluorescence associated with pellet fractions. The amount of FtsZ (pmol) shown is corrected for background. (g-i) FtsZ-MinC-MinD SUV-sedimentation assays were performed as described in *Materials and Methods* in reactions containing FtsZ (10 μM) or FtsZ(ΔC4) (10 μM), FtsZ(ΔC9) (10 μM), FtsZ(ΔC18) (10 μM), FtsZ(I374V) (10 μM), where indicated, SUVs (0.25 mg ml^{-1}), MinD (4 μM), MinC (4 μM), ATP (4 mM), GTP (1 mM) and a GTP regenerating system, GMPCPP (0.2 mM), or GDP (2 mM), where indicated. SUV-associated FtsZ (pmol) was collected by centrifugation

as described in *Materials and Methods* and quantified by measuring fluorescence and corrected for background. Data from at least three replicates are shown as mean $\pm$ SEM (* is $p < .01$; ** is $p < .001$; *** is $p < .0001$)

Fig 6. Model for MinC engaging FtsZ assemblies. (a) Relative position of the FtsZ subunit interface and the MinC-FtsZ interaction sites. The distance between adjacent FtsZ protofilament interfaces (42 angstroms) and between MinC N-domain protomer clefts (76 angstroms) is shown. Although there is likely considerable flexibility in the position of the MinC NTD relative to the CTD, distances suggest that both MinC NTD clefts in a dimer would be unable to engage adjacent FtsZ protomers in a single polymer. *E. coli* FtsZ is modeled onto *M. jannaschii* FtsZ dimer PDB 1W59 ⁷⁵ and *E. coli* MinC is modeled onto *T. maritima* MinC PDB 1HF2 ⁴⁹. GTP is indicated by wheat CPK spheres, FtsZ(N280) and FtsZ(E276) are indicated by light blue and dark blue CPK spheres, respectively. MinC G10, S12, S16, R172, E193, L194 and Y201 are shown as yellow CPK spheres. (b) The MinC C-domain site recruits the FtsZ CTE of stable GMPCPP-FtsZ polymers. (c) Both the MinC N- and C-domain site mediate binding to free GDP-FtsZ. (d) FtsZ polymers assembled with GTP are recruited by MinC. Recruitment does not require the CTE of FtsZ, although it may stabilize the interaction. Although neither the MinC NTD or CTD site identified here is essential for recruitment, both are important for robust severing of FtsZ polymers.

S1 Table: Phenotypes of *E. coli* strains examined and reactivity to MinC antibodies

Strain	Phenotype ^a	Expression detected by MinC antisera
MG1655	+++	yes
$\Delta minC::kan-Prha-parE$	-	no
MG1655 $\Delta minC::minC+$	+++	yes
MG1655 <i>minC</i> (G10D)	-	yes
MG1655 <i>minC</i> (S11D)	+++	not tested
MG1655 <i>minC</i> (S12D)	++	yes
MG1655 <i>minC</i> (S16D)	-	yes
MG1655 <i>minC</i> (V18D)	++	yes
MG1655 <i>minC</i> (S69P)	+	no
MG1655 <i>minC</i> (A70P)	+	no
MG1655 <i>minC</i> (S134F)	+	yes
MG1655 <i>minC</i> (V147D)	-	no
MG1655 <i>minC</i> (V151D)	++	no
MG1655 <i>minC</i> (A155P)	+	no
MG1655 <i>minC</i> (E156A)	+++	not tested
MG1655 <i>minC</i> (V165D)	-	no
MG1655 <i>minC</i> (R172A)	-	yes
MG1655 <i>minC</i> (L174N)	+++	not tested
MG1655 <i>minC</i> (E193K)	++	yes
MG1655 <i>minC</i> (L194P)	+	no
MG1655 <i>minC</i> (L194N)	++	yes
MG1655 <i>minC</i> (G199D)	+++	not tested
MG1655 <i>minC</i> (Y201A)	++	yes
MG1655 <i>minC</i> (L203N)	+++	not tested
MG1655 <i>minC</i> (L218Q)	++	no

^a ‘+++’ denotes not filamentous, ‘+’ and ‘++’ denote mild and moderate filamentation, respectively. ‘-’ refers to severe filamentation.

Fig. S1. Cell morphology of strains expressing *minC* mutant proteins from the chromosome. Strains expressing chromosomal *minC* mutants were grown to log phase and examined by DIC microscopy as described in *Materials and Methods*. Size bar is 2 μm .

Fig. S2. Inhibition of FtsZ polymerization by MinC and MinC mutant proteins.

Inhibition of FtsZ polymerization by MinC or MinC mutant proteins was monitored by FtsZ polymer sedimentation. FtsZ polymerization assays were performed in reactions containing FtsZ (6 μ M), MinC (0 to 4 μ M), or MinC mutant proteins (0 to 4 μ M), in the presence of GTP (1 mM) and a GTP regenerating system. The amount of pellet-associated FtsZ in reactions containing MinC or MinC mutant proteins is shown by SDS-PAGE.

Fig. S3. Mapping the FtsZ-MinC CTD interaction site on the Aa MinCD co-crystal structure. (a) *E. coli* MinC modeled onto *T. maritima* MinC PDB 1HF2⁴⁹. Residues on the surface of the MinC CTD featured in this study are indicated by red CPK spheres. The MinD binding site is indicated by an arrow. (b) *A. aeolicus* MinCD cocrystal⁴¹. *E. coli* MinC CTD residues important for function were aligned with the *A. aeolicus* amino acid sequence and are indicated in red. (c) Clustal omega alignment of *E. coli* MinC and *A. aeolicus* MinC. Residues mapping to the MinC CTD are highlighted in red.

Fig. S4. MinD copolymerization with MinC or MinC mutant proteins. (a)

MinC-MinD copolymer formation was visualized by negative staining electron microscopy. Reactions containing MinD (8 μ M), ATP (4 mM), MinC (4 μ M) or MinC mutant proteins (4 μ M), where indicated, were applied to carbon-coated grids, fixed with glutaraldehyde, and stained with uranyl-acetate. Scale bar is 100 nm. (b) MinC-MinD copolymer formation was monitored by 90°-angle light scatter as described in *Materials and Methods*. Reactions contained MinD (8 μ M), ATP (4 mM), and MinC (4 μ M) or MinC mutant proteins (4 μ M), where indicated. Light scattering was monitored for 5 min, ATP was added, and light scattering was monitored for an additional 30 min. Each curve is representative of at least two replicates. (c) MinC-MinD copolymer formation was monitored by ultracentrifugation as described in *Materials and Methods* in reactions containing MinD (8 μ M), MinC (4 μ M) or MinC mutant protein (4 μ M), where indicated, and ATP (4 mM). The amount of MinD in pellet fractions was quantified by SDS-PAGE and densitometry, and normalized to MinD densitometry with wildtype MinC. Data from at least three replicates are plotted as mean $\pm$ SEM (* is $p < .01$; ** is $p < .001$; *** is $p < .0001$).

Fig. S5. Gfp recruitment to SUVs by MinCD complexes. Gfp (10 μ M) was added to reactions containing MinD (4 μ M), MinC (4 μ M), ATP (4 mM), and GTP (1 mM), GDP (2 mM), or GMPCPP (0.2 mM), where indicated, in assembly buffer. SUV-associated protein complexes were collected by centrifugation as described in *Materials and Methods*, and Gfp fluorescence was measured with excitation and emission wavelengths set to 395 nm and 510 nm, respectively. Data from at least three replicates are plotted as mean $\pm$ SEM.

Fig. S6 Circular dichroism of MinC and MinC mutant proteins. Circular dichroism spectra were measured for reactions containing MinC (1 μ M), MinC(S16D) (1 μ M), and MinC(L194N) (1 μ M), where indicated, as described in *Materials and Methods*. The traces shown are representative of least three replicates.

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Title: The ATP-dependent protease ClpXP targets and degrades the MinD

ATPase and modulates assembly with MinC.

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Abstract

The essential cytoskeletal protein FtsZ forms the basis of the highly conserved Z-ring, which together with additional cell division proteins facilitates cytokinesis. Numerous proteins interact with FtsZ to spatially and temporally regulate Z-ring assembly, including ClpXP and the Min system. In *Escherichia coli*, the ATP-dependent protease ClpXP degrades FtsZ to remodel the Z-ring. The Min system, including the proteins MinC, MinD, and MinE, restricts the assembly of the Z-ring at midcell as it oscillates between the cell poles and destabilizes FtsZ polymerization. MinD was previously identified as a potential ClpXP substrate in vivo. Here, we found that MinD is a direct ClpXP substrate in vitro. We tested MinD for ClpXP-mediated degradation in vitro and observed that MinD is subject to proteolysis by ClpXP. Our results indicate that the N-terminal region of MinD and the ClpX N-domain are important for recognition and degradation of MinD in vitro, and furthermore that ClpXP prevents copolymerization of MinD with MinC and promotes copolymer disassembly in vitro.

Keywords: Cell division, Min system, MinD, ClpXP, AAA+ ATPase, *Escherichia coli*

Abbreviations

Clp, caseinolytic protease; FtsZ; filamentous temperature sensitive mutant Z; Gfp, green fluorescent protein; Min, minicell-forming; SUV, small unilamellar vesicle; TEM, transmission electron microscopy.

Introduction

Cytokinesis in prokaryotes is a highly organized cellular process wherein a network of widely conserved cell division proteins function together to divide a single bacterial cell into two identical daughter cells.¹ In *E. coli*, cell division commences with the assembly of a large ring-like protein structure termed the “Z-ring”, which contains bundled polymers of the GTPase FtsZ, and FtsZ-interacting proteins including FtsA and ZipA, at the division site². Following Z-ring assembly, additional proteins are recruited to the division septum to remodel the Z-ring and peptidoglycan architecture, leading to septation²⁻⁴. The Z-ring is a highly dynamic structure wherein FtsZ subunits are rapidly exchanged with a cytoplasmic pool via cycles of GTP binding and hydrolysis⁵. Many proteins interact with FtsZ to spatially and temporally regulate Z-ring assembly, and a number of these proteins modulate FtsZ dynamics in the Z-ring⁶.

Many fundamental processes in prokaryotes, including cytokinesis, are regulated by targeted proteolysis. In *E. coli*, the two-component ATP-dependent protease ClpXP degrades FtsZ to modulate Z-ring dynamics during the division process^{6,7}. ClpXP comprises two subunits: ClpX, a AAA+ (ATPase Associated with diverse cellular Activities) ATPase that undergoes ATP-dependent hexamerization, and ClpP, a serine protease composed of two stacked heptameric rings⁸. The ClpX subunit contains an N-terminal Zinc-binding domain (ZBD) that dimerizes, and a AAA+ module that is capable of

hexamerization. Both the ZBD and AAA+ domains are important for engaging substrates, but have different substrate binding properties⁹. ClpX recognizes and unfolds protein substrates, primarily through short polypeptide sequences located at the extreme N- and C-terminal regions of proteins¹⁰; these recognition motifs are known as degrons. Substrates may contain multiple degrons, such as FtsZ, and the degrons may be present at internal regions of a substrate in addition to the termini^{11,12}. ClpX utilizes ATP hydrolysis to unfold and translocate substrates through its axial channel into the central chamber of ClpP for degradation^{13,14}.

In addition to FtsZ, several *E. coli* cell division proteins have been identified as potential ClpXP substrates in proteomic studies that evaluated substrate association with a protease-defective ClpP mutant in vivo^{10,15}. Among the substrates identified were the Z-ring associated proteins ZapC and FtsA, and the division-site selection protein MinD^{2,16}. Subsequent work by the Janakiraman group confirmed that ZapC is a direct ClpXP substrate in vivo¹⁷; however, whether FtsA or MinD are direct substrates of ClpXP in vitro remains to be determined.

MinD is a component of the Min system of proteins, including MinC, MinD and MinE, that oscillates between the poles of the cell to prevent Z-ring assembly at the cell poles³. The Min system is used in several groups of organisms to regulate division-site selection; however, the oscillation observed

in *E. coli* is not preserved across all organisms that contain a Min system, and some organisms lack a Min system entirely³. MinC directly interacts with FtsZ to disrupt GTP-dependent polymerization in vitro^{18,19}. The cellular distribution of MinC is determined by MinD via a direct protein-protein interaction. MinD is a member of the Walker A cytoskeletal ATPases (WACA) protein family and contains a deviant Walker A motif^{20,21}. MinD associates with the cytoplasmic membrane in the ATP-bound dimer conformation via a C-terminal membrane targeting sequence (MTS). MinE binds to MinD, stimulating ATP hydrolysis and displacement of MinD from the membrane^{22,23}.

MinC and MinD assemble into ATP-dependent filaments in vitro. The Lowe group solved a crystal structure of the *Aquifex aeolicus* MinCD co-complex, which supports a model in which *A. aeolicus* copolymers contain alternating MinC and MinD dimers^{24,25}. In *E. coli*, residues on the surface of the MinC C-domain (CTD) have been identified that are important for copolymerization with MinD¹⁹. In addition to *E. coli* and *A. aeolicus*, *Pseudomonas aeruginosa* MinC and MinD have recently been reported to undergo ATP-dependent assembly in vitro²⁶. Although several groups have reported copolymer formation in vitro, the physiological consequences of MinCD assembly in vivo are currently under investigation. The Lutkenhaus group reported that MinD mutants that fail to assemble with MinC, but still interact with MinC at the membrane, do not result in functional defects in

vivo²⁷. Although copolymers may not be required for division in vivo, they may modulate Min patterning in vivo¹⁹.

Regulation of MinD by protein-protein interactions is still under investigation. The Baker group identified MinD as a putative ClpXP substrate, and subsequently demonstrated that intracellular MinD turnover was impaired in *clpX* deletion strains, suggesting that MinD levels are regulated by ClpXP¹⁵. Consistent with these findings, the amino acid sequence of MinD contains a putative ClpX N-terminal recognition sequence¹⁰.

Here, using in vitro degradation assays, we confirm that MinD is directly recognized and degraded by ClpXP, and that the MinD N-terminal region and the ClpX Zinc-binding (ZBD) domain are important for recognition and degradation of MinD in vitro. Additionally, we further demonstrate that ClpXP modulates the assembly of MinD with MinC and disassembles copolymers. Our results implicate ClpXP as an important factor in controlling MinD abundance and assembly with MinC.

Results

ClpXP degrades MinD in vitro

In vivo, the half-life of MinD in a wildtype strain of *E. coli* was reported to be ~5 min, but in a *clpX* deletion strain the half-life was 3-fold longer (~15 min), indicating that MinD levels are regulated in a ClpX-dependent manner¹⁵.

To determine if MinD is a bona fide ClpXP substrate, we tested whether ClpXP could degrade MinD in vitro. MinD (6 μ M) was incubated with ClpX (1.0 μ M), ClpP (1.2 μ M), and ATP (8 mM) with an ATP regenerating system for 180 min and degradation was monitored by loss of MinD levels with time using SDS-PAGE (Fig. 1a). We detected ~50% less MinD after 180 min in reactions containing ClpXP and ATP (Fig. 1a); however, when either ATP or ClpP was omitted from reactions, MinD levels did not change after 180 min (Fig. 1a). Since SDS-PAGE is not quantitative, we tested degradation of fluorescent MinD (FL-MinD) that was labeled with Alexa-Fluor 488 by detecting degradation products using an ultrafiltration assay. Degradation reactions containing ClpXP (0.7 μ M), ATP (8 mM), and FL-MinD (10 μ M) were terminated by the addition of EDTA (50 mM) and fluorescent peptides were quantified. We observed that the amount of degraded MinD (pmol) increased linearly over the course of 30 min (Fig. 1b). The rate of FtsZ degradation by ClpXP has previously been shown to increase with substrate concentration, and FtsZ polymers are degraded cooperatively by ClpXP^{6,7}. Therefore, we examined the rate of MinD degradation by ClpXP with increasing MinD concentration (4-16 μ M) by measuring the amount of MinD that was degraded by ClpXP (0.7 μ M) in 30 min. We observed a concentration-dependent increase in the rate of MinD degradation, with a $V_{\max}$ occurring above 20 μ M MinD, and moreover found that degradation displayed apparent cooperativity ($n_h=2$) (Fig. 1c). The rate of degradation of 12 μ M MinD was $.08 \pm .01$ pmol MinD min^{-1} ClpX $^{-1}$ (Fig. 1c).

Next, to confirm that degradation of MinD is ClpX- and ATP-dependent, we omitted either ATP or ClpP from degradation reactions containing ClpX and calculated the rate of MinD degradation. We detected no MinD proteolysis without ClpP or ATP (Fig. 1d). Together, these results demonstrate that MinD is degraded in a ClpXP- and ATP-dependent manner, and that the rate of degradation increases with MinD concentration until a maximum is reached (~12 μ M).

Degradation of MinD in the presence of phospholipids and MinC

MinD associates with *E. coli* small unilamellar vesicles (SUVs) in the presence of ATP and recruits MinC to SUVs in vitro. MinE binds to membrane-associated MinD in vitro and stimulates ATP hydrolysis and subsequent release from SUVs^{28,29}. Since membrane-associated MinD binds MinC and MinE, we tested if the addition of SUVs (0.25 mg ml⁻¹), or SUVs with added MinC (10 μ M) or MinE (10 μ M) altered the rate of MinD (10 μ M) degradation by ClpXP (0.70 μ M). We observed that the addition of *E. coli* SUVs to degradation reactions did not alter the rate of MinD proteolysis (Fig. S1a). Similarly, the addition of SUVs and MinC or MinE also had no effect on degradation rates (Fig. S1a).

Next, we tested whether addition of MinC (10 μ M) or MinE (10 μ M) without SUVs altered the rate of MinD degradation by ClpXP (0.70 μ M). We

observed that the addition of MinC or MinE to degradation reactions did not alter the rate of MinD proteolysis (Fig. S1a). Lastly, to confirm that ClpXP does not degrade MinC or MinE, we incubated ClpXP (1.0 μ M) with MinC (6 μ M) or MinE (6 μ M) and monitored degradation after 180 min. We detected no degradation of MinC or MinE (Fig. S1b).

The ClpX N-terminal domain is required for MinD recognition and degradation

The ClpX N-terminal domain is a zinc-binding domain (ZBD) that forms a dimer independently of the ClpX AAA⁺ ATPase domain, and shares a high degree of sequence homology across bacterial taxa³⁰. ClpX(Δ N61) is a mutant of ClpX that lacks the N-terminal 61 amino acids containing the ZBD, but retains the ability to form a functional complex with ClpP^{31,32}. ClpX(Δ N61)P catalyzes degradation of some substrates, including ssrA-tagged substrates; however, other substrates, like MuA tetramers and λ O utilize the ClpX N-domain for recognition and degradation^{30,31}.

To ascertain whether the ClpX N-terminal domain is important for recognition and degradation of MinD, we purified ClpX(Δ N61) and compared the rates of MinD (10 μ M) degradation between ClpXP (0.70 μ M) and ClpX(Δ N61)P (0.70 μ M). We observed no detectable MinD degradation by ClpX(Δ N61)P, compared to ClpXP (Fig. 2a). Furthermore, both purified ClpX (0.30 μ M) and

ClpX(Δ N61) (0.30 μ M) supported degradation of Gfp-ssrA (1 μ M) with ClpP in vitro (Fig. S2).

SspB is an adaptor protein that facilitates recognition of ssrA-tagged substrates by ClpX³³. The N-terminal region of SspB is important for interactions with ssrA-tagged substrates, while the C-terminal 10 amino acids interacts directly with the ClpX N-domain. Thus, SspB recruits ssrA-tagged proteins to ClpX for recognition and degradation. A peptide containing the ClpX binding site of SspB, termed the ClpX-binding (XB) peptide is capable of binding to ClpX and a crystal structure of the XB-ClpX N-domain complex has been solved that reveals the specific binding site on ClpX for XB³³. Importantly, the XB peptide competitively inhibits the degradation of some substrates that are recognized by the ClpX N-domain, including the phage replication protein λ O, the MuA transposase, and the cytoskeletal protein FtsZ^{12,34}.

Structural NMR studies revealed that the binding sites for XB and λ O overlap on the ClpX N-domain, which explains why a molar excess of XB inhibits degradation of λ O in vitro³⁴. Since the ClpX N-domain was required for recognition and degradation of MinD in vitro (Fig. 2a), we tested if the XB peptide competitively inhibits MinD degradation. We measured MinD (10 μ M) degradation rates in the presence of increasing amounts of XB peptide (0-40 μ M) and observed a concentration-dependent inhibition of degradation (Fig. 2b), suggesting that the MinD and SspB binding sites overlap on the ClpX N-domain.

Together, our results suggest that the ClpX N-domain mediates recognition and degradation of MinD.

The MinD N-terminal region contains residues that are important for ClpXP-mediated degradation.

Substrate recognition by ClpX is mediated by peptides at the extreme N- or C-terminal regions of protein substrates³⁵. Several N- and C-terminal decons have been identified in *E. coli* that are important for recognition and degradation by ClpXP¹⁰. We examined the amino acid sequence of *E. coli* MinD and identified a putative ClpX N motif-2 consensus motif (M-b- ϕ - ϕ - ϕ -X₅- ϕ) in the MinD N-terminal region (¹MARIIV-X₅-G¹²)¹⁰ (Fig. 3a). Previously, fusing the N-motif-2 consensus sequence of IscS (¹MKLPI-X₅-A¹¹) to Arc, which is not a ClpXP substrate, was sufficient to promote Arc degradation by ClpXP¹⁰. Mutagenesis of the conserved positively charged (b) amino acid of the IscS N-motif 2 decons to aspartic acid, IscS(K2D), drastically impaired degradation of the IscS¹⁻¹¹-Arc fusion protein¹⁰. This corresponding residue in MinD, Arg 3, is surface-exposed (Fig. 3b). We mutagenized MinD Arg 3 to Glu, purified and tested MinD(R3E) (6 μ M) for degradation by ClpXP (1 μ M) in vitro by SDS-PAGE. We observed that while MinD was degraded with a $t_{1/2}$ of ~60 min, MinD(R3E) was severely impaired for degradation under the conditions tested (Fig. 3c).

To ensure that MinD(R3E) behaved like wildtype MinD and confirm that impaired degradation did not arise from a MinD(R3E) functional defect, we

measured the ability of MinE to stimulate ATP hydrolysis of MinD(R3E) in the presence of SUVs. We observed that the ATP hydrolysis rate of MinD(R3E) (8 μ M) was stimulated 10-fold by MinE (16 μ M) and SUVs (1 mg ml⁻¹), similarly to MinD (8 μ M) (Fig. S3a). Additionally, we tested MinD(R3E) for copolymer formation with MinC by 90° light scatter, and for SUV-recruitment of MinC. Copolymer formation of MinD(R3E) (8 μ M) with MinC (4 μ M) was similar to wild type MinD (8 μ M) (Fig. S3b). Furthermore, MinD(R3E) (4 μ M) recruited similar levels of MinC (4 μ M) to SUVs by MinD(R3E) compared to MinD (4 μ M) (Fig. S3c). Thus, mutagenizing Arg 3 does not perturb MinD function in vitro (Fig. S3).

The MinD N-terminal region contains a highly conserved lysine residue, K11, which is important for dimerization and the interaction with MinE³⁶. Mutagenesis of this lysine residue to alanine prevents dimerization, copolymer formation, and stimulation of ATP hydrolysis by MinE and SUVs in vitro²⁵. Lysine 11 is also present near the N-terminal region of MinD (Fig. 3a). Therefore, we purified and tested MinD(K11A) (6 μ M) for degradation by ClpXP (1 μ M) in vitro. Similarly to MinD(R3E), we observed no degradation of MinD(K11A) under the conditions tested (Fig. 3c). Together, our results suggest that the MinD N-terminal region may be important for recognition and degradation of MinD by ClpXP.

ClpXP prevents MinCD copolymer formation in vitro

MinC and MinD form copolymers in the presence of ATP^{24,25}, and the co-crystal structure of the *A. aeolicus* MinCD complex has been reported²⁴.

Next, to test if ClpXP modulates copolymerization of MinD with MinC *in vitro*, we monitored 90° angle light scatter associated with reactions containing MinD (8 μ M), MinC (4 μ M), ClpX (0.75 μ M), and ClpP (0.9 μ M). Light scatter was monitored for 5 min to collect a baseline reading, and ATP (8 mM) was added with ClpX (0.75 μ M) and ClpP (0.9 μ M) to stimulate copolymer formation. Light scatter was monitored for an additional 30 min. The addition of ATP without ClpXP stimulated robust copolymer formation; however, the addition of ATP with ClpXP lead to a small increase in light scatter that rapidly decreased with time (Fig. 4a).

Since copolymer inhibition could be a result of MinD unfolding, but not degradation, we tested if ClpX inhibited MinCD assembly without ClpP. Interestingly, we found that the addition of ClpX resulted in a 45% inhibition of copolymerization (Fig. 4b), and that ClpX(E185Q), which hexamerizes and binds substrates with ClpP but does not unfold them, similarly impaired copolymer formation (45% inhibition). Together, these results suggest that ClpX is capable of impairing MinCD assembly independently of ClpP and ATP hydrolysis, but that ClpXP is more effective in preventing assembly (Fig. 4a and 4b). Next, to confirm that the addition of ClpXP reaction buffer does not inhibit copolymer formation, we monitored MinCD copolymer formation following the addition of ATP with an equivalent volume of ClpXP buffer and ATP used in reactions. The addition of buffer did not impair copolymer formation (Fig. 4c).

Lastly, to confirm that ClpP did not inhibit light scatter without ClpX, we monitored 90° angle light scatter of MinCD copolymers in the presence of ClpP (0.9 μ M) and ATP (4 mM). We observed that ClpP alone did not inhibit MinCD copolymer assembly (Fig. 4d). Together, our results suggest that MinCD copolymer formation is prevented in a ClpX and ClpXP-dependent manner.

ClpXP disassembles MinCD polymers

Since we observed that ClpXP prevented the ATP-dependent assembly of MinCD copolymers, we tested if ClpXP could destabilize copolymers after they were pre-assembled with ATP using light scattering assays. MinCD copolymerization was stimulated by the addition of ATP (4 mM), and increasing amounts of ClpXP (0.5-0.9 μ M) with ATP (4 mM) and ATP regenerating system were added to assembled copolymers after 30 min. The addition of ClpXP resulted in a stable, concentration-dependent decrease in light scatter (Fig. 5a). In contrast, addition of equivalent volumes of ClpXP buffer with ATP (4 mM) and regenerating system to assembled MinCD copolymers failed to stimulate disassembly (Fig. S4).

Next, we investigated whether ClpX promotes copolymer disassembly without ClpP. We monitored light scatter associated with MinCD copolymers after addition of ClpX (0.75 μ M). The addition of ClpX led to a small decrease in light scatter, but light scatter rapidly recovered to levels observed before the addition of ClpX (Fig. 5b). To confirm that ClpP alone did not disassemble MinCD

copolymers, we monitored light scatter associated with fully assembled copolymers after the addition of ClpP (0.9 μ M). We observed that ClpP alone did not disassemble copolymers (Fig. 5c). Since MinD(R3E) was impaired for recognition and degradation by ClpXP, but formed copolymers with MinC, we investigated whether copolymers formed with MinD(R3E) and MinC would still be sensitive to ClpXP. We found that the addition of ClpXP (0.9 μ M) to assembled MinCD(R3E) polymers failed to induce disassembly, in contrast to copolymers formed with MinC (4 μ M) and MinD (8 μ M) (Fig. 5a and Fig. 5d). Our results suggest that ClpX promotes disassembly of MinCD copolymers with ClpP, and that MinD(R3E) is important for ClpXP-dependent disassembly.

Lastly, we directly visualized copolymers via negative staining transmission electron microscopy (TEM), and compared them to copolymers incubated with ClpXP. Copolymer formation by MinC and MinD was stimulated with ATP (4 mM). ClpXP (0.9 μ M) was pre-assembled with ATP (4 mM) and a regenerating system, and was added to reactions containing copolymers. After 15 min, reactions were fixed with glutaraldehyde, applied to copper grids and analyzed by negative staining TEM. MinC and MinD formed dense, bundled networks of copolymers without ClpXP (Fig. 6a); however, copolymers appeared less bundled, shorter, and were more isolated in the presence of ClpXP (Fig. 6b). Furthermore, in many instances the ends of copolymers were associated with small round structures, which were not observed without ClpXP (Fig. 6a and 6b). To confirm that polymers were not observed in reactions containing ClpXP alone,

we visualized ClpXP (0.9 μ M) assembled with ATP (4 mM) and a regenerating system. We detected a homogenous population of ClpXP (Fig. 6c), and did not observe polymers. Since copolymers formed with MinD(R3E) were not destabilized by ClpXP, we visualized MinD(R3E)-dependent copolymers incubated in the presence or absence of ClpXP (0.9 μ M). The appearance of copolymers incubated without ClpXP was similar compared to incubation with ClpXP, consistent with a failure to be disassembled (Fig. 6d and 6e). A structure of the MinCD co-crystal has been solved in *A. aeolicus*, and was used to build a filament structural model where copolymers are comprised of alternating MinC and MinD dimers²⁴. Since the formation of copolymers requires incorporation of MinC, it is possible that copolymer disassembly by ClpXP could arise from degradation of MinC or a failure of MinC to dimerize and/or interact with MinD. We observed no degradation of MinC by SDS-PAGE (Fig. S1c), therefore copolymer disassembly is likely not mediated by MinC degradation.

Discussion

Here, we report that the two component ATP-dependent protease ClpXP degrades the cell division protein MinD in vitro. We measured the rate of ClpXP-mediated proteolysis using a quantitative degradation assay, and observed that recognition and degradation of MinD requires the ClpX N-domain and the MinD N-terminal region (Fig. 1c, Fig. 2 and Fig. 3). We identified two residues in the MinD N-terminal region that were important for recognition and degradation by ClpXP: Arg 3 and Lys 11. MinD Arg 3 is

located in a ClpX N-motif-2 consensus sequence on MinD (Fig. 3a). A MinD mutant, MinD(R3E) was not degraded in vitro but behaved similarly to wildtype MinD in vitro (Fig. 3c and Fig. S3); therefore, impaired degradation of MinD(R3E) is likely a result of impaired recognition by ClpX. MinD(K11A) was similarly defective for degradation in vitro (Fig. 3c). However, although MinD(K11A) binds ATP, it is impaired for dimerization and the interaction with MinE and MinC³⁶. Furthermore, MinD K11 is within the variable region of the ClpX N-motif-2 (Fig. 3a); thus, impaired degradation of MinD(K11A) is likely due to the inability of MinD(K11A) to form dimers.

ClpX undergoes ATP-dependent oligomerization into ring-like hexamers, and uses the energy derived from ATP hydrolysis to unfold and translocate substrates through the axial channel³⁵. ClpX hexamers interact with ClpP via docking of 'IGF' pore loops onto the hydrophobic surface of ClpP tetradecamers³⁷. The resulting ClpXP complex is a robust proteasome. Although ClpX functions with ClpP to regulate protein turnover in vivo, ClpX regulates essential cellular processes through ClpP-independent protein remodeling. In *E. coli*, ClpX remodels MuA to regulate phage transposition, and functions as a protein disaggregase^{38–40}. Given that ClpX is capable of remodeling protein complexes with and without ClpP, and that ClpXP degrades MinD in vitro (Fig. 1), we investigated the ability of ClpXP to modulate MinCD copolymer assembly in vitro. We found that ATP-driven coassembly of MinCD was impaired in the presence of ClpX hexamers without

ClpP, and abolished in the presence of ClpX hexamers with ClpP. However, ClpP alone or ClpXP buffer did not impair assembly (Fig. 4a-d). Furthermore, copolymer formation was similarly impaired by ClpX compared to ClpX (E185Q), which contains a mutation in the Walker B motif and is defective for ATP hydrolysis, but still oligomerizes and binds substrates⁴¹. Thus, ClpX impairs MinCD copolymer assembly via an ATP hydrolysis-independent mechanism. Consistent with these results, previous studies demonstrated that in *B. subtilis*, ClpX impairs FtsZ polymer assembly through an ATP-independent mechanism^{42,43}.

ATP-hydrolysis by ClpX is required for substrate unfolding and translocation, but not substrate binding. Therefore, our results are consistent with ClpX inhibiting copolymerization via a holdase mechanism, where binding of MinD by ClpX without unfolding may be sufficient to impair the assembly of copolymers.

In *E. coli*, ClpXP degrades FtsZ to modulate Z-Ring dynamics in vivo^{6,7}. ClpXP cooperatively degrades FtsZ polymers, and polymer disassembly likely shifts the equilibrium of polymer dynamics towards net disassembly⁷. Eukaryotic members of the AAA+ protein family, including spastin and katanin, are also capable of polymer disassembly⁴⁴. Since we observed that ClpXP prevented ATP-dependent MinCD copolymer assembly (Fig. 4a), and ClpXP is known to disassemble polymers, we tested if ClpXP could induce disassembly

of assembled MinCD copolymers. We observed that the addition of ClpXP to MinCD copolymers resulted in a concentration-dependent loss of light scatter (Fig. 5a). In contrast, when ClpX, ClpP, or equivalent volume of ClpXP buffer were added to copolymers, we observed a mild reduction in light scatter that rapidly re-equilibrated to initial levels (Fig. 5b, 5c, and S4).

We observed that MinD(R3E) was impaired for recognition and degradation by ClpXP (Fig. 3c), but functioned normally *in vitro* (Fig. S3), suggesting that this residue is important in the context of recognition by ClpXP but not normal MinD function. Therefore, we tested if copolymers assembled with MinD(R3E) would remain susceptible to disassembly. We observed that the addition of ClpXP did not induce a sustained loss of light scatter, in contrast to copolymers formed with MinD (Fig. 5a and 5d). These results suggest that MinD Arg 3 is not important for copolymer formation with MinC, but is required for ClpXP-mediated disassembly of copolymers.

MinCD copolymers from several organisms, including *E. coli*, form large linear filaments, which are readily observed by electron microscopy^{19,24–26}. We directly visualized MinCD copolymers by TEM and compared them to copolymers with the addition of ClpXP. Consistent with our light scattering results, copolymers formed with MinC and MinD or MinD(R3E) were highly bundled and branched in the absence of ClpXP; however, while MinCD copolymers appeared less bundled, shorter and more isolated following

addition of ClpXP, MinCD(R3E) copolymers did not appear to be affected by the addition of ClpXP (Fig. 6a, 6b, 6d, and 6e). Moreover, in many instances we observed many large ring-like structures (30-50 nm) near the ends of filaments in reactions containing MinCD copolymers with added ClpXP (Fig. 6b). Although assembled ClpXP in control reactions appeared homogenous and smaller (15 nm), ClpXP complexes containing two full-length ClpX hexamers docked onto a single ClpP tetradecamer may account for the larger size. However, since ClpP is in excess over ClpX, this is unlikely to be the case. Together, our results suggest that ClpXP promotes the disassembly of MinCD copolymers, and that disassembly requires Arg 3 of MinD (Fig. 5 and 6). Since MinC is not degraded by ClpXP (Fig. S1c), and MinCD(R3E) copolymers are not disassembled by ClpXP (Fig. 5d and 6e), copolymer destabilization is mediated by the interaction of ClpXP with MinD and not MinC.

MinD was initially identified as a potential ClpXP substrate in *E. coli* using ClpP trap assays¹⁵. Here, for the first time, we report that MinD is a direct substrate of ClpXP in vitro. Future studies will investigate the physiological role of MinD degradation in vivo. A recent study revealed that the local stoichiometry of MinD and MinE impacts the stabilization and release of MinD from the membrane⁴⁵. By degrading MinD, ClpXP may modulate Min oscillation in vivo through modifying the stoichiometry of MinD and MinE.

Polar oscillation of Gfp-tagged MinD and MinC has been visualized in live cells^{18,19,46}. Gfp-MinD oscillation still occurs in the absence of MinC, and expressing MinD mutations impaired for copolymerization with MinC does not result in cell division defects in vivo^{27,46}. However, we recently reported that MinC mutant proteins impaired for the FtsZ interaction exhibit slow oscillation in vivo¹⁹. Copolymerization with MinC may limit the available population of MinD through concentration on the membrane or by sequestration in the cytoplasm. Therefore, copolymer formation may modulate the oscillation in vivo. We observed that ClpXP alters copolymer assembly and abundance (Fig. 4, Fig. 5, and Fig. 6). Thus, ClpXP may also control the accessible population of MinD by modifying copolymer formation.

Materials and Methods

Expression and purification of proteins

MinC, MinD, MinD(R3E), MinE, FtsZ, ClpX, ClpX(Δ N61), ClpX(E185Q), and ClpP, were overexpressed in *E. coli* BL21 (λ DE3) and purified as described^{7,12,19,25,40,47}. Gfp-ssrA was purified as previously described⁴⁸. Protein concentrations are reported as MinC dimers, MinD dimers, MinE dimers, ClpX hexamers, ClpP dodecamers, and Gfp-tagged monomers.

Mutagenesis

MinD and ClpX mutant proteins were constructed by site-directed mutagenesis of plasmids containing *minD* or *clpX* using the QuickChange II XL Site-directed mutagenesis kit (Agilent).

Phospholipid recruitment assays with MinC and MinD

MinD (4 μ M) or MinD(R3E) (4 μ M), where indicated, were added to reactions containing SUVs (.25 mg ml⁻¹), MinC (4 μ M), and ATP (4 mM) in assembly buffer containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)-pH 6.5, 100 mM KCl, 10 mM MgCl₂. Small Unilamellar vesicles from *E. coli* extracts (Avanti Polar lipids) were prepared as described in Conti, et al., 2018⁴. Following the addition of ATP, reactions were incubated for 10 min at 30°C, and SUV-containing protein complexes were collected by centrifugation at 14,000 x *g* at 23°C, resuspended in LDS loading buffer, and analyzed by SDS-PAGE and densitometry.

ATP hydrolysis assays with MinD

ATP hydrolysis was measured by monitoring the amount of inorganic phosphate released after 10 min in reactions containing ATPase buffer (20 mM Tris 7.5, 50 mM KCl, 10 mM MgCl₂) with ATP (4 mM), MinD (8 μ M), MinE (16 μ M), and SUVs (1 mg ml⁻¹), where indicated. Phosphate released in reactions was detected using Biomol green (Enzo Life Sciences) and comparing to a phosphate standard curve.

Degradation assays

FL-MinD (10 μ M), or FL-MinD (4-16 μ M), where indicated, was added to reactions containing ClpX (0.7 μ M), ClpX(Δ N61) (0.7 μ M), ClpP (0.84 μ M), MinC (5 μ M), MinE (10 μ M), and ATP (10 mM) with an ATP regenerating system containing creatine phosphokinase (50 μ g ml⁻¹) and creatine phosphate (30 mM), where indicated, in degradation buffer (50 mM MES 6.5, 100 mM KCL, 10 mM MgCl₂, 2 mM TCEP). Degradation reactions were incubated for 30 min at 30°C and reactions were terminated by the addition of EDTA (50 mM). Degradation products were applied to pre-washed 3000 Da Nanosep® spin-filters with Omega™ membrane (Pall). Samples were centrifuged at 14,000 x *g* for 20 min at 23°C, and the fluorescence of the filtrate was measured with excitation and emission wavelengths set for 490 nm and 525 nm, respectively. Fluorescent MinD was generated by labeling with Alexa Fluor-488 succinimidyl ester (Life Technologies). Labeled MinD was tested for the ability to form ATP-dependent copolymers with MinC.

Degradation reactions using non-labeled MinD was monitored in reactions containing MinD (6 μ M), MinD(R3E) (6 μ M) or MinD(K11A) (6 μ M), where indicated, MinC (6 μ M), MinE (6 μ M), where indicated, ClpX (1.0 μ M), ClpP (1.2 μ M), ATP (4 mM) and an ATP regenerating system containing creatine phosphokinase (50 μ g ml⁻¹) and creatine phosphate (30 mM), where indicated, in degradation buffer. Immediately following the addition of ATP, and after 30 min, 60 min, 120 min, and 180 min, or after 180 min, where

indicated, samples were removed from degradation reactions and added to LDS loading buffer. Protein amounts were analyzed by SDS-PAGE and densitometry.

Degradation of Gfp-ssrA was monitored in reactions containing Gfp-ssrA (1 μ M), ClpX (0.3 μ M) or ClpX(Δ N61) (0.3 μ M), ClpP (0.4 μ M) ATP (4 mM) and an ATP regenerating system containing creatine phosphokinase (25 μ g ml⁻¹) and creatine phosphate (15 mM) in degradation buffer. Fluorescence was monitored with time using excitation and emission wavelengths set to 395nm and 509 nm, respectively.

Copolymer assays with MinC and MinD

To monitor MinCD copolymer formation by light scatter, MinD (8 μ M) was added to reactions containing MinC (4 μ M) in copolymer assembly buffer (20 mM MES, 100 mM KCl, 10 mM MgCl₂). 90°-angle light scatter was monitored with excitation and emission wavelengths set to 450nm. After 5 min, ATP (8 mM) or ClpX (0.75 μ M), ClpX(E185Q) (0.75 μ M), ClpP (0.9 μ M), or ClpX (0.75 μ M) and ClpP (0.9 μ M), where indicated, and ATP (8 mM) and ATP regenerating system containing creatine phosphokinase (50 μ g ml⁻¹) and creatine phosphate (30 mM), were added and 90°-angle light scatter was monitored for an additional 30 min. In reactions monitoring copolymer disassembly, 90°-angle light scatter was initially monitored in reactions containing MinD (8 μ M) or MinD(R3E) (8 μ M) where indicated, MinC (4 μ M),

and ATP (4 mM) for 30 min at 23°C. Then, ClpX (0.75 μ M), ClpP (0.9 μ M) or ClpX (0.4-0.75 μ M) or and ClpP (0.5-0.9 μ M), where indicated, ATP (4 mM) and ATP regenerating system containing creatine phosphokinase (50 μ g ml⁻¹) and creatine phosphate (30 mM), were added and 90°-angle light scatter was monitored for an additional 15 min.

Electron Microscopy

Reactions containing MinC (4 μ M), MinD (8 μ M) and MinD(R3E) (8 μ M), where indicated, ClpX (0.75 μ M) and ClpP (0.90 μ M), where indicated, ATP (8 mM) and ATP regenerating system containing creatine phosphokinase (50 μ g ml⁻¹) and creatine phosphate (30 mM) in copolymer assembly buffer were applied to 300-mesh carbon/formvar-coated grids, fixed with glutaraldehyde (1%), and stained with uranyl acetate (2%). Samples were imaged by transmission electron microscopy using a JEM-2100 80Kv instrument.

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Fig. 1: ClpXP degrades MinD in vitro

(a) MinD degradation was visualized by loss of protein after 180 min by SDS-PAGE. The amount of MinD after 0 min and 180 min was visualized in reactions containing MinD (6 μ M), ClpX (1.0 μ M), ClpP (1.2 μ M), ATP (8 mM) and a regenerating system, where indicated, as described in *Materials and Methods*. (b) The amount of FL-MinD degraded with time was monitored in reactions containing ClpX (0.75 μ M) and ClpP (.9 μ M), MinD (10 μ M), and ATP (8 mM) with a regenerating system, as described in *Materials and Methods*. Following the addition of ATP, reaction samples were removed over a 30 min period and MinD degradation products were collected by centrifugation as described in *Materials and Methods*. The amount of MinD degraded (pmol) was quantified by measuring the fluorescence of filtrate and correcting for background. (c) The rate of MinD degradation was determined for reactions containing ClpX (0.75 μ M) and ClpP (0.9 μ M), MinD (0-16 μ M) and ATP (8 mM) and a regenerating system, as described in *Materials and Methods*. (d) The rate of degradation by ClpXP was determined for reactions containing ClpX (0.75 μ M), ClpP (0.9 μ M), ATP (8 mM) and a regenerating system, where indicated, and MinD (10 μ M), as described in *Materials and Methods*. In B-D, data from at least 3 replicates are shown as mean $\pm$ SEM.

Fig. 2: The N-terminal domain of ClpX is important for recognition and degradation of MinD.

(a) The rate of MinD degradation by ClpXP or ClpX(Δ N61)P was determined for reactions containing MinD (10 μ M), ATP (8 mM), ClpX (0.75 μ M) and ClpP (0.9 μ M), or ClpX (Δ N61) (0.75 μ M) and ClpP (0.9 μ M), where indicated. Data from at least 3 replicates are shown as mean $\pm$ SEM (b) MinD degradation rate by ClpXP was determined in reactions containing ClpX (0.75 μ M) and ClpP (0.9 μ M), MinD (4 μ M), ATP (8 mM), and various concentrations of XB (0-40 μ M). Data from at least three replicates are shown as mean $\pm$ SEM. In A-B, FL-MinD peptides were collected by centrifugation and the rate of degradation was calculated as described in *Materials and Methods*.

Fig. 3: Residues in the MinD N-terminal region are important for recognition and degradation by ClpXP.

(a) Alignment of the N-terminal region of known ClpX substrates with the MinD N-terminal region. Sequences belong to the N-motif 2 family of ClpX recognition tags (M-b- ϕ_3 -x $_5$ - ϕ) where b represents a basic amino acid residue and ϕ represents a hydrophobic amino acid residue, and x represents any amino acid¹⁰. (b) Structure of *E. coli* dimeric MinD bound to ATP (PDB 3Q9L)⁴⁹. MinD Arg 3 is indicated by red surface mapping. (c) Degradation of MinD was monitored by SDS-PAGE in reactions containing ClpX (0.75 μ M) and ClpP (0.9 μ M), ATP (4 mM) and MinD (6 μ M), MinD(R3E) (6 μ M), or MinD(K11A) (6 μ M), where indicated. Samples were removed from degradation reactions and the amount of MinD was visualized as described in *Materials and Methods*.

(a)

N motif-2	NH ₂	M	b	Φ	Φ	Φ	XXXXX	Φ
IcsS	NH ₂	M	K	L	P	I	YLDYS	A
OmpA	NH ₂	M	K	K	T	A	AIAIA	V
MinD	NH ₂	M	A	R	I	I	V	VTSGK

(b)

(c)

Fig. 4: ClpXP modulates MinCD copolymer assembly

(a-d) MinC-MinD copolymerization was monitored by 90°-angle light scatter in reactions containing MinC (4 μ M) and MinD (8 μ M), ATP (8 mM), ClpX (0.75 μ M), ClpP (0.9 μ M), ClpX (0.75 μ M) and ClpP (0.9 μ M), or equivalent ClpXP buffer, where indicated, as described in *Materials and Methods*. Light scatter associated with MinC and MinD was monitored for 5 min, and either ATP or ATP with ClpX, ClpP, ClpXP, or equivalent ClpXP buffer was added, where indicated, and light scatter was monitored for an additional 30 min.

Fig. 5: Monitoring disassembly of MinCD copolymers by ClpXP

(a-c) MinC-MinD copolymers were monitored by 90°-angle light scatter in reactions containing MinC (4 μ M), MinD (8 μ M), ClpXP (0.5-0.9 μ M) where indicated, ATP (8 mM) and regenerating system, as described in *Materials and Methods*. (d) MinC-MinD copolymer formation was monitored by 90°-angle light scatter as described in *Materials and Methods* in reactions containing ClpX (0.75 μ M), ClpP (0.9 μ M), ClpX (0.75 μ M) or ClpP (0.9 μ M), where indicated, and MinC (4 μ M), MinD (8 μ M) or MinD(R3E) (8 μ M), where indicated. In reactions a-d, light scatter was monitored for 5 min, ATP was added, and light scatter was monitored for an additional 30 min. Following assembly, either ClpXP, ClpX, ClpP, was added with ATP and regenerating system, where indicated, to reactions and light scattering was monitored for an additional 15 min. Curves are representative of at least three replicates.

Fig. 6: Visualizing the effect of ClpXP on the appearance of copolymers

(a) MinC-MinD copolymer formation was visualized by negative staining electron microscopy. Reactions containing MinC (4 μ M), MinD or MinD(R3E) (8 μ M), where indicated, ClpX (0.75 μ M) and ClpP (0.9 μ M), where indicated, ATP (8 mM) and regenerating system, where indicated, were applied to carbon-coated grids, fixed with glutaraldehyde and stained with uranyl-acetate. Scale bar is 200 nm.

(a)
MinCD

(b)
MinCD + ClpXP

(c)
ClpXP

(d)
MinCD(R3E)

(e)
MinCD(R3E) + ClpXP

Fig. S1: MinD degradation in the presence of SUVs, MinC, and MinE

(a) The rate of MinD degradation by ClpXP was determined for reactions containing ClpX (0.75 μM), ClpP (0.9 μM), ATP (8 mM) and a regenerating system, MinD (10 μM), and MinC (5 μM), MinE (10 μM), and SUVs (.25 mg ml⁻¹), as described in *Materials and Methods*. (b) MinC or MinE degradation was visualized by loss of protein after 180 min by SDS-PAGE. The amount of MinC or MinE after 0 min and 180 min was visualized in reactions containing MinC (6 μM), MinE (6 μM), where indicated, ClpX (1.0 μM), ClpP(1.2 μM), ATP (8 mM) and a regenerating system, as described in *Materials and Methods*.

Fig. S2: Gfp-ssrA degradation

(a) Gfp-ssrA degradation was monitored in reactions containing Gfp-ssrA (1 μ M), ATP (4 mM), ClpX (0.3 μ M) or ClpX(Δ N61), where indicated, and ClpP (0.9 μ M). Fluorescence was monitored for 30 min using 395nm and 510nm excitation and emission filters, respectively. Curves are representative of at least 3 replicates.

Fig. S3: Activity of MinD(R3E) in vitro

(a) The ability of MinD(R3E) to be stimulated for ATP hydrolysis by MinE was examined in the presence of SUVs. Release of inorganic phosphate was monitored over 10 min in reactions containing SUVs (1 mg ml^{-1}), ATP (4 mM), and MinD (8 μM), MinD(R3E) (8 μM), and MinE (16 μM), as where indicated, as described in *Materials and Methods* and the MinE-stimulated increase in ATP hydrolysis rate was compared to the basal rate. Data from at least two replicates are plotted as mean $\pm$ SEM. (b) Copolymer formation by MinD and MinD(R3E) with MinC was compared by monitoring 90-angle light scatter as described in *Materials and Methods*. Reactions contained MinC (4 μM), ATP (4 mM), and MinD (8 μM) or MinD(R3E) (8 μM), where indicated. Light scatter was monitored for 5 min, ATP was added, and monitored for an additional 30 min. Each curve is representative of at least three replicates. (c) The ability of MinD(R3E) to recruit MinC to SUVs was measured and compared to MinD. Reactions contained SUVs (0.25 mg ml^{-1}), ATP (4 mM), MinC (4 μM), MinD (4 μM) or MinD(R3E) (4 μM), as described in *Materials and Methods*.

Fig. S4: measuring the effect of buffer on MinCD copolymer stability in vitro

MinC-MinD copolymers were monitored by 90°-angle light scatter in reactions containing MinC (4 μ M), MinD (8 μ M), and equivalent amounts of buffer corresponding to ClpXP concentrations (0.5-0.9 μ M) where indicated, ATP (8 mM) and regenerating system, as described in *Materials and Methods*.

Manuscript IV

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Title: The protein chaperone ClpX targets native and non-native aggregated substrates for remodeling, disassembly and degradation with ClpP

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Abstract

ClpX is a member of the Clp/Hsp100 family of ATP-dependent chaperones and partners with ClpP, a compartmentalized protease, to degrade protein substrates bearing specific recognition signals. ClpX targets specific proteins for degradation directly or with substrate-specific adaptor proteins. Native substrates of ClpXP include proteins that form large oligomeric assemblies, such as MuA, FtsZ and Dps in *Escherichia coli*. To remodel large oligomeric substrates, ClpX utilizes multivalent targeting strategies and discriminates between assembled and unassembled substrate conformations. Although ClpX and ClpP are known to associate with protein aggregates in *E. coli*, a potential role for ClpXP in disaggregation remains poorly characterized. Here, we discuss strategies utilized by ClpX to recognize native and non-native protein aggregates and the mechanisms by which ClpX alone, and with ClpP, remodels the conformations of various aggregates. We show that ClpX promotes the disassembly and reactivation of aggregated Gfp-ssrA through specific substrate remodeling. In the presence of ClpP, ClpX promotes disassembly and degradation of aggregated substrates bearing specific ClpX recognition signals, including heat-aggregated Gfp-ssrA, as well as polymeric and heat-aggregated FtsZ, which is a native ClpXP substrate in *E. coli*. Finally, we show that ClpX is present in insoluble aggregates and prevents the accumulation of thermal FtsZ aggregates in vivo, suggesting that ClpXP participates in the management of aggregates bearing ClpX recognition signals.

Introduction

Maintaining cellular proteostasis relies on chaperone pathways that promote native protein folding. Typical strategies include targeting misfolded, unfolded, and aggregated polypeptides for reactivation or degradation (Bukau and Horwich, 1998; Wickner et al., 1999; Stoecklin and Bukau, 2013). Misfolded proteins are generated during polypeptide elongation and as a complication of environmental stress (Powers and Balch, 2013). The challenges imposed on chaperone systems by proteotoxic stress are especially relevant in pathogenic organisms like *E. coli*, which experience extreme fluctuations in environmental conditions leading to accumulation of protein aggregates and subsequent proteotoxicity (Mogk et al., 2011). Protein quality control systems reactivate, degrade and remove damaged and aggregated proteins. Under thermal stress in *E. coli*, the heat shock response provides a cellular defense mechanism and upregulates heat shock protein and chaperone levels to restore proteostasis (Mogk et al., 2011).

In addition to preventing protein aggregation, chaperone proteins mediate aggregate clearance through proteolysis of non-native proteins and aggregation reversal (Hartl et al., 2011; Mogk et al., 2011). Clearance of misfolded proteins in *E. coli* is carried out by AAA+ (ATPases Associated with diverse cellular Activities) proteins, which initiate substrate recognition, unfolding, and translocation into a proteolytic chamber (ClpP, HslV) (Snider and Houry, 2008; Sauer and Baker, 2011). Several AAA+ proteins, such as

Lon and FtsH, contain both AAA+ chaperone and proteolytic domains within a single protomer (Sauer and Baker, 2011). The chaperone-protease Lon recognizes exposed aromatic and hydrophobic residues, which may contribute to less stringent substrate selectivity and favor degradation of unfolded or misfolded proteins (Gur and Sauer, 2008).

The Clp ATPases of the AAA+ superfamily can be separated into two functional categories: degradation or disaggregation machines. Degradation machines, including ClpX, ClpA, and HslU form complexes with peptidases ClpP or HslV to remove misfolded proteins or specific substrates (Zolkiewski, 2006). Disaggregation machines, including Hsp104 and its bacterial homolog ClpB, disaggregate and reactivate aggregated proteins by an ATP-dependent mechanism and can function in cooperation with the Hsp70/DnaK system independent of protein degradation (Zolkiewski, 1999; Dougan et al., 2002; Doyle et al., 2007; Sweeny and Shorter, 2016). Through a collaborative mechanism, Hsp70, with Hsp40, binds first to a polypeptide segment of an aggregated protein and then the substrate is remodeled by Hsp104/ClpB (Zietkiewicz et al., 2004; Zietkiewicz et al., 2006; Acebron et al., 2009).

E. coli substrates that are degraded by ClpXP include a variety of cellular proteins, metabolic enzymes and several proteins capable of forming large conformational assemblies, including FtsZ, Dps and MinD (Flynn et al.,

2003;Stephani et al., 2003;Neher et al., 2006;Camberg et al., 2009;Camberg et al., 2014;Conti et al., 2015). ClpXP can associate with cellular aggregates in *E. coli* and can promote removal of cellular inclusions, but direct protein disaggregation in vitro is not well characterized for ClpX (Vera et al., 2005;Winkler et al., 2010). An early study suggested that ClpX, in the absence of ClpP, could protect the lambda O phage protein from aggregation and resolubilize lambda O aggregates (Wawrzynow et al., 1995). In *Bacillus subtilis*, ClpX also localizes to protein aggregates, suggesting that it may be involved in protein disaggregation (Kruger et al., 2000;Kain et al., 2008;Kirstein et al., 2008)Simmons et al., 2008). ClpX and ClpX substrates are present in polar protein aggregates in *E. coli* under stress in vivo, suggesting that ClpX associates with aggregated proteins and participates in their removal (Kain et al., 2008;Maisonneuve et al., 2008;Simmons et al., 2008).

ClpXP comprises an asymmetric, hexameric ring of ClpX docked to two stacked heptameric rings of the ClpP serine protease (Wang et al., 1997;Glynn et al., 2009). Although ClpX has been shown to independently remodel substrates, such as MuA, in the presence of ClpP, hydrophobic 'IGF' loops on the bottom surface of the ClpX hexamer contact hydrophobic pockets on the ClpP tetradecamer, allowing unfolded substrates to access the ClpP proteolytic chamber (Kim et al., 2001;Abdelhakim et al., 2010;Baker and Sauer, 2012). Nucleotide binding by ClpX protomers, in the cleft between the

large and small AAA+ subdomains, regulate the position of the subdomains relative to each other; these conformational changes enable ClpX to couple substrate translocation to ATP hydrolysis (Glynn et al., 2009; Baker and Sauer, 2012). Substrates are then translocated into the ClpP chamber for degradation (Baker and Sauer, 2012).

Substrates bind to the ClpX N-domain and to residues in the ClpX central channel (pore-loops) (Bolon et al., 2004; Park et al., 2007; Martin et al., 2008; Baker and Sauer, 2012). The N-domain of ClpX is separated from the AAA+ domain by a flexible linker and can dimerize independently. The N-domain is important for direct recognition of some substrates, including FtsZ and MuA, as well as adaptor proteins, but is not required for direct recognition of the ssrA-tag (Abdelhakim et al., 2008; Martin et al., 2008; Camberg et al., 2009; Baker and Sauer, 2012). Adaptor proteins, such as RssB or SspB, promote the interaction and engagement of specific substrates, such as RpoS or ssrA-tagged substrates, respectively (Sauer and Baker, 2011). The ssrA tag is an 11-residue degron appended to a nascent polypeptide when the ribosome stalls during protein synthesis, targeting the misfolded protein for subsequent degradation (Gottesman et al., 1998; Levchenko et al., 2000).

ClpXP is implicated in the degradation of diverse cellular substrates and more than 100 substrates have been reported (Flynn et al., 2003; Neher et al.,

2006). Native substrates of ClpX contain recognition motifs at the N- or C-termini (Flynn et al., 2003). Notably, the essential cell division protein FtsZ in *E. coli* has two distinct ClpX motifs: one in the flexible linker region and one near the C-terminus (Camberg et al., 2014). FtsZ is a tubulin homolog that assembles into linear polymers in vitro and forms the septal ring critical for division in vivo, called the Z-ring (Erickson et al., 2010). ClpXP degrades approximately 15% of FtsZ proteins during the cell cycle in *E. coli* and is capable of degrading both monomers and polymers in vitro (Camberg et al., 2009). ClpXP degrades polymers more efficiently, which is consistent with a common strategy of multivalent recognition of substrates by AAA+ ATPases (Davis et al., 2009; Camberg et al., 2014; Ling et al., 2015). In addition to FtsZ, several other ClpXP substrates form large oligomeric structures, including the tetrameric phage protein MuA, the dodecameric bacterial protein Dps, and the bacterial cell division ATPase MinD (Stephani et al., 2003; Neher et al., 2006; Abdelhakim et al., 2010; Conti et al., 2015). Like FtsZ, alternate monomeric and oligomeric conformations of MuA are also differentially recognized by ClpX (Abdelhakim et al., 2008; Abdelhakim et al., 2010; Ling et al., 2015).

In this study, we use engineered and native substrates to investigate the role of ClpX and ClpXP in the disassembly and degradation of protein aggregates that bear specific ClpX recognition signals. We observed that ClpX, with and without ClpP, destabilizes Gfp-ssrA aggregates in vitro. The

native ClpXP substrate FtsZ forms several discrete conformations, including linear ordered polymers and also heat-induced aggregates. Our results show that ClpXP disassembles both heat-induced and linear polymers containing FtsZ. Finally, we also demonstrate that thermal stress promotes aggregation of FtsZ, which is exacerbated in cells deleted for *clpX* or *clpP*. Together, these results show bona fide chaperone activity for ClpX in vitro and suggest that ClpX, with or without ClpP, may play a broader role in rescue and disassembly of protein aggregates.

Materials and Methods

Bacterial strains and plasmids

E. coli strains and plasmids used in this study are described in Table 1. An expression plasmid encoding FtsZ(Δ C67) was constructed by introducing a TAA stop codon (at residue 317 of FtsZ) into pET-FtsZ by site-directed mutagenesis (Camberg et al., 2009).

Expression and purification of proteins

Gfp-ssrA was purified as previously described (Yakhnin et al., 1998). ClpX, ClpP, FtsZ, and FtsZ(Δ C67) were each overexpressed in *E. coli* BL21 (λ DE3) and purified as described (Maurizi et al., 1994; Grimaud et al., 1998; Camberg et al., 2009; Camberg et al., 2014). ClpX(E185Q) was purified as described for wild type ClpX, except the expression strain, *E. coli* MG1655 Δ *clpX* carrying plasmid pClpX(E185Q), was induced with 1% arabinose (Table

1) (Camberg et al., 2011). Gfp(uv) containing an N-terminal histidine tag was overexpressed in *E. coli* BL21 (λ DE3) and grown to an OD₆₀₀ of 1.0 and induced for 3 hours at 30 °C. Cells were lysed by French press in purification lysis buffer (20 mM HEPES, pH 7.5, 5 mM MgCl₂, 50 mM KCl, and 10% glycerol). Soluble extracts were bound to TALON metal affinity resin (GE Healthcare), eluted with an imidazole gradient, and imidazole was removed by buffer exchange. Protein concentrations are reported as FtsZ monomers, ClpX hexamers, ClpP tetradecamers, and Gfp or Gfp-tagged monomers. For polymerization assays, FtsZ was labeled with Alexa Fluor 488 and active protein (FL-FtsZ) was collected after cycles of polymerization and depolymerization as described (Gonzalez et al., 2003; Camberg et al., 2014).

Dynamic light scattering

Dynamic light scattering (DLS) measurements were made using a Zetasizer Nano ZS (Malvern Instruments). To determine size distribution, FtsZ (5 μ M), aggFtsZ (5 μ M), Gfp-ssrA (1.5 μ M) and aggGfp-ssrA (1.5 μ M) in reaction buffer (50 mM HEPES, pH 7.5, 100 mM KCl and 10 mM MgCl₂) were added to polystyrene cuvettes and scanned at 23 °C with a detector angle of 173° and a 4 mW, 633 nm He–Ne laser. The reported intensity-weighted hydrodynamic diameters are based on 15 scans.

Heat denaturation, aggregation, disassembly and reactivation of aggregated substrates

To heat-inactivate Gfp substrates, Gfp-ssrA (1.5 μ M) or Gfp(uv) (1.5 μ M) was added, where indicated, to buffer containing HEPES (50 mM, pH 7.5), KCl (100 mM), $MgCl_2$ (10 mM), glycerol (10%) and dithiothreitol (DTT) (2 mM) in a volume of 800 μ l and incubated at 85 °C for 15 minutes. Immediately following heat-treatment, the denatured substrate was placed on ice for 2 minutes and added to a reaction (50 μ l) containing ClpX, (0.3 μ M), ClpX (E185Q) (0.3 μ M), ClpP (0.3 μ M), ATP (4 mM), ATP γ S (1 mM), or ADP (2 mM), where indicated. Samples containing ATP were supplemented with an ATP-regenerating system containing phosphocreatine (5 mg ml⁻¹) and creatine kinase (CK) (60 μ g ml⁻¹). Fluorescence recovery was monitored by measuring fluorescence in a Cary Eclipse fluorometer with excitation and emission wavelengths set at 395 nm and 510 nm, respectively. Readings were corrected for background signal by subtracting the fluorescence of buffer. Rates were calculated by fitting to a one-phase association model in GraphPad Prism (version 6.0b). Disaggregation was monitored by 90°-angle light scatter with excitation and emission wavelengths set to 550 nm. Readings were corrected for background signal by subtracting the scatter of the buffer and then plotted as percent of the initial turbidity. Heat-induced aggregation of Gfp-ssrA with time was monitored by 90°-angle light scatter with the temperature of the cuvette holder set to 80 °C using a circulating water bath.

To inactivate native FtsZ substrates, FtsZ and FtsZ(Δ C67) (5 μ M) were heated for 15 minutes in reaction buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 10 mM MgCl_2) in a volume of 120 μ l at 65 $^{\circ}\text{C}$, then cooled on ice for 40 seconds, and held at 23 $^{\circ}\text{C}$ until addition to reactions (60 μ l volume) containing ClpX (0.5 μ M or 1 μ M), ClpX(E185Q) (0.5 μ M), ClpP (1 μ M), ATP (4 mM) and an ATP-regenerating system (phosphocreatine at 5 mg ml^{-1} and creatine kinase at 60 $\mu\text{g ml}^{-1}$), where indicated. Disaggregation was monitored by 90 $^{\circ}$ -angle light scatter with excitation and emission wavelengths set to 450 nm. Readings were corrected for background signal by subtracting the scatter of the buffer and then plotted as percent of the initial turbidity. Heat-induced aggregation of FtsZ with time was monitored by 90 $^{\circ}$ -angle light scatter with the temperature of the cuvette holder set to 65 $^{\circ}\text{C}$ using a circulating water bath.

Polymerization and GTP hydrolysis assays

FL-FtsZ was incubated with the GTP analog GMPCPP (0.5 mM) in the presence of increasing concentrations of ClpX and ClpP (0, 0.25, 0.5 or 1 μ M) as indicated and in the presence of phosphocreatine at 5 mg ml^{-1} and creatine kinase at 60 $\mu\text{g ml}^{-1}$. Samples were incubated for 3 minutes in buffer containing MES (50 mM, pH 6.5), KCl (100 mM) and MgCl_2 (10 mM) at 23 $^{\circ}\text{C}$, then centrifuged at 129,000 $\times g$ in a Beckman TLA 120.1 rotor for 30 minutes. Pellets were resuspended in 0.2 M NaCl with 0.01% Triton X-100 (100 μ l) and the fluorescence associated with FL-FtsZ for supernatants and pellets was measured using a Cary Eclipse spectrophotometer. GTP hydrolysis rates for

FtsZ and FtsZ(Δ C67) were measured before and after aggregation using the Biomol Green (Enzo Life Sciences) detection reagent as described (Camberg et al., 2014).

Heat shock of wild type and deletion strains

E. coli wild type and deletion strains were grown overnight, diluted 1:100 in fresh Lennox broth the next day and grown at 30 °C to an OD of 0.4. All strains were incubated in a water bath at 50 °C for 1 hour, followed by recovery at 30 °C for 35 minutes. Cells were harvested by centrifugation and lysed with Bacterial Protein Extraction Reagent (B-PER) (ThermoFisher Scientific) (2 ml) and lysozyme (25 μ g ml⁻¹). Insoluble fractions were collected by centrifugation at 15,000 x *g* for 5 minutes at 4 °C, resuspended in lithium dodecyl sulfate sample buffer and analyzed by reducing SDS-PAGE. Total proteins were transferred to a nitrocellulose membrane and visualized by Ponceau (Fisher Scientific) staining and membranes were immunoblotted using antibodies to ClpX and FtsZ (Camberg et al., 2009;2011). Band intensities were analyzed by densitometry (NIH ImageJ), normalized to the intensity of the average of the 'no heat' sample, and evaluated for significance by the Mann-Whitney test. Where indicated, to test a mild heat shock condition, cells were incubated in a water bath at 42 °C for 30 minutes, followed by recovery at 30 °C for 35 minutes, and analyzed as described.

Results

ClpXP degrades aggregates in vitro

To determine if ClpX can remodel protein substrates from the aggregated state, we used the fusion protein, Gfp-ssrA, which forms aggregates upon heat treatment (Zietkiewicz et al., 2004; Zietkiewicz et al., 2006). Gfp-ssrA is rapidly degraded by ClpXP and has been extensively studied to understand substrate targeting by ClpXP. The Gfp moiety is widely used in protein disaggregation assays because it forms non-fluorescent aggregates when heated, but is disaggregated and reactivated by several chaperone systems (Zietkiewicz et al., 2004; Zietkiewicz et al., 2006). Therefore, we heated Gfp-ssrA at 85 °C for 15 minutes to induce aggregation (aggGfp-ssrA), resulting in an 86% loss of fluorescence emitted (Figure 1A). Next, to measure the distribution of aggregates by size after heating, we performed dynamic light scattering (DLS) of untreated and heat-denatured Gfp-ssrA. We observed that without heating, the particle sizes are uniform with an average hydrodynamic diameter of 8-10 nm (Figure 1B). After heating, aggregates are approximately 500-600 nm, and there is a narrow distribution of particle sizes and no small particles (i.e., less than 100 nm) (Figure 1C). Upon heat-treatment, aggregation of Gfp-ssrA (1.5 μ M) occurs rapidly and plateaus by 10 minutes by 90°-angle light scattering (Figure 1D). The heat inactivation is irreversible since incubation of aggregated Gfp-ssrA (aggGfp-ssrA) alone does not lead to appreciable fluorescence reactivation, which is consistent with previous reports using Gfp (Figure S1) (Zietkiewicz et al.,

2004). To determine if ClpXP can bind to aggregates and degrade them, we incubated aggGfp-ssrA with ClpXP and monitored turbidity by 90°-angle light scattering. Incubation of aggGfp-ssrA with ClpXP led to a 35% loss of turbidity in 2 hours (Figure 1E). However, when ClpXP was omitted from the reaction, there was very little change in turbidity over time (5% loss in 2 hours) (Figure 1E). This suggests that ClpXP targets aggregated substrates for degradation. To determine if degradation is required to reduce turbidity, we omitted ClpP and observed that ClpX is capable of reducing sample turbidity by 15% in 2 hours (Figure 1E). Finally, when ATP was omitted from the reaction containing ClpXP, we observed a less than 10% reduction in the turbidity of the reaction (Figure 1E). To confirm that ClpXP degrades aggGfp-ssrA, we incubated aggGfp-ssrA with combinations of ClpX, ClpP and ATP, and sampled degradation reactions after 2 hours. We observed that in the presence of ClpXP, aggGfp-ssrA is degraded, but not when ClpP or ATP was omitted (Figure 1F). Together, these results demonstrate that ClpXP targets aggregates for ATP-dependent degradation and that ClpX is also capable of promoting disassembly in the absence of ClpP.

FtsZ is a well-characterized ClpXP substrate that is essential for cell division and forms linear polymers *in vitro* in the presence of GTP (Erickson et al., 2010). We previously showed that ClpXP binds to GTP-stimulated FtsZ polymers and promotes FtsZ degradation (Camberg et al., 2009). ClpXP also recognizes and degrades non-polymerized FtsZ, but less efficiently than

polymerized FtsZ (Camberg et al., 2009). In vitro, FtsZ rapidly aggregates when heated at 65 °C and this aggregation is associated with an increase in overall light scatter and a 97% loss of GTPase activity (Figure 2A and 2B). FtsZ, which purifies as a mixture of monomers (40.4 kDa) and dimers (80.8 kDa), has an average hydrodynamic diameter of 10-15 nm by DLS (Figure 2C). Heat treatment of FtsZ (5 µM) at 65 °C produces several particle sizes, including small (30-40 nm) and large aggregates (>300 nm) (Figure 2D). To determine if ClpXP reduces the turbidity associated with aggregated FtsZ (aggFtsZ), we incubated aggFtsZ with ClpXP and ATP and observed a 40% loss of turbidity after incubation with ClpXP for 2 hours (Figure 2E). However, in the absence of ClpXP, the light scatter signal remained stable for aggFtsZ (Figure 2E). Incubation of ClpX with aggFtsZ also resulted in a 25% loss in light scatter, suggesting that ClpX also promotes disassembly of aggregates similar to what we observed for aggGfp-ssrA (Figure 2E and 1E).

Next, to confirm that aggFtsZ is degraded by ClpXP, we assembled reactions containing combinations of aggFtsZ, ClpX, ClpP and ATP and sampled these reactions at 0 and 120 minutes for analysis by SDS-PAGE. We observed that in the presence of ClpXP and ATP, 50% of the total aggFtsZ in the reaction is lost to degradation after 120 minutes (Figure 2F). Omission of either ClpP or ATP from the reaction prevents loss of aggFtsZ (Figure 2F). These results indicate that ClpXP degrades aggFtsZ. Furthermore, the amount of aggFtsZ after incubation with ClpX is unchanged despite the decrease in

light scatter detected, suggesting that ClpX can disaggregate aggFtsZ (Figure 2E and 2F).

In addition to forming aggregates upon heating, FtsZ also assembles into a linear head-to-tail polymer, which is a native, ordered aggregate, and distinct from the disordered aggregates which are induced by heating (aggFtsZ). We compared the loss of aggFtsZ by ClpXP to a similar reaction monitoring loss of native polymerized FtsZ, which is a known substrate of ClpXP. Like aggFtsZ, we also observed a ~50% loss of polymeric FtsZ, stabilized by the GTP analog GMPCPP, after 120 minutes in reactions containing ClpXP and ATP (Figure 2F). GMPCPP promotes the assembly of stable polymers that are far less dynamic than polymers assembled with GTP (Lu et al., 2000). To test if ClpXP disassembles GMPCPP-stabilized FtsZ polymers, we incubated pre-assembled polymers with ClpXP and ATP. Then, we collected polymers by high-speed centrifugation. In these assays, we used active fluorescent FtsZ, labeled with Alexa fluor 488 (FL-FtsZ), to quantify the amount of polymerized FtsZ in the pellet fraction and soluble FtsZ in the supernatant. We observed that after incubation of GMPCPP-stabilized FtsZ polymers with increasing concentrations of ClpXP (0 μ M to 1 μ M), few FtsZ polymers were recovered in the pellet fractions containing ClpXP (26% of the total FtsZ was recovered in the reaction containing 1 μ M ClpXP), indicating that ClpXP is highly effective at promoting the disassembly of GMPCPP-stabilized FtsZ polymers (Figure 2G).

ClpX reactivates heat-aggregated Gfp-ssrA

Incubation of ClpX with aggGfp-ssrA resulted in loss of turbidity, suggesting that ClpX may function independently of ClpP to reactivate substrates (Figure 1E). Reactivation of misfolded proteins may occur through binding and stabilization of intermediates enabling proteins to adopt the native folded conformation, or through ATP-dependent chaperone-assisted unfolding. To determine if ClpX, which recognizes the ssrA amino acid sequence, is able to reactivate aggGfp-ssrA, we monitored fluorescence of aggGfp-ssrA in the presence and absence of ClpX and ATP. AggGfp-ssrA regains very little fluorescence alone, approximately 20 units, which is 8% of the initial fluorescence lost upon heating; however, in the presence of ClpX, fluorescence recovers rapidly in the first 10 minutes of the reaction and then plateaus, regaining approximately 85 units, which is 27% of the initial fluorescence lost upon heating (Figure 3A).

ClpX catalyzes ATP-dependent unfolding of substrates (Kim et al., 2000; Singh et al., 2000). To determine if ATP is essential for reactivation, we incubated aggGfp-ssrA with ClpX under various nucleotide conditions including with ATP, the ATP analog ATP γ S, ADP and omission of nucleotide. We observed an 82% slower rate of fluorescence reactivation when ClpX and aggGfp-ssrA were incubated with ATP γ S than with ATP (0.02 AU min⁻¹ and

0.11 AU min⁻¹, respectively), and no recovery over background with ADP or without nucleotide (Figure 3B). Reactivation by ClpX and ATP is prevented in the presence of ClpP, and the residual fluorescence after heat treatment is lost upon degradation (Figure S2). Together, these results indicate that ClpX requires ATP to reactivate Gfp-ssrA and, surprisingly, that ATPγS is also capable of promoting reactivation, although at a much slower rate than ATP (Figure 3B).

Reactivation and disaggregation by ClpX requires a specific recognition sequence

Next, we determined if a ClpX recognition motif is important for efficient recognition of aggregated substrates by ClpX. We compared reactivation of aggGfp-ssrA with heat-aggregated Gfp (aggGfp) without an ssrA tag. We observed that after incubation with ClpX and ATP for 60 minutes, approximately 30 units of fluorescence were recovered, which is 8% of the initial pre-heat fluorescence, indicating that aggGfp is a poor substrate for reactivation by ClpX (Figure 4A). In contrast, aggGfp-ssrA recovered 33% (>100 units) of the initial pre-heat fluorescence after incubation with ClpX (Figure 4A).

Two regions of FtsZ are important for promoting degradation of *E. coli* FtsZ by ClpXP, one in the unstructured linker region (amino acids 352-358)

and one near the C-terminus (residues 379 through 383) (Camberg et al., 2014). Using a truncated FtsZ mutant protein, FtsZ(Δ C67), which is deleted for 67 C-terminal amino acid residues, including both regions involved in ClpX recognition, we tested if ClpXP reduces the light scatter in reactions containing heat-aggregated FtsZ(Δ C67) [aggFtsZ(Δ C67)]. We heated FtsZ(Δ C67) at 65 °C for 15 minutes, the condition that promotes aggregation of full length FtsZ, and confirmed that heat treatment resulted in an 84% loss of GTP hydrolysis activity and an increase in light scatter, which is stable over time (Figure 4B and 4C). In the presence of ClpXP, we observed no decrease in light scatter for aggFtsZ(Δ C67) after incubation for 120 minutes (Figure 4C), which is expected since FtsZ(Δ C67) is a poor substrate for ClpXP degradation (Figure S3). Together, these results demonstrate that for ClpX to recognize aggregates and promote disaggregation, disassembly and/or reactivation, a ClpX recognition motif is required.

Impaired reactivation by ClpX(E185Q)

ATP is required for reactivation of aggGfp-ssrA, however, it is unknown if this event requires ATP-hydrolysis and substrate unfolding. Therefore, we used the ClpX mutant protein ClpX(E185Q), which has a mutation in the Walker B motif and is defective for ATP-hydrolysis, but interacts with substrates (Hersch et al., 2005; Camberg et al., 2014). We observed that ClpX(E185Q) is defective for disaggregation of aggGfp-ssrA by monitoring

turbidity by 90°-angle light scatter of reactions containing aggGfp-ssrA, ClpX(E185Q) and ATP (Figure 5A). We also tested if aggFtsZ is disassembled by ClpX(E185Q), and observed no reduction in light scatter in reactions containing aggFtsZ, ClpX(E185Q) and ATP after 120 minutes compared to ClpX (Figure 5B). Finally, we tested if reactivation of aggGfp-ssrA requires ATP hydrolysis using ClpX(E185Q) instead of ClpX. We observed that ClpX(E185Q) promotes a small amount of reactivation of aggGfp-ssrA and restores fluorescence, but to a much lesser extent than the level observed for wild type ClpX (Figure 5C). These results suggest that ATP hydrolysis by ClpX is required to promote efficient reactivation of aggGfp-ssrA and disassembly of large complexes containing aggFtsZ or aggGfp-ssrA (Figure 5A, 5B and 5C).

ClpXP prevents accumulation of FtsZ aggregates in vivo under extreme thermal stress

ClpX and ClpP were previously reported to localize to protein aggregates in *E. coli*, suggesting that ClpXP may target aggregates in vivo for direct degradation (Winkler et al., 2010). We used the native ClpXP substrate FtsZ, which aggregates upon heat treatment, to determine if ClpX and/or ClpXP modulates FtsZ aggregate accumulation after thermal stress by comparing the levels of FtsZ present in insoluble cell fractions (Figure 2A and 6A). Wild type cells and cells deleted for *clpX*, *clpP*, *clpB*, *clpA*, *dnaK*, *lon*, *hslU* and *hslV* were exposed to heat shock and insoluble protein fractions were collected and

analyzed by immunoblot. We observed that FtsZ was present in the insoluble fraction of wild type cells (BW25113), and this amount was 42% higher in cells exposed to heat shock at 50 °C (Figure 6A and S4A). However, FtsZ levels were even higher in the insoluble fractions of $\Delta clpX$ and $\Delta clpP$ strains compared to the parental strain (2.4-fold and 2.3-fold, respectively), although the amount of total protein was similar to the wild type strain exposed to heat shock (Figure S4B). We detected less protein overall in the $\Delta dnaK$ strain after recovery, but this strain also had poor viability after heat shock and recovery (Figure S4C). In addition, we also detected ClpX in the insoluble fraction in all strains except the *clpX* deletion strain (Figure S4A). Next, we conducted a mild heat shock, 42 °C for 30 minutes, followed by recovery, and observed that deletion of *clpB* had a larger effect on the accumulation of insoluble FtsZ than deletion of *clpX* (Figure S4D). To determine the relative contributions of either *clpB* or *clpX* during a 40 minute recovery period after incubation at 50 °C, we analyzed insoluble FtsZ levels at 20 minute time intervals during recovery (Fig. 6B). Notably, we observed that in cells deleted for *clpX*, insoluble FtsZ was present immediately after heat treatment and continued to accumulate throughout the recovery period to a greater extent than in wild type or *clpB* deletion cells. These results suggest that ClpXP prevents accumulation of FtsZ aggregates in cells exposed to extreme thermal stress. Since we observed that insoluble FtsZ levels were elevated in $\Delta clpB$ strains exposed to mild heat shock (Figure S4D), we repeated the recovery time course in *clpX* and *clpB* deletion strains after mild heat shock, 42 °C for 30 minutes, to

monitor insoluble FtsZ levels (Figure S4E). We observed that insoluble FtsZ accumulates during the recovery period in *clpB* deletion strains after mild heat shock (Figure S4E).

Finally, if ClpXP is active in cells after severe heat shock, then it should not be a thermolabile protein. To determine if ClpXP remains active after exposure to 50 °C in vitro, we incubated ClpXP in buffer at 50 °C for one hour, and then measured activity after addition of Gfp-ssrA by monitoring the loss of Gfp-ssrA fluorescence. We observed that ClpXP remained active for unfolding and degradation of Gfp-ssrA after incubation at 50 °C for one hour (Figure S4F). As a control, ClpXP was also incubated in buffer at 30 °C for one hour and then assayed for activity. We observed that ClpXP incubated at 30 °C was more active than ClpXP incubated at 50 °C, suggesting that a partial loss of activity had occurred at high temperature (Figure S4F). However, this assay was performed in the complete absence of other cellular chaperones or substrates and suggests that some ClpXP likely continues to retain activity after exposure to heat stress, while some may become inactivated.

Discussion

Here, using both a native and an engineered aggregated substrate, we demonstrate that ClpXP has the operational capacity to disassemble and degrade large aggregates that have ClpX degrons. In this study, FtsZ, a native

substrate of ClpXP in *E. coli*, was aggregated in vitro by thermal stress, and we further show that FtsZ also aggregates in vivo when cells are exposed to high temperature (Figure 2A and 6A). The observation that FtsZ is aggregation prone is in agreement with a prior study reporting the presence of FtsZ in intracellular aggregates of $\Delta rpoH$ cells incubated at 42 °C by mass spectrometry (Tomoyasu et al., 2001). FtsZ aggregates are cleared in vitro and in vivo by ClpXP, and ClpXP does not require the assistance of additional chaperones (Figure 2E, 2F and 6A). Moreover, in the absence of ClpP, ClpX also promotes disassembly of FtsZ and Gfp-ssrA aggregates indicating that disassembly can also occur by a proteolysis-independent mechanism, although disaggregation is more efficient in the presence of ClpP. ClpXP-mediated disassembly of Gfp-ssrA aggregates requires ATP in experiments monitoring turbidity (Figure 1E). In addition, the Walker B mutation in ClpX, E185Q, which impairs ATP hydrolysis, also impairs disaggregation of aggGfp-ssrA and, to a lesser extent, aggFtsZ. Aggregate disassembly and resolubilization by ClpX was previously described using the substrate lambda O protein, and here we show disassembly of aggregates and kinetic monitoring using two additional substrates, as well as reactivation of Gfp-ssrA fluorescence (Wawrzynow et al., 1995). Reactivation of Gfp-ssrA is largely dependent on ATP hydrolysis (Figure 3B), since ClpX(E185Q) only weakly promotes reactivation of aggregated Gfp-ssrA (Figure 5C), yet ClpX(E185Q) is capable of stable interactions with substrates in the presence of ATP, although they are not unfolded (Hersch et al., 2005; Camberg et al., 2014). It is unlikely

that there are soluble, unfolded Gfp-ssrA monomers in solution after heating, since we did not detect them by DLS and it has been demonstrated that soluble, unfolded Gfp rapidly refolds, in 20 to 30 seconds, by a spontaneous reaction that does not require chaperones (Figure 1C) (Makino et al., 1997; Tsien, 1998; Zietkiewicz et al., 2004). Therefore, it is likely that large aggregates contain loosely associated unfolded proteins, which can be removed and reactivated by ClpX and, in the case of Gfp-ssrA, allowed to spontaneously refold. As expected, recognition by ClpX is highly specific, as Gfp without an ssrA-tag is not reactivated (Figure 4A).

We also detected partial disaggregation of aggFtsZ by ClpX, but not by ClpX(E185Q) (Figure 5B). Aggregation of FtsZ is induced at 65 °C, but the aggregates formed by FtsZ are smaller than those formed by Gfp-ssrA (30 nm and 600 nm, respectively) (Figure 1C and 2D). FtsZ aggregates likely contain 8-10 monomers, based on the average size of a folded FtsZ monomer, which is approximately 40 Å in diameter (Figure 2D) (Oliva et al., 2004). In contrast, Gfp aggregates in this study likely contain more than 120 subunits, based on an average size of a folded Gfp monomer, which is approximately 50 Å across the long axis (van Thor et al., 2005). The small size of the FtsZ aggregate may allow it to be more susceptible to disassembly by ClpX than a larger aggregate.

In the model for disassembly of aggregates by ClpXP, ClpX binds to exposed recognition tags on the surface of the aggregate and promotes removal, unfolding and degradation of protomers from within the aggregate (Figure 7A). Removal of protomers eventually leads to destabilization and fragmentation of the aggregate as well as degradation (Figure 1F and 2F). Although this process does not require ClpP, it occurs more robustly when ClpP is present than when ClpP is omitted (Figure 1E and 2E). For aggregated substrate reactivation, ClpX likely engages unfolded protomers from the aggregate, which may be internal or loosely bound to the exterior of the aggregate, unfolds and release them. For small aggregates, this activity may be sufficient to lead to fragmentation and capable of promoting reactivation of substrates such as Gfp-ssrA (Figure 7B).

Finally, we observed large increases in insoluble FtsZ when cells were exposed to two different temperatures, 50 °C, which represents extreme heat shock, and 42 °C, which represents a mild heat shock (Figure 6A, 6B and S4D). At 42 °C, deletion of *clpB* was associated with a large accumulation of insoluble FtsZ, suggesting that under mild heat stress, ClpB is the major factor that ensures FtsZ solubility (Figure S4D and S4E). However, we observed a remarkably different result after heat shock at 50 °C and throughout the recovery period. Specifically, in a *clpX* deletion strain, large amounts of insoluble FtsZ accumulate during the recovery period to a greater extent than in a *clpB* deletion strain (Figure 6A and 6B). It is unknown if ClpXP and ClpB

are processing FtsZ aggregates directly in vivo, because we did not observe a reduction of aggregated FtsZ during the recovery period for any strain. FtsZ is typically present at very high levels (5,000 to 20,000 copies per cell) and is essential for cell division in *E. coli* (Bramhill, 1997). Interestingly, FtsZ also forms linear polymers as part of its normal biological function to promote cell division, and polymers are efficiently recognized, disassembled and degraded by ClpXP (Figure 2F and 2G) (Camberg et al., 2009; Camberg et al., 2014; Viola et al., 2017). Given the diverse conformational plasticity of FtsZ, its use as a model disaggregation and remodeling substrate will be informative for studies of targeting and processing of multisubunit substrates by AAA+ proteins. As with FtsZ, many other ClpXP substrates are detectable in protein aggregates in cells (Flynn et al., 2003; Maisonneuve et al., 2008). Moreover, a previous study showed that ClpXP is important for cell viability under thermal stress conditions in cells depleted of DnaK (Tomoyasu et al., 2001). Given that it is estimated that 2-3% of *E. coli* proteins are ClpXP substrates, ClpXP likely serves as an additional mechanism to manage accumulation of aggregation-prone proteins in vivo, particularly under extreme stress conditions (Flynn et al., 2003; Maisonneuve et al., 2008).

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or Plasmid	Genotype	Source, reference or Construction
Strains		
BW25113 2000	F ⁻ , DE(<i>araD-araB</i>)567, <i>lacZ</i> 4787(del)(::rrnB-3),	Datsenko and Wanner,
	<i>LAM</i> ⁻ , <i>rph</i> -I, DE(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW0429 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>lon</i> -725::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW0428 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>clpX</i> 724::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW0427 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>clpP</i> 723::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW2573 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>clpB</i> 757::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW0866 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>clpA</i> 783::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW3902 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>hsl</i> U790::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW3903 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>hsl</i> V720::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW0013 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>dna</i> K734::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JW0462 2006	F ⁻ , Δ(<i>araD-araB</i>)567, Δ <i>lacZ</i> 4787(::rrnB-3),	Baba et al.,
	Δ <i>htp</i> G757::kan, λ, <i>rph</i> -I, Δ(<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	
JC0259 2011	MG1655 Δ <i>clpX</i> ::kan	Camberg et al.,
Plasmids		
pET-ClpX 2009	<i>kan</i>	Camberg et al.
pET-ClpP 1994	<i>kan</i>	Maurizi et al.
pET-FtsZ 2009	<i>kan</i>	Camberg et al.
pET-FtsZ(ΔC67)	<i>kan</i>	This study
pET-H ₆ -Gfp(uv)	<i>kan</i>	This study
pBad-Gfp-ssrA 2000	<i>amp</i>	Singh et al.,
pClpX(E185Q) 2011	<i>amp</i>	Camberg et al.,

Figure 1 – Disaggregation and degradation of aggregated Gfp-ssrA by ClpXP.

(A) The fluorescence emission spectra (450-600 nm) of Gfp-ssrA (1.0 μ M) (green) and heat-treated Gfp-ssrA (1.0 μ M) (black) (85 °C for 15 minutes) were measured using an excitation wavelength of 395 nm. Plotted curves are representative of three traces.

(B) DLS was performed for Gfp-ssrA (1.0 μ M) (green) as described to determine particle size (nm) distribution.

(C) DLS was performed for heat-treated Gfp-ssrA (aggGfp-ssrA) (1.0 μ M) (black) as described to determine particle size (nm) distribution.

(D) Aggregation by 90°-angle light scatter was measured for Gfp-ssrA (1.5 μ M) (open circles) in a cuvette attached to a circulating water bath held at 80 °C. Light scattering was monitored for 15 minutes.

(E) Disaggregation of aggGfp-ssrA (1 μ M) was monitored by 90°-angle light scatter as described in *Materials and Methods*. Disaggregation reactions contained aggGfp-ssrA (1 μ M) (black circles), ClpX (0.5 μ M) and ATP (blue circles), ClpX (0.5 μ M) and ClpP (0.6 μ M) (gold circles), ClpX (0.5 μ M), ClpP (0.6 μ M), and ATP (4 mM) (red circles), and a regenerating system, where indicated. Light scattering was monitored for 120 minutes. Curves shown are representative of at least three replicates.

(F) Degradation of Gfp-ssrA and aggGfp-ssrA was monitored as described in *Materials and Methods* in reactions containing Gfp-ssrA (1 μ M) or aggGfp-

ssrA (1 μ M), where indicated, and ClpX (0.5 μ M), ClpP (0.6 μ M), ATP (4 mM), and a regenerating system, where indicated. Reactions were incubated at 23 °C for 120 minutes and samples were analyzed by SDS-PAGE and Coomassie stain.

Figure 2 - Aggregation and disaggregation of native ClpXP substrate FtsZ.

(A) Aggregation by 90°-angle light scatter was measured for FtsZ (5 μ M) (black circles) in a cuvette attached to a circulating water bath at 65 °C for 30 minutes as described in *Materials and Methods*. The curve shown is representative of at least three replicates.

(B) Rates of GTP hydrolysis were measured for FtsZ (5 μ M) (gray) and aggFtsZ (5 μ M) (black) with GTP (1 mM) for 15 minutes at 30 °C, as described in *Materials and Methods*. The average rate was determined from at least four replicates.

(C) DLS was performed for FtsZ (5 μ M) (gray) as described to determine particle size (nm) distribution.

(D) DLS was performed for aggFtsZ (5 μ M) (black) as described to determine particle size (nm) distribution.

(E) Disaggregation of aggFtsZ (5 μ M) was monitored by 90°-angle light scatter as described in *Materials and Methods*. Disaggregation reactions contained aggFtsZ (5 μ M) (black circles) or aggFtsZ (5 μ M) and ClpX (1 μ M) (blue circles), or aggFtsZ (5 μ M), ClpX (1 μ M), and ClpP (1 μ M) (red circles), with ATP (4 mM) and a regenerating system. Light scattering was monitored for 120 minutes. The curves shown are representative of at least three replicates.

(F) Degradation was monitored for FtsZ and aggFtsZ as described in *Materials and Methods* in reactions containing FtsZ (6 μ M), aggFtsZ (6 μ M), ClpX (0.5

μM), ClpP (0.5 μM), ATP (4 mM) and a regenerating system, where indicated. For degradation of FtsZ, GMPCPP (0.5 mM) was included to promote the assembly of stable polymers. Degradation reactions were incubated at 23 °C for 120 minutes. To detect protein loss due to degradation, samples from 0 and 120 minutes were analyzed by SDS-PAGE to solubilize any remaining aggregates.

(G) Degradation was monitored for FL-FtsZ (125 pmol) incubated in the presence of GMPCPP (0.5 mM) for 3 minutes, then ATP (4 mM), a regenerating system, and increasing concentrations of ClpXP (0, 0.25, 0.5 and 1 μM as shown) were added and reactions were incubated for an additional 30 minutes at 23 °C. Reactions were centrifuged at 129,000 x *g* for 30 minutes at 23 °C. Pellet-associated FtsZ was quantified by fluorescence, and each data point is an average of at least three replicates.

Figure 3 - Reactivation of aggregated Gfp-ssrA in the presence of ClpX.

(A) Reactivation of aggGfp-ssrA (1.0 μ M) was monitored as described in *Materials and Methods* in the absence (black circles) and presence (blue circles) of ClpX (0.3 μ M), ATP (4 mM), and a regenerating system. Fluorescence emission (AU) was monitored for 30 minutes. The curves shown are representative of at least three replicates.

(B) Reactivation of aggGfp-ssrA (1.0 μ M) was monitored in the absence (black circles) or presence of ClpX (0.3 μ M), ATP (4 mM) and a regenerating system (blue circles), ATP γ S (2 mM) (orange circles), ADP (2 mM) (green circles), or no nucleotide (gray circles), where indicated. Fluorescence emission (AU) was monitored for 60 minutes. The curves shown are representative of at least three replicates.

Figure 4 - Aggregation and disaggregation of ClpXP substrates with and without recognition motifs.

(A) Reactivation of aggGfp-ssrA (1.0 μ M) alone (dark gray circles) or in the presence of ClpX (0.3 μ M) (blue circles), and reactivation of aggGfp(uv) alone (1.0 μ M) (light gray circles) or in the presence of ClpX (0.3 μ M) (green circles), where indicated, was monitored with ATP (4 mM) and a regenerating system as described in *Materials and Methods*. Fluorescence emission (AU) was monitored for 60 minutes. The plotted curves are representative of at least three replicates.

(B) Rates of GTP hydrolysis were determined as described in *Materials and Methods* for FtsZ(Δ C67) (5 μ M) (gray) and aggFtsZ(Δ C67) (5 μ M) (black), where indicated, incubated with GTP (1 mM) for 15 minutes at 30 °C. The average rate was determined from at least four replicates.

(C) Disaggregation was monitored by 90°-angle light scatter for aggFtsZ(Δ C67) (5 μ M) alone (black), aggFtsZ(Δ C67) (5 μ M) in the presence of ClpXP (0.5 μ M), ATP (4 mM) and a regenerating system (red), or aggFtsZ(5 μ M) in the presence of ClpXP (0.5 μ M), ATP (4 mM) and a regenerating system (blue) where indicated as described in *Materials and Methods*. Light scattering was monitored for 120 minutes. The curves shown are representative of at least three replicates.

Figure 5 – Disaggregation and reactivation of ClpX substrates in the presence of ClpX(E185Q).

(A) Disaggregation was monitored by 90°-angle light scatter, as described in *Materials and Methods* for aggGfp-ssrA (1.0 μ M) alone (black circles) or in the presence of ClpX (0.5 μ M) (blue circles) or ClpX (E185Q) (0.5 μ M) (open circles), where indicated, with ATP (4 mM), and a regenerating system. Light scattering was monitored for 120 minutes. The curves shown are representative of at least three replicates.

(B) Disaggregation was monitored by 90°-angle light scatter for aggFtsZ (5 μ M), ClpX (0.5 μ M) or ClpX(E185Q) (0.5 μ M) where indicated, ATP (4 mM), and a regenerating system for 120 minutes as described in *Materials and Methods*. The curves shown are representative of at least three replicates.

(C) Reactivation was monitored as described in *Materials and Methods* for aggGfp-ssrA (1.0 μ M) alone (black circles) or in the presence of ClpX (0.3 μ M) (blue circles) or ClpX (E185Q) (0.3 μ M) (open circles), with ATP (4 mM) and a regenerating system, where indicated. Fluorescence emission (AU) was monitored for 90 minutes. The curves shown are representative of at least three replicates.

Figure 6 – FtsZ aggregation in deletion strains after heat shock.

(A) FtsZ levels were compared in insoluble cell extracts prepared from single gene deletion strains (Table 1) after heat shock at 50 °C for 1 hour and recovery (30 °C) as described in *Materials and Methods*. Cells were collected and insoluble protein extracts were analyzed by immunoblotting using anti-FtsZ antibodies. Relative FtsZ levels were quantified by densitometry from four independent experiments. Where indicated, ‘*’ represents a *p*-value of 0.03.

(B) Insoluble FtsZ levels were monitored during the 30 °C recovery period (0, 20 and 40 minutes) after heat shock at 50 °C for 60 minutes in wild type, $\Delta clpB$ and $\Delta clpX$ deletion strains.

Figure 7 – Model of aggregate disassembly.

(A) ClpXP binds to aggregated substrates bearing a ClpX-recognition motif.

ClpXP unfolds and degrades protomers from within the aggregate, leading to fragmentation and disassembly in an ATP-dependent manner.

(B) ClpX binds to aggregates that contain unfolded proteins bearing a ClpX-recognition motif. Unfolded proteins loosely associated with the aggregate surface are reactivated by ClpX through a direct protein interaction that requires ATP-dependent unfolding.

Supplemental Figure S1 – Heat-aggregation of Gfp-ssrA.

The fluorescence emission of aggGfp-ssrA (1.0 μ M) (black circles) was monitored as described in *Materials and Methods* for 90 minutes.

Supplemental Figure S2 – Unfolding and degradation of aggregated Gfp-ssrA by ClpXP.

Unfolding and degradation were monitored for aggGfp-ssrA (1.0 μ M) alone (black circles) or in the presence of ClpP (0.3 μ M) (gold circles), ClpX (0.3 μ M) and ClpP (0. μ M) (red circles) with ATP (4 mM), where indicated. Fluorescence emission (AU) was monitored as described in *Materials and Methods*.

Supplemental Figure S3 – Degradation of FtsZ and FtsZ(Δ C67) by ClpXP.

Degradation was monitored for FtsZ (6 μ M) and FtsZ(Δ C67), ClpXP (0.5 μ M), ATP (4 mM), GMPCPP (0.5 mM), and a regenerating system where indicated at 23 °C for 120 minutes as described in *Materials and Methods*, and samples were analyzed by SDS-PAGE and Coomassie stain.

Supplemental Figure S4 – Insoluble FtsZ in deletion strains after heat-treatment.

(A) Single gene deletion strains (Table 1) were incubated at 50 °C for 1 hour and recovered as described in *Materials and Methods*. Cells from deletion strains were collected and insoluble protein extracts were collected as described and analyzed by reducing SDS-PAGE. Immunoblots were performed with antibodies to FtsZ or ClpX as described.

(B) Total protein present in insoluble cell extracts shown in (A) after heat shock at 50 °C and recovery was detected by transferring proteins to a nitrocellulose membrane and staining with Ponceau.

(C) Cell viability for all strains in (A) was determined by measuring colony forming units (CFU ml⁻¹) of cultures before heating ('pre-HS'), after heat treatment at 50 °C for 1 hour ('post-HS'), and after 35 minutes of recovery at 30 °C ('post-rec').

(D) FtsZ levels were compared in single gene deletion strains after heat shock at 42 °C for 30 minutes and recovery (30 °C) as described in *Materials and Methods*. Cells were collected and insoluble protein extracts were analyzed by immunoblotting with antibodies to FtsZ as described.

(E) Insoluble FtsZ levels were monitored in wild type, $\Delta clpX$ and $\Delta clpB$ deletion strains before heat shock (50 °C for one hour or 42 °C for 30 minutes, where indicated) and during the 30 °C recovery period (0, 20 and 40 minutes). At the indicated times, cells were collected from cultures and insoluble protein

extracts were analyzed by immunoblotting with antibodies to FtsZ as described.

(F) Thermal stability of ClpXP was assayed by incubation of ClpX (0.5 μ M) and ClpP (0.7 μ M) in phosphate buffered saline supplemented with ATP (4 mM) $MgCl_2$ (10 mM), glycerol (15%), Triton X-100 (0.005%), and TCEP (1 mM). Reactions containing ClpXP were added to a preheated quartz cuvette attached to a circulating water bath set to 50 °C or 30 °C, where indicated, and incubated for one hour. The circulating water bath was rapidly cooled to 30 °C, the reactions were supplemented with ATP and regenerating system, Gfp-ssrA (0.2 μ M) was added, and fluorescence was monitored with time in the absence (black) or presence of ClpXP, treated at 50 °C (red) or 30 °C (aqua).