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Cytoskeletal proteins in *Caulobacter crescentus*: spatial orchestrators of cell cycle progression, development, and cell shape

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Abstract

Caulobacter crescentus, an aquatic Gram-negative α -proteobacterium, is dimorphic, as a result of asymmetric cell divisions that give rise to a free-swimming swarmer daughter cell and a stationary stalked daughter. Cell polarity of vibrioid *C. crescentus* cells is marked by the presence of a stalk at one end in the stationary form and a polar flagellum in the motile form. Progression through the cell cycle and execution of the associated morphogenetic events are tightly controlled through regulation of the abundance and activity of key proteins. In synergy with the regulation of protein abundance or activity, cytoskeletal elements are key contributors to cell cycle progression through spatial regulation of developmental processes. These include: polarity establishment and maintenance, DNA segregation, cytokinesis, and cell elongation. Cytoskeletal proteins in *C. crescentus* are additionally required to maintain its rod shape, curvature, and pole morphology. In this chapter, we explore the mechanisms through which cytoskeletal proteins in *C. crescentus* orchestrate developmental processes by acting as scaffolds for protein recruitment, generating force, and/or restricting or directing the motion of molecular machines. We discuss each cytoskeletal element in turn, beginning with those important for organization of molecules at the cell poles and chromosome segregation, then cytokinesis, and finally cell shape.

1. The *Caulobacter crescentus* life cycle

Caulobacter crescentus is an aquatic, Gram-negative α -proteobacterium with a curved rod morphology that undergoes cell cycle-regulated morphogenesis and an obligate asymmetric cell division (Stove & Stanier 1962; Shapiro et al. 1971). Its dimorphic life cycle and overt cell polarity, along with readily available genetic tools and easy methods of synchronization make *C. crescentus* an excellent model organism for studying cell cycle-regulated processes and the role of the cytoskeleton in directing these events.

C. crescentus has two distinct cell types resulting from asymmetric cell division –a motile daughter or “swarmer” cell that possesses a polar flagellum and adhesive pili, and a sessile daughter or “stalked” cell that bears a slender, polar extension of the cell body called the stalk (Fig. 1) (Stove & Stanier 1962). An adhesive polysaccharide-rich matrix (holdfast) is secreted at the end of the stalk, and serves to attach the stalked cell to surfaces in the aquatic environments *C. crescentus* inhabits (Curtis & Brun 2010). Though the precise function of the stalk is debated, it likely serves to maximize nutrient access and/or uptake when *C.*

C. crescentus is growing within a community in nutrient-poor aquatic environments (Klein et al. 2013). The flagellum and pili in swarmer and the stalk in stalked cells serve as morphological markers of cell polarity, and their polar locations are maintained from generation to generation.

Under laboratory growth conditions, the swarmer cell, which is non-replicating, differentiates into a stalked cell shortly after birth (Fig. 1). In nutrient-limited conditions in the wild, the swarmer cell stage is prolonged until a signal is received that there are enough nutrients available to produce progeny (Lesley & Shapiro 2008; England et al. 2010; Britos et al. 2011; Boutte et al. 2012). The swarmer-to-stalked cell differentiation is characterized by morphological changes including elongation of the cell, ejection of the flagellum, retraction of the pili, and biogenesis of a stalk at the pole where the flagellum previously resided (Jenal 2000; Curtis & Brun 2010).

Accompanying the morphological changes associated with the swarmer-to-stalked transition, the stalked cell initiates replication and segregation of its single, circular chromosome. The chromosome is precisely positioned in *C. crescentus*, with each chromosomal locus localizing to a particular cellular address (Viollier et al. 2004). Notably, the chromosomal centromere (*parS*, a locus close to the origin of replication (*ori*)) is anchored at the old cell pole and the terminus (*ter*) is found near the new cell pole. Upon duplication, one copy of the centromere is rapidly segregated to the opposite pole and other loci follow into the opposite daughter compartment as they are replicated. The anchoring of the centromeres to opposite poles after segregation leads to an *ori-ter-ori* orientation of the chromosome in predivisional cells (Viollier et al. 2004). Following DNA segregation, a flagellum is built at the pole opposite the stalk, the envelope begins to constrict near midcell, and cytokinesis yields biochemically and morphologically distinct stalked and swarmer daughters (Fig. 1). The stalked cell immediately enters another round of division, while the swarmer first must differentiate to become a stalked cell.

Progression of *C. crescentus* through the cell cycle and execution of the associated morphogenetic events described above are tightly controlled through regulation of the abundance, activity, and localization of key proteins. Interconnected transcriptional circuits lead to sequential, cell cycle-dependent expression of over 500 genes (Laub et al. 2000; Zhou et al. 2015). Layered on top of transcriptional regulation, post-transcriptional and post-translational mechanisms, for example regulated proteolysis and phosphorylation, are in place to increase the robustness of the cell cycle program (Schrader et al. 2014; Zhou et al. 2015; Goley et al. 2007; Jenal 2009). In synergy with the regulation of protein abundance or activity, cytoskeletal elements are key contributors to cell cycle progression through spatial regulation of key developmental processes. These include: polarity establishment and maintenance, DNA segregation, cytokinesis, and cell elongation. Cytoskeletal proteins in *C. crescentus* are additionally required to maintain its rod shape, curvature, and pole morphology. In this chapter, we explore the mechanisms through which cytoskeletal proteins in *C. crescentus* orchestrate developmental processes by acting as scaffolds for protein recruitment, generating force, and/or restricting or directing the motion of molecular machines. We will discuss each cytoskeletal element in turn, beginning with those important

for organization of molecules at the cell poles and chromosome segregation, then cytokinesis, and finally cell shape.

PopZ: centromere anchoring and polar organization

In *C. crescentus*, the cell poles serve as sites of assembly for molecules and activities involved in cell cycle progression and development, including the chromosomal centromere, proteins required for stalk and flagellar biosynthesis, and factors involved in the specification of stalked or swarmer cell fate. PopZ (Pole-organizing protein that affects FtsZ or Polar organizing protein Z) is a polar cytoskeletal protein that is conserved in nearly all α -proteobacteria and plays a key role in recruiting diverse proteins to the cell pole(s).

PopZ was first identified in *C. crescentus* as a polymerizing protein important for polar anchoring of the chromosomal centromere by two independent methods: a bioinformatic screen for proteins that are predicted to interact functionally with known polar proteins (Bowman et al. 2008) and a genetic screen for genes that cause cell division defects on overexpression (Ebersbach et al. 2008). It is a small acidic protein (177 amino acids in *C. crescentus*, pI = 3.88) that forms a scaffold for the recruitment of factors involved in stalk morphogenesis and centromere segregation and anchoring. While it is non-essential, deletion of *popZ* leads to cell elongation, minicell formation and prevention of stalk synthesis (Fig. 3B) (Bowman et al. 2008; Ebersbach et al. 2008).

PopZ localization and function in cells

The first indication for PopZ's role in polar organization came from investigating its localization over the cell cycle (Fig. 1, 3I) (Bowman et al. 2008; Ebersbach et al. 2008). PopZ – visualized as a functional fluorescent fusion to YFP – forms a unipolar focus at the old pole in newly divided cells (Fig. 3I.1). As chromosome replication is initiated and centromere segregation proceeds, PopZ transitions from a unipolar focus to bipolar foci (Fig. 3I.2). PopZ oligomerizes and forms a matrix that excludes large macromolecules including the nucleoid and ribosomes. This matrix can be seen by phase contrast microscopy as regions of low refractive index and by electron cryotomography (ECT) as ribosome-free zones (Fig. 4A) (Ebersbach et al. 2008; Bowman et al. 2010). The PopZ matrix has been proposed to function as a multivalent platform for the recruitment and/or retention of specific proteins at the cell pole(s) (Bowman et al. 2008; Ebersbach et al. 2008; Bowman et al. 2010).

A critical function of PopZ is in regulating the localization of the chromosomal centromere (*parS*) through its interactions with the ParA/ParB chromosome segregation machinery (Bowman et al. 2008; Ebersbach et al. 2008; Bowman et al. 2010; Ptacin et al. 2014). At the old pole of newborn cells, the PopZ matrix binds to ParB, which binds to *parS*, thereby anchoring the *parS*-proximal regions of the chromosome (including the *ori*) close to the old pole. Following replication of the *parS* locus, one of the two ParB-*parS* complexes moves towards the new cell pole where it is captured by a newly assembled PopZ matrix (Fig. 1). Thus, the polar PopZ matrices anchor the duplicated centromeres at the poles and stabilize the *ori-ter-ori* orientation of the replicating chromosome. *In vitro* observations and heterologous expression studies indicate that PopZ can directly bind free ParB or ParB

bound to *parS*, suggesting that PopZ and ParB are sufficient for centromere anchoring (Bowman et al. 2008; Ebersbach et al. 2008). In addition to anchoring the centromere, PopZ further contributes to chromosome organization by regulating the localization of ParA – the ATPase that directs migration of one copy of ParB-*parS* to the new pole (Bowman et al. 2008; Ebersbach et al. 2008; Bowman et al. 2010; Ptacin et al. 2014). Regulation of ParA localization by PopZ is described in the section on ParA.

Upon the swarmer-to-stalked cell transition, PopZ at the old pole undergoes an apparent change in function (Ebersbach et al. 2008; Bowman et al. 2010). Roughly coincident with the initiation of DNA replication, PopZ releases the ParB-*parS* complex at the old pole and begins to recruit factors important for stalk morphogenesis and other developmental events at the old pole (Fig. 1). SpmX, a periplasmic muramidase that is the earliest recruit to the developing stalked pole, is localized to the old pole in a PopZ-dependent manner. SpmX recruitment is presumed to be indirect: the muramidase domain of SpmX, which is thought to reside in the periplasm, is sufficient for its localization, whereas PopZ is exclusively cytoplasmic (Bowman et al. 2010). Following localization of SpmX at the pole, PopZ recruits histidine kinases, DivJ and CckA, which regulate cell fate and stalk synthesis, and CpdR, ClpX, and RcdA, factors required for the regulated proteolysis of the master cell cycle regulator, CtrA (Ebersbach et al. 2008; Bowman et al. 2010). Co-immunoprecipitation experiments indicate that PopZ directly interacts with DivJ and CckA, further supporting PopZ's role as a scaffold for the localization of these proteins (Ebersbach et al. 2008; Bowman et al. 2010). PopZ could recruit the other factors indirectly through its interaction with DivJ, CckA or other, as yet unknown, proteins.

Assembly properties of PopZ

In vitro, PopZ forms large oligomers as evidenced by its behavior in native polyacrylamide gel electrophoresis (PAGE) and size exclusion chromatography. Although PopZ is a 17 kDa protein, it migrates in complexes as large as 650 kDa in a native PAGE (Bowman et al. 2008; Ebersbach et al. 2008). Size exclusion chromatography studies of PopZ mutants indicate that PopZ forms dimers of trimers, which then associate to form higher order structures (Bowman et al. 2013). Electron micrographs of purified PopZ show that PopZ polymers are 5 nm in diameter and vary widely in length – ranging from short filaments of 25 – 50 nm to long, branched filaments that are over 200 nm in length (Fig. 5A) (Bowman et al. 2008). Mutants of PopZ that are defective in forming higher order structures do not form a polar focus *in vivo* and are incapable of bipolar localization, suggesting that the ability to polymerize is required for PopZ localization (Laloux & Jacobs-Wagner 2013; Bowman et al. 2013).

PopZ is dissimilar in sequence and structure to other known polymerizing proteins, thus it establishes a new class of cytoskeletal proteins. Sequence comparison of PopZ homologs across α -proteobacterial species reveals that PopZ has 3 main regions – (i) an N-terminal region containing an α -helix (amino acids 1–26 in *C. crescentus* PopZ), (ii) a proline-rich linker region (amino acids 26 – 106) and (iii) an α -helix-rich C-terminal region (amino acids 106 – 177) (Laloux & Jacobs-Wagner 2013; Bowman et al. 2013). The N-terminal and C-terminal regions have distinct functions in regulating the assembly and polar localization of

PopZ. The C-terminal α -helices are required for polar localization of PopZ, as mutants of PopZ that do not contain the C-terminal region fail to self-assemble and are diffuse throughout the cell (Laloux & Jacobs-Wagner 2013; Bowman et al. 2013). Size exclusion chromatography, multi-angle laser light scattering and native PAGE analyses indicate that the C-terminal region is sufficient for oligomerization (Bowman et al. 2013). This is further supported by observations in *C. crescentus* and upon heterologous expression in *E. coli* that fluorescent fusions to the C-terminal region of PopZ localize as a unipolar focus (Bowman et al. 2013).

While the C-terminal region is sufficient for polymerization of PopZ and its localization at the old pole in *C. crescentus*, PopZ requires its N-terminal α -helix for the transition from a unipolar focus to bipolar foci and for directing stalk biogenesis (Laloux & Jacobs-Wagner 2013). Mutations in the N-terminus lead to reduced frequency of bipolar PopZ foci, cell elongation and poor SpmX localization (Laloux & Jacobs-Wagner 2013). Moreover, isolation of N-terminal mutants of PopZ that prevent its interaction with ParA *in vitro* but do not affect PopZ bipolar localization *in vivo* suggests that the N-terminus is also required for binding ParA (Ptacin et al. 2014). Cells expressing these mutants have delayed or incomplete migration of the ParB-*parS* complex, emphasizing the need for PopZ-ParA interaction for efficient chromosome segregation (see ParA section for more detail).

PopZ: unanswered questions

While the details of PopZ's function in regulating chromosome organization and polar development are beginning to become clear, how PopZ localizes to the poles in the first place has not yet been explained. PopZ does not rely on any other known polar protein for its localization and can localize to polar foci when expressed in *E. coli*, which lacks a PopZ homolog, suggesting that polar assembly is an intrinsic property of PopZ (Bowman et al. 2008; Ebersbach et al. 2008). One model argues that PopZ localizes to any nucleoid free region – which is the new pole in the case of swimmers – unless there is a pre-existing PopZ focus. The strongest evidence for this model comes from heterologous expression studies in *E. coli*. PopZ-YFP localizes as a single focus in *E. coli*, irrespective of expression levels (Bowman et al. 2008; Ebersbach et al. 2008; Laloux & Jacobs-Wagner 2013). Moreover, heterologous expression of *popZ-yfp* in *E. coli* following treatment with cephalixin, a cell division inhibitor, leads to PopZ foci between nucleoids (Laloux & Jacobs-Wagner 2013). It therefore seems likely that the nucleoid physically excludes the large PopZ matrix, and vice versa, leading to PopZ assembly only in nucleoid-free regions. Whether these observations in *E. coli* are sufficient to explain polar PopZ localization in *C. crescentus* cells, which are smaller than *E. coli* and in which the nucleoid fills a larger portion of the cytoplasm, is unclear. Moreover, it is possible that *C. crescentus* has additional factors that assist in polar localization of the PopZ matrix. Inhibition of the MreB cytoskeleton or loss of the polar marker TipN, for example, each disrupts the normal distribution of PopZ (Bowman et al. 2008; Laloux & Jacobs-Wagner 2013). However, it has not been established if their effects on PopZ are direct.

Another unanswered question is how PopZ forms a new matrix at the new pole after DNA replication initiation. Unlike ParB and MipZ, which transition from unipolar to bipolar

distribution by moving across the cell with the segregating centromere, PopZ forms a matrix at the new pole *de novo*, without disassembly or movement of the old matrix (Bowman et al. 2008; Ebersbach et al. 2008). PopZ overproduction in both *C. crescentus* and *E. coli* leads to expansion of a single PopZ matrix at one pole (Ebersbach et al. 2008; Laloux & Jacobs-Wagner 2013), therefore changes in PopZ levels cannot explain its appearance at the new pole. Initiation of DNA replication was originally suggested to be a signal for the formation of the new PopZ matrix (Bowman et al. 2008; Ebersbach et al. 2008). However, more recent evidence from cells treated with the replication inhibitor novobiocin suggests that it is the segregation of chromosome, specifically ParA-dependent migration of the ParB-*parS* complex and not replication itself, that is required for the bipolarization of PopZ (Laloux & Jacobs-Wagner 2013). It is estimated that 40% of total PopZ is not in a polar matrix, but rather is diffuse in the cytoplasm (Bowman et al. 2008; Laloux & Jacobs-Wagner 2013). It is possible that ParA, bound to TipN at the new pole, recruits cytoplasmic PopZ and increases its local concentration such that PopZ can form a new matrix (Laloux & Jacobs-Wagner 2013). How this series of interactions is triggered only upon centromere segregation remains to be determined.

ParA and the chromosome segregation machinery

The *parABS* locus encodes a tripartite DNA partitioning system that functions in diverse bacteria to segregate plasmid or chromosomal DNA. It comprises a *cis*-acting DNA sequence, *parS*, and two proteins: ParB, which binds *parS* and *parS*-proximal sites along the DNA, and ParA, a Walker-type ATPase required for directed movement of ParB-*parS* complexes. As discussed below, there is conflicting data as to whether ParA assembles into cytoskeletal filaments or, alternatively, forms a nucleoprotein complex made of DNA-bound ParA dimers to promote DNA segregation (Howard & Gerdes 2010; Mierzejewska & Jagura-Burdzy 2012). We include ParA in our discussion of cytoskeletal proteins in *C. crescentus*, as one of the models proposed for chromosome segregation invokes ParA assembly into filaments.

ParABS: localization and function in cells

ParA and ParB were first identified as proteins required for plasmid segregation in *E. coli* (van den Elzen et al. 1983). Since then, numerous studies have demonstrated their roles in chromosome segregation in diverse bacteria (Mohl & Gober 1997; H. J. Kim et al. 2000; Mohl et al. 2001; Godfrin-Estevenson et al. 2002; Fogel & Waldor 2006; Saint-Dic et al. 2006; Jakimowicz et al. 2007; Toro et al. 2008; Bartosik et al. 2009; Donovan et al. 2010; Iniesta 2014; Mierzejewska & Jagura-Burdzy 2012). *parA* and *parB* are essential in *C. crescentus* (Mohl & Gober 1997), and ParB depletion causes DNA segregation defects and cell elongation (Easter & Gober 2002).

ParB dimers bind specific AT-rich sites near the chromosomal origin of replication (*ori*), known as *parS*, through a conserved helix-turn-helix motif (Toro et al. 2008; Figge et al. 2003). In pre-divisional cells, ParB bound to *parS* forms a polar focus that partially overlaps with PopZ at the old pole (Fig. 1) (Mohl & Gober 1997; Bowman et al. 2008; Ebersbach et al. 2008). Following initiation of replication, *parS* is one of the first loci to be duplicated, as

it lies only 8 kb away from *ori. parS* functions as the chromosomal centromere and is invariably the first locus to be segregated (Toro et al. 2008). *parS* duplication leads to formation of two ParB-*parS* complexes. One ParB-*parS* focus migrates rapidly to the new pole, while the other focus is retained near the old pole (Mohl & Gober 1997; Viollier et al. 2004; Bowman et al. 2008; Toro et al. 2008; Shebelut et al. 2010). ParA is required for the completion of directed movement of the ParB-*parS* focus to the new pole (Mohl & Gober 1997; Toro et al. 2008).

ATP-bound ParA dimers bind sequence non-specifically to the nucleoid (Easter & Gober 2002) and may also polymerize (Ptacin et al. 2010). In swarmer cells imaged by widefield fluorescence microscopy, ParA forms a dynamic, cloud-like localization, with its highest concentration at the new pole opposite the ParB-*parS* complex (Fig. 3J) (Schofield et al. 2010). As DNA replication progresses, one of the two ParB-*parS* complexes comes in contact with the ParA cloud and is translocated rapidly across the cell to the new pole (Shebelut et al. 2010). ParB activates the ATPase activity of ParA and converts ParA-ATP – bound to DNA and/or assembled as a polymer – to free ParA-ADP monomers (Easter & Gober 2002; Figge et al. 2003). ParB, due to its affinity for ParA-ATP, moves up the concentration gradient of ParA towards the new pole (Toro et al. 2008; Ptacin et al. 2010; Shebelut et al. 2010). Thus, a tight coordination between ATPase activity, dimerization, and differential DNA binding affinities of the ParAB system drives chromosome segregation.

Mechanism of ParABS-mediated chromosome segregation

While it is clear that ParA is required for directing the centromere to the new pole, whether ParA generates a pulling force on the centromere, like microtubules in a eukaryotic spindle, or if ParB follows the retracting ParA cloud through directed diffusion is unclear. Based on existing data, there are two models for ParA function and DNA translocation in *C. crescentus*. In the first model, ATP-bound ParA is proposed to form a linear, bundled polymer that is anchored at the new pole (Ptacin et al. 2010; Banigan et al. 2011). As the ParB-*parS* complex binds this filament at its unanchored end, it activates the ATPase activity of ParA and leads to depolymerization of the ParA filament. As ParA depolymerizes, it pulls ParB-*parS* along due to the affinity of ParB for ATP-bound ParA. Thus, ParABS system forms a “burning bridge” that helps translocate *parS*.

Evidence for this ParA depolymerization-based burning bridge model comes from the observation that ParA can form bundled filaments *in vitro* (Ptacin et al. 2010), and that ParA from other species have been observed to form filaments (Leonard et al. 2005; Ebersbach et al. 2006; Ringgaard et al. 2009). However, there is limited evidence for such a filament structure *in vivo*. While early super-resolution images of ParA-eYFP suggested a ~40 nm thick linear filament of ParA that stretches from the new pole to the ParB focus (Ptacin et al. 2010), more recent super-resolution microscopy of ParA-Dendra2 revealed a cloud-like localization that is distinct from a structured filament (Lim et al. 2014). Moreover, an estimation of ParA concentration in cells suggests that there are far fewer ParA molecules than would be required to form a linear polymer that can stretch across the cell from the new pole to where ParB first interacts with ParA (Lim et al. 2014). Furthermore, single molecule

tracking of the ParB focus revealed that ParB-*parS* complex does not follow a linear path, as would be predicted for a burning bridge model (Lim et al. 2014).

For the above reasons, a diffusion-based “DNA-relay” model has been proposed wherein ParA-ATP dimers on the DNA form a cloud-like nucleoprotein complex that drives the motion of ParB-*parS* through directed diffusion coupled with a Brownian ratchet (Lim et al. 2014). Observations of the movement of chromosomal loci suggest that these loci are in constant oscillations about their equilibrium point. Thus, ParA dimers bound to DNA also undergo oscillation. When the ParB-*parS* complex binds a DNA-bound ParA dimer out of its equilibrium point, the elastic nature of the chromosome brings ParA/ParB-*parS* complex closer to the equilibrium point. Simultaneously, ParB stimulates ParA’s ATPase activity leading to release of ParA monomers from the chromosome. As this cycle of ParB binding to ParA dimers and their subsequent removal repeats, the centromere moves to the new pole following the DNA bound ParA-dimer concentration gradient.

Ensuring robust chromosome segregation: ParABS and beyond

Maintaining a concentration gradient of DNA-bound ParA that increases towards the new pole is central to efficient segregation through the proposed DNA relay mechanism. Interaction of ParA with PopZ and the new pole marker TipN are required to establish this gradient (Ptacin et al. 2010; Schofield et al. 2010; Ptacin et al. 2014) (Fig. 1). Heterologous expression of PopZ and ParA in *E. coli* suggests that PopZ interacts directly with ParA (Laloux & Jacobs-Wagner 2013). Moreover, in otherwise wild-type *C. crescentus* cells, a DNA binding mutant of ParA (ParA_{G16V}) localizes specifically as bipolar foci in complex with the PopZ matrix, whereas in *popZ* cells, this mutant is distributed throughout the cytoplasm with no apparent concentration gradient (Ptacin et al. 2014). These observations illustrate that binding to both PopZ and DNA are required to maintain the ParA gradient.

While necessary, the PopZ-ParA interaction is not sufficient to maintain the ParA gradient with its highest concentration near the new cell pole, as PopZ is enriched at the old pole or both poles depending on cell cycle status. For this, TipN, which is localized at the new cell pole, is required. In *tipN* cells, the ParA concentration gradient redistributes to a cloud enriched at both poles, leading to inefficient ParB-*parS* migration (Ptacin et al. 2010; Schofield et al. 2010). Moreover, whereas the DNA-binding mutant ParA_{G16V} forms bipolar foci in otherwise wild-type cells, it localizes to the old pole in *tipN* cells. In addition, ParA and TipN have been shown to interact directly by FRET and *in vitro* pull down experiments (Schofield et al. 2010). From these observations, TipN has been suggested to restrict the ParA concentration maximum to the new pole, possibly by titrating away free ParA monomers that result from ParB-activated ATP hydrolysis by ParA and subsequent release of ParA from the DNA (Schofield et al. 2010). Simultaneous deletion of *popZ* and *tipN* is synthetic lethal, further supporting the cooperative contributions of these factors to regulating ParA localization and function.

While it is clear that ParA is required for efficient centromere migration, there appear to be other compensatory mechanisms for chromosome segregation in the absence of a functional ParA. These include MreB-driven cell elongation (Shebelut et al. 2009; Shebelut et al. 2010; Schofield et al. 2010) and bulk segregation mechanisms mediated by the motor activities

associated with DNA replication and transcription (Shebelut et al. 2010). In fact, ParA is required only for the late stages of centromere translocation (Shebelut et al. 2010). Nevertheless, cells lacking a functional ParABS system are not viable and have severe cell division defects. The cytokinesis defects associated with *parABS* mutants are attributed to the mislocalization of MipZ, a ParB-associated factor that coordinates DNA segregation with assembly of the cytokinetic FtsZ ring (Thanbichler & Shapiro 2006).

FtsZ: the orchestrator of cytokinesis

Shortly after segregation of the chromosomal centromere, the multi-protein machinery that will ultimately divide the cell begins to assemble near midcell (Thanbichler & Shapiro 2006). At the core of this machinery is FtsZ, a tubulin-like GTPase that is conserved in most bacteria with a peptidoglycan cell wall. It polymerizes on binding GTP and forms a ring like structure at the division site (Z-ring) that serves to coordinate invagination of the cell envelope during cytokinesis (Erickson et al. 2010; Meier & Goley 2014). In the absence of FtsZ, cells fail to divide, elongate, and eventually lyse (Fig. 3D). FtsZ is proposed to act as a scaffold for the recruitment of the division machinery (divisome), and as a potential source of constrictive force for envelope invagination. In addition to its role in cell division, *C. crescentus* FtsZ also directs elongation of the cell prior to initiating constriction of the envelope for cytokinesis (Aaron et al. 2007). The conserved features of FtsZ assembly and function are discussed elsewhere in this book. Here, we focus on the characteristics of FtsZ that have been specifically observed for *C. crescentus*.

FtsZ domain architecture and function

Based on sequence homology across species, FtsZ is divided into four regions – (i) a poorly conserved N-terminal peptide of unknown function (ii), a GTPase domain that adopts the tubulin fold, (iii) an intrinsically disordered C-terminal linker (CTL), and (iv) a C-terminal conserved (CTC) peptide (Vaughan et al. 2004). The GTPase domain is highly conserved and is sufficient for polymerization of FtsZ *in vitro* (X. Wang et al. 1997; Din et al. 1998; Vaughan et al. 2004). The CTC is the binding site for several essential regulators of FtsZ function, including membrane-anchoring proteins and the ClpXP protease (Ma et al. 1997; Din et al. 1998; Ma & Margolin 1999; Król et al. 2012; Duman et al. 2013; Williams et al. 2014). The disordered CTL is the least conserved region of FtsZ: it is highly variable in length and sequence across species and is particularly long (100–400 residues) in α -proteobacteria (Vaughan et al. 2004; Margolin 2005). Despite its poor conservation, the CTL plays an essential, length- and disorder-dependent, but sequence-independent function in *E. coli* and *B. subtilis* (Buske & Levin 2013; Gardner et al. 2013). In *C. crescentus*, however, the linker plays both sequence- and length-dependent roles in executing cytokinesis (Sundararajan et al. 2015). The mechanisms by which the length and sequence of the CTL contribute to efficient cytokinesis are not yet clear, although both of these features affect steady state FtsZ levels and Z-ring structure (Sundararajan et al. 2015).

Z-ring structure *in vivo*

Various studies have characterized the structure and dynamics of the Z-ring *in vivo* as a means to understand its function both as a scaffold and as a potential force generator. ECT

and polarized fluorescence microscopy imaging of *C. crescentus* cells revealed relatively sparse, single-stranded FtsZ protofilaments oriented perpendicular to the long axis of the cell (Fig. 4B) (Z. Li et al. 2007; Si et al. 2013; Szwedziak et al. 2014). On average, protofilaments are positioned 16 nm from the inner membrane (Z. Li et al. 2007; Szwedziak et al. 2014). Whether these filaments assemble into a continuous ring, however, is currently subject to debate (Fig. 3K, 4B) (Z. Li et al. 2007; Holden et al. 2014; Szwedziak et al. 2014). An early ECT study and later super-resolution light microscopy favor a model of FtsZ protofilaments organized into discontinuous clusters (Z. Li et al. 2007; Holden et al. 2014), but a more recent ECT study revealed protofilaments that appear to completely encircle the cell at its waist (Szwedziak et al. 2014). The diameter of the Z-ring gradually decreases with cell envelope invagination, starting at ~500 nm and decreasing to ~350 nm at a constant rate of 0.8 nm/min (Holden et al. 2014). This gradual constriction of the Z-ring then switches to a rapid constriction stage, during which the diameter decreases to ~150 nm towards the end of cytokinesis (Holden et al. 2014).

FtsZ function over the cell cycle

Following DNA replication initiation in stalked cells, FtsZ begins to assemble approximately at the middle of the cell into a circumferential Z-ring (Fig. 2) (Quardokus et al. 2001; Thanbichler & Shapiro 2006). The assembly of the Z-ring triggers the sequential recruitment of more than two dozen factors that function together to effect cytokinesis and other FtsZ-dependent events (Goley et al. 2011). These factors include (i) FtsZ-binding proteins that regulate Z-ring structure, (ii) proteins that anchor FtsZ to the membrane, (iii) factors that stabilize the division machinery (divisome), (iv) peptidoglycan remodeling enzymes, (v) DNA translocation factors, (vi) outer membrane proteins, and (vii) pole morphogenesis factors. Although all of these proteins require FtsZ for their midcell localization, only a subset – FtsA, FzlA, FzlC, ZapA, FtsE, KidO, GdhZ, and ClpX – interact directly with FtsZ (Ma et al. 1997; X. Wang et al. 1997; Din et al. 1998; Goley et al. 2010; Radhakrishnan et al. 2010; Beaufay et al. 2015). FtsZ and the divisome together cause local remodeling of the cell envelope which results in cytokinesis (Meier & Goley 2014). While it is clear that FtsZ acts as a scaffold for the local assembly of the divisome, recent evidence suggests that FtsZ also contributes to the regulation of peptidoglycan enzyme activities through a CTL-dependent mechanism beyond its scaffolding function (Sundararajan et al. 2015).

Surprisingly, FtsZ directs a significant portion of cell wall synthesis for cell elongation in *C. crescentus* (Aaron et al. 2007; Kuru et al. 2012). Before the initiation of constriction, FtsZ recruits MreB, the actin homolog important for directing elongation-mode cell wall synthesis, and MurG, a critical component of the peptidoglycan precursor synthesis pathway, and directs longitudinal peptidoglycan synthesis at the midcell (Aaron et al. 2007; Goley et al. 2011). Midcell elongation continues until the arrival of the late divisome proteins FtsW and FtsB, after which peptidoglycan remodeling switches from an elongation mode to a division mode (Aaron et al. 2007; Goley et al. 2011).

Force generation and FtsZ filament curvature

In vitro observations of *E. coli* FtsZ polymers reconstituted on liposomes have suggested that, in addition to acting as a scaffold, FtsZ is capable of generating force to deform

membranes (Osawa et al. 2008; Osawa et al. 2009). A curvature-based force generation mechanism has been proposed based on several observations. First, electron microscopy of FtsZ protofilaments indicates that FtsZ filaments become more curved upon GTP hydrolysis (Lu et al. 2000) and GTP hydrolysis mutants of FtsZ fail to complete cytokinesis (Y. Wang et al. 2001; Stricker & Erickson 2003). In addition, the geometry of membrane deformation by FtsZ *in vitro* depends on the orientation of the membrane anchor relative to the direction of filament curvature (Osawa et al. 2009; Osawa & Erickson 2011). Based on these observations and the presence of a discontinuous Z-ring, an “iterative pinching” model of FtsZ function has been proposed (Z. Li et al. 2007; Erickson et al. 2010; Y. Li et al. 2013; Holden et al. 2014). According to this model, membrane-anchored, curved FtsZ filaments cause local deformation of the membrane that is coupled with peptidoglycan remodeling. Repeated rounds of FtsZ filament assembly, membrane deformation, and peptidoglycan remodeling at the division site lead to coordinated cell envelope invagination.

Regulating the curvature of FtsZ filaments is central to the proposed “iterative pinching model”. Interestingly, in *C. crescentus*, FzlA, a glutathione S-transferase family protein that is conserved in α -proteobacteria, binds directly to FtsZ and induces formation of highly curved, stable filament bundles (Goley et al. 2010). FzlA, an early recruit to the division site, is the only known FtsZ-binding factor that affects filament curvature and is essential for cytokinesis. Depletion of FzlA results in smooth elongated cells with no envelope invaginations, whereas overexpression of FzlA results in aberrant assembly of FtsZ as a tight focus instead of a ring-like structure (Goley et al. 2010). It has been proposed that FzlA-mediated curved filaments contribute to FtsZ-mediated force generation in *C. crescentus* and its relatives.

Cell cycle regulation of FtsZ levels

In *C. crescentus*, assembly of the Z-ring is temporally regulated, in part, through transcriptional and post-translational regulation of FtsZ levels (Quardokus et al. 1996; Kelly et al. 1998; Quardokus et al. 2001; Williams et al. 2014). FtsZ levels are lowest in swarmer cells (Quardokus et al. 1996; Kelly et al. 1998; Quardokus et al. 2001). As swarmer mature to stalked cells, FtsZ is upregulated to approximately 1000 molecules/cell. This number further increases following DNA replication initiation to ~6400 molecules/cell and finally reaches a maximum of ~7600 molecules during active envelope constriction in pre-divisional cells. Just before the completion of cytokinesis, FtsZ levels drop back down to ~1700 molecules (Quardokus et al. 2001). This cycling of FtsZ levels is achieved by the cell cycle-controlled transcription of *ftsZ* through the regulated activity of three cell cycle master regulators: CtrA, which represses *ftsZ* transcription (Kelly et al. 1998); DnaA, which activates *ftsZ* transcription (Hottes et al. 2005); and CcrM, which regulates the methylation state and activity of the *ftsZ* promoter (Gonzalez & Collier 2013). In addition, FtsZ has a shorter half-life in swarmer cells than in stalked cells, and is degraded by the ClpXP and ClpAP protease complexes (Quardokus et al. 1996; Kelly et al. 1998; Quardokus et al. 2001; Williams et al. 2014). Notably, ClpX recognizes a degradation signal on the C-terminal helix of FtsZ and is recruited to the division plane towards the end of cytokinesis to promote FtsZ turnover (Williams et al. 2014).

Positioning of the Z-ring in coordination with DNA segregation

In addition to the regulation of cytokinesis through alteration of FtsZ levels, the timing and location of Z-ring assembly in *C. crescentus* is coordinated with segregation of the chromosomal centromere through the action of a negative regulator of FtsZ assembly, MipZ (Fig. 1,2) (Quardokus et al. 2001; Quardokus & Brun 2002; Thanbichler & Shapiro 2006; Kiekebusch et al. 2012). MipZ is a Walker-type ATPase that binds FtsZ and promotes disassembly of FtsZ filaments by increasing its GTP hydrolysis activity (Thanbichler & Shapiro 2006). MipZ interacts with the ParB-*parS* complex and with DNA, depending on its nucleotide-bound and dimerization state, forming a concentration gradient that is highest at the cell poles and lowest at midcell following *parS* segregation (Thanbichler & Shapiro 2006; Kiekebusch et al. 2012). This gradient establishes a region of low MipZ concentration at midcell where FtsZ can polymerize, thereby determining the position of the Z-ring and the future division site (Thanbichler & Shapiro 2006; Kiekebusch et al. 2012). MipZ is the only known spatial regulator of FtsZ localization in *C. crescentus*. Unlike *E. coli* and *B. subtilis*, nucleoid occlusion of Z-ring assembly has not been observed in *C. crescentus* and, in fact, Z-rings readily form over regions of high DNA concentration (Thanbichler & Shapiro 2006).

Another connection between FtsZ and DNA segregation exists through FtsK, a component of the divisome that translocates the chromosomal terminus (*ter*) (S. C. E. Wang et al. 2006). FtsK is required for *ter* partitioning and it is recruited to the Z-ring before the beginning of invagination (S. C. E. Wang et al. 2006; Goley et al. 2011). Depletion of FtsK results in formation of multiple Z-rings and disruption of division (S. C. E. Wang et al. 2006). Based on its association with the nucleoid, and its effect on the Z-ring, FtsK may play a role functionally analogous to nucleoid occlusion in *C. crescentus* (S. C. E. Wang et al. 2006).

Metabolic regulation of FtsZ function

Recent work in numerous organisms has demonstrated that cytokinesis must not only be regulated in a cell cycle-dependent fashion, but also in response to environmental and metabolic cues (Kirkpatrick & Viollier 2012). This is true in *C. crescentus* as well: KidO and GdhZ are metabolic enzymes implicated in the regulation of Z-ring assembly in response to metabolic state of the cell (Radhakrishnan et al. 2010; Beaufay et al. 2015). These two factors are proposed to work synergistically to prevent premature Z-ring assembly and promote Z-ring disassembly during constriction. On binding to its substrate NADH, KidO reduces lateral interaction between FtsZ filaments (Radhakrishnan et al. 2010; Beaufay et al. 2015). GdhZ, on the other hand, binds NAD⁺ and glutamate, and increases the GTP hydrolysis rate of FtsZ, thereby favoring depolymerization (Beaufay et al. 2015). As both KidO and GdhZ are regulated by the metabolic coenzyme, NAD, it is hypothesized that they function as metabolic checkpoints for cell cycle progression through FtsZ. In addition, KidO and GdhZ are upregulated towards the end of cytokinesis, and are recruited to the division site. Hence, they could be involved in the disassembly of the cytokinetic ring at the end of the cell cycle, in parallel to proteolytic degradation of FtsZ (Beaufay et al. 2015; Williams et al. 2014). The significance of this mode of regulation remains to be fully elucidated (Radhakrishnan et al. 2010; Beaufay et al. 2015).

MreB: regulating cell shape and polarity

FtsZ contributes to cell shape maintenance primarily by maintaining consistent cell length. Uniform cell width, on the other hand, is maintained by MreB, a membrane-associated bacterial homologue of actin that is present in most rod-shaped bacteria (Daniel & Errington 2003; Figge et al. 2004; Shaevitz & Gitai 2010; Harris et al. 2014). *C. crescentus* cells depleted of MreB widen, become lemon-shaped, and eventually lyse (Fig. 3E) (Figge et al. 2004; Gitai et al. 2004). Beyond its role in defining rod shape, *C. crescentus* MreB has been implicated in the regulation of chromosome segregation, polar morphogenesis, and cell curvature (Gitai et al. 2004; Wagner et al. 2005; Gitai et al. 2005; Divakaruni et al. 2007; Shebelut et al. 2009; Charbon et al. 2009; Dye et al. 2011). The assembly properties of MreB and its conserved role in maintaining rod shape are discussed elsewhere in this book. Here, we will focus on insights into MreB regulation and roles for MreB specific to cell cycle progression and development derived from studies in *C. crescentus*.

Regulation and dynamics of MreB localization

Like many proteins implicated in cell shape and polar development, the localization of MreB is dynamic over the cell cycle in *C. crescentus* (Fig. 2). In recently divided cells, MreB localizes in dynamic patches dispersed along the length of the cell that move circumferentially along the short cell axis (Fig. 2, 3L) (Figge et al. 2004; Gitai et al. 2004; Wagner et al. 2005; Gitai et al. 2005; S. Y. Kim et al. 2006; Harris et al. 2014). Following assembly of the cytokinetic FtsZ ring in early stalked cells, MreB is recruited to midcell in an FtsZ-dependent manner and is thought to help direct cell wall metabolism for elongation of the cell prior to cytokinesis (Figge et al. 2004; Gitai et al. 2004; Divakaruni et al. 2007; Goley et al. 2011). However, the localization of MreB to midcell is not required for normal growth and morphology, as MreB mutants have been isolated that fail to accumulate at midcell, yet have apparently normal cell shape (Aaron et al. 2007; Dye et al. 2011). Roughly coincident with cytoplasmic compartmentalization in late predivisional cells, MreB is dispersed from midcell and once again adopts a dynamic patchy localization (Gitai et al. 2004; Goley et al. 2011).

While FtsZ is required for midcell recruitment of MreB, it is not clear if the two interact directly in *C. crescentus*, as reported for *E. coli* (White et al. 2010; Fenton & Gerdes 2013). However, MbiA, a factor identified in *C. crescentus* and conserved in a subset of α -proteobacteria, does bind MreB directly and regulate its localization (Yakhnina & Gitai 2012). While *mbiA* is not essential, overexpression of *mbiA* causes cell shape defects that resemble the effects of MreB depletion. Surprisingly, MbiA overproduction also leads to mislocalization of MreB: in the presence of excess MbiA, MreB condenses into a thick band at midcell. This occurs even in swarmer cells in which FtsZ has not yet assembled into a ring, implying FtsZ-independent midcell localization. Overexpression of *mbiA* also promotes midcell localization of a mutant of MreB - MreB_{Q26P} - that is otherwise defective for midcell recruitment (Yakhnina & Gitai 2012). As *mbiA* is dispensable for growth in the laboratory, the physiological significance and mechanism of MbiA-dependent regulation of MreB localization and function remain to be elucidated.

MreB and *C. crescentus* morphogenesis

Investigations into the physiological functions of MreB have been facilitated by identification of small molecule inhibitors of its assembly, including A22 (Gitai et al. 2004) and its less toxic derivative, MP265 (Takacs et al. 2010). A22 was first described as a molecule affecting *E. coli* cell shape (Iwai et al. 2002), but MreB was confirmed as its primary target through the identification of mutations in *C. crescentus* MreB that confer resistance to A22 (Gitai et al. 2005; Charbon et al. 2009; Dye et al. 2011). Indeed, A22 and MP265 cause MreB to become diffusely localized *in vivo* (Gitai et al. 2004; Takacs et al. 2010) and inhibit its assembly into antiparallel double filaments *in vitro* (Fig. 5D) (van den Ent et al. 2014).

While it is clear that the conserved function of MreB is the maintenance of cell width through the regulation of cell wall metabolism, in *C. crescentus*, MreB also influences cell curvature. A subset of A22-resistant MreB mutant strains have defects in cell curvature and are straight (Dye et al. 2011). This is attributed to a role for MreB in the membrane attachment of crescentin, the curvature-defining intermediate filament-like protein discussed later in this chapter (Charbon et al. 2009; Dye et al. 2011).

A second MreB-dependent morphogenetic process specific to *C. crescentus* and its relatives is the elaboration of a polar stalk. Stalk synthesis and elongation require specialized peptidoglycan metabolism to create a compartment with a smaller diameter than the cell body. Despite their different dimensions, elongation and synthesis of the stalk, like elongation of the cell body, requires MreB (Wagner et al. 2005). In *C. crescentus* cells depleted of MreB, stalks are either short, absent, or misshapen, appearing as polar “bulbs” much wider in diameter than wild-type stalks. When MreB is added back to these cells, stalks form at ectopic poles (i.e. emanating from the sides of cells) and are frequently branched (Wagner et al. 2005). These findings suggest that stalk elongation is a modified version of cell elongation, and that MreB is important for developing stalks of the appropriate diameter and for maintaining cell polarity so that stalks are assembled in the appropriate place.

MreB and polar development

In addition to its role in stalk placement, MreB is proposed to play a general role in cell polarity, as it influences the polar localization of the chromosomal centromere and of numerous proteins involved in cell cycle progression and development (Gitai et al. 2004; Gitai et al. 2005; Bowman et al. 2008). Alterations in MreB levels cause severe defects in chromosome segregation (Gitai et al. 2004). Cells treated with A22 experience growth condition-specific defects in segregation of origin-proximal regions of the chromosome: cells grown in liquid media have delayed *parS* segregation, while those grown on solid media frequently fail to segregate *parS* entirely (Gitai et al. 2005; Shebelut et al. 2009). Moreover, chromatin immunoprecipitation experiments demonstrated physical association of MreB with multiple origin-proximal loci, including *parS* (Gitai et al. 2005). These observations initially led to the proposition that MreB forms a mitotic spindle-like segregation machine. However, the lack of a strict requirement for MreB for chromosome segregation under all tested growth conditions (Shebelut et al. 2009) and absence of a large-

scale polar MreB structure *in vivo* (S. Y. Kim et al. 2006; Swulius et al. 2011; van den Ent et al. 2014) argue against this possibility.

In addition to defects in chromosome segregation, PopZ is completely delocalized in lemon-shaped cells depleted of MreB, and its assembly at the new cell pole is delayed in cells treated with A22 (Bowman et al. 2008; Laloux & Jacobs-Wagner 2013). MreB depletion also leads to mislocalization of polar developmental regulators including DivK, DivJ, and CckA (Gitai et al. 2004). However, since PopZ provides a platform for recruitment of these factors (Ebersbach et al. 2008; Bowman et al. 2010), the effects of MreB on their localization may be indirect. Indeed, as assembly of PopZ at the new cell pole requires chromosome segregation (Laloux & Jacobs-Wagner 2013), one possibility is that inhibition of MreB causes delayed or inhibited *parS* segregation, which prevents timely bipolarization of PopZ, which in turn prevents proper recruitment of other polar proteins. In general, MreB inhibition with A22 slows cell growth and cell cycle progression, providing a possible explanation for the observed delay in chromosome segregation and accumulation of PopZ at the new cell pole (Sliusarenko et al. 2011; Laloux & Jacobs-Wagner 2013).

MreB: unanswered questions

The use of small molecule inhibitors to assess the acute effects of MreB inhibition circumvents many of the caveats associated with the large-scale cell shape changes associated with long term MreB depletion. However, questions about the mechanisms by which MreB participates in polarity establishment and morphogenesis still remain. How is the dynamic localization of MreB to and its release from midcell achieved, and for what purpose? Does MreB directly interact with crescentin to anchor it to the membrane and promote cell curvature? How does MreB help to maintain both the width of the cell and the width of the stalk, when their diameters are vastly different? And does MreB play a specific role in defining cell polarity, or are its effects on polarity secondary to its effects on cell physiology and shape?

Crescentin and cell curvature

In addition to maintaining a rod shape through the action of MreB, *C. crescentus* cells adopt a gentle curvature as a result of another cytoskeletal protein, crescentin. Encoded by *creS*, crescentin is the first and best described bacterial intermediate filament (IF) protein. It was identified in a visual screen for cell shape mutants (Ausmees et al. 2003). *creS* is not essential: *creS* mutant cells grow as straight rods, but are otherwise indistinguishable from wild-type (Fig. 3F) (Ausmees et al. 2003) suggesting that this protein evolved specifically for determining cell shape. Emphasizing its role in defining cell shape, overexpression of *creS* causes hypercurvature of cells (Cabeen et al. 2009). While deleting *creS* has no effect on fitness in the laboratory, the vibrioid morphology of wild-type *C. crescentus* enhances its ability to colonize surfaces in conditions of fluid flow (Persat et al. 2014). This enhanced surface colonization is likely to confer a fitness advantage in the wild, where *C. crescentus* populates surfaces in aquatic environments such as streams and lakes.

Crescentin localization and assembly properties

Crescentin forms a membrane-proximal, filamentous structure along the inner curvature of the cell when visualized by immunofluorescence microscopy or by imaging fluorescently-tagged crescentin variants (Fig. 2, 3M) (Ausmees et al. 2003; Cabeen et al. 2009). As the cell cycle progresses, the crescentin structure elongates, then separates into two at the incipient division plane prior to completion of cytokinesis (Charbon et al. 2009). Several lines of evidence indicate that crescentin is membrane associated *in vivo*. In cells treated with inhibitors of the actin homolog, MreB, or with inhibitors of cell wall synthesis, the crescentin filament appears to dislodge from the inner curvature and recoil into a helical, cytoplasmic structure (Cabeen et al. 2009; Charbon et al. 2009). Similarly, an N-terminal truncation variant of crescentin (CreS_{N27}) assembles into a helical, cytoplasmic filament, indicating that membrane attachment is likely mediated by the N-terminus (Cabeen et al. 2009). Fluorescence microscopy of CreS-GFP in elongated cells revealed that crescentin helices have a uniform pitch of $1.6 \pm 0.1 \mu\text{m}$ (Ausmees et al. 2003). This is likely a stretched conformation, as the CreS_{N27} truncation mutant that is presumed to be dissociated from the membrane has a shorter pitch of $1.4 \pm 0.15 \mu\text{m}$ (Cabeen et al. 2009).

Based on sequence identity and biochemical properties, crescentin is classified as an IF protein (Ausmees et al. 2003). Like eukaryotic IF proteins, crescentin contains 4 coiled-coil regions with seven-amino-acid (heptad) repeats separated by short linkers. Also like other IF proteins, crescentin bears a conserved stutter, an interruption in the heptad repeat, in the C-terminal coiled-coil region (Ausmees et al. 2003). Solubilized crescentin spontaneously assembles into filaments *in vitro* (Ausmees et al. 2003; Esue et al. 2010; Cabeen et al. 2011). While self-assembly is largely unaffected by salt concentrations, crescentin filaments form most readily at a low pH of 6.5 (Fig. 5E). At higher pH of up to 8.5, divalent cations (e.g. 5 mM Mg^{2+}) are required to stabilize polymers (Cabeen et al. 2011). At pH 6.5, crescentin structures are typically 8 – 10 nm (single filaments) or 17 – 20 nm (paired bundles) in width, but can form thicker bundles, particularly in the presence of divalent cations (Fig. 5E) (Ausmees et al. 2003; Cabeen et al. 2011). A study of the mechanical properties of crescentin *in vitro* determined that crescentin filamentous structures are elastic and solid-like, and can recover their network elasticity after shear stress (Esue et al. 2010). Both the self-assembly and mechanical properties of crescentin are consistent with its designation as an IF protein. It is worth noting that crescentin polymers have not been observed by ECT *in vivo* except when a membrane-dissociated form of crescentin was overproduced (Cabeen et al. 2010), so the physiologically relevant structure of crescentin awaits demonstration.

Crescentin-mediated cell curvature: mechanical regulation of peptidoglycan synthesis

A growing body of evidence supports a model in which crescentin plays a mechanical role in regulating peptidoglycan synthesis to elicit a curved cell shape. First, crescentin-induced cell curvature depends on its polymerization and membrane attachment (Cabeen et al. 2009; Charbon et al. 2009; Cabeen et al. 2010; Cabeen et al. 2011). Crescentin variants that fail to polymerize under physiological pH and salt conditions *in vitro* (e.g. pH 7.5, 200 mM KCl) also fail to assemble into filaments *in vivo*, and cells producing these non-polymerizing variants are straight (Cabeen et al. 2011). Cells expressing membrane-dissociated CreS_{N27} are also straight, even though the polymerization properties of this mutant are similar to

wild-type (Cabeen et al. 2009; Cabeen et al. 2011). A mutation in the lipopolysaccharide biosynthesis pathway (*wbqL*) also causes loss of cell curvature by disrupting membrane attachment of crescentin by a yet unknown mechanism (Cabeen et al. 2010).

Second, using a dominant negative variant of crescentin, Cabeen and colleagues demonstrated that loss of cell curvature upon crescentin inactivation requires cell growth (Cabeen et al. 2009). These data argue that crescentin does not bend the cell directly, but that it modulates synthesis of the peptidoglycan cell wall in a manner that leads to cell curvature. Consistent with that hypothesis, sacculi (isolated peptidoglycan) from wild-type, *creS*, or *creS*-overexpressing cells imaged by electron microscopy have the same relative degrees of curvature as the cells from which they are isolated (Cabeen et al. 2009). Moreover, when crescentin is present, a gradient in the rates of cell wall synthesis is present across the short axis of the cell: peptidoglycan is synthesized more slowly proximal to crescentin than it is distal to crescentin (Cabeen et al. 2009). These differential rates in peptidoglycan synthesis are thought to be sufficient to lead to the cell curvature observed. It is interesting to note that heterologous expression of *creS* in *E. coli* is sufficient to induce curvature (Cabeen et al. 2009), consistent with the idea that crescentin uses conserved pathways, i.e. those involved in cell wall metabolism, to alter cell shape.

Third, given the reduced helical pitch of membrane-dissociated crescentin, the physiologically relevant, membrane attached form is likely to be constrained and, therefore, exerts a strain on the cell envelope (Cabeen et al. 2009). This strain imposed on the inner curvature of the cell is proposed to locally compress the peptidoglycan, making bond hydrolysis less favorable and thereby locally altering the rates of cell wall hydrolysis and insertion (Cabeen et al. 2009). In support of the concept that peptidoglycan synthesis can be modulated by application of force, constraining growth of otherwise straight cells (*E. coli* or *C. crescentus creS*) in a curved chamber induces curved morphology that is maintained when the cells are released (Cabeen et al. 2009). Collectively, these observations suggest that the physical constraint imposed on cells by crescentin is central to the mechanism of inducing curvature, and that it does so by modulating the kinetics of cell wall remodeling in a spatially regulated fashion. This hypothesis is upheld in a mechanochemical computational model of the influence of the crescentin filament on cell growth (Jiang & Sun 2012).

Crescentin: unanswered questions

How does crescentin assemble into functional structures *in vivo* and locally disassemble during cytokinesis? FRAP and FLIP experiments on fluorescently labeled crescentin suggest that these structures are nearly static (half-time of fluorescence recovery of 26 ± 2 min), and there is slow exchange between assembled and free crescentin in the cytoplasm (rate of loss of fluorescence recovery following photobleaching 0.5 ± 0.18 /h) (Esue et al. 2010). *De novo* assembly of crescentin structures in cells depleted of native crescentin shows biphasic growth: initial rapid assembly causing longitudinal extension, followed by a shift to a gradual incorporation of monomers and apparent thickening of structures as the filament reaches the cell poles (Charbon et al. 2009). Crescentin monomers are added laterally to a crescentin structure that extends bidirectionally, along the inner membrane, as the cell grows (Charbon et al. 2009). Curiously, as the structures extend to the poles, longitudinal

elongation ceases despite continued lateral addition of monomers. This is true even in *creS*-overexpression conditions, arguing against limiting crescentin levels being responsible for its polar exclusion (Charbon et al. 2009). It is possible that crescentin assembly is negatively regulated by an unknown mechanism involving polar proteins.

The slow turnover of the crescentin structure poses a challenge for cell division. This is evident during heterologous expression of *creS* in *E. coli* and in overexpression of *creS* in *C. crescentus*. In the former, expression of *creS* causes curvature in *E. coli* but at the same time results in cell division defects. In the latter, overexpression of cytoplasmic *creS* Δ_{27} disrupts cytokinesis, probably due to stable crescentin structures physically stalling constriction of cell envelope (Fig. 4C) (Cabeen et al. 2010). Since such a disruption of cytokinesis is only evident in cases where crescentin may not have all of its interacting partners (as in *E. coli*) or is not attached to the membrane (as in the overexpression of *creS* Δ_{27}), it is proposed that an unidentified membrane-bound factor and/or activity is important for disassembly of crescentin structure during cell division.

CTP synthase: co-opting a metabolic enzyme to define cell shape

CTP synthase (CtpS) is a universally conserved enzyme that generates CTP from ATP, UTP and glutamine (Long et al. 1970). Surprisingly, this essential metabolic enzyme was simultaneously discovered to form filaments in bacteria, yeasts, flies, and mammals (Ingerson-Mahar et al. 2010; Noree et al. 2010; Liu 2010; Carcamo et al. 2011). Polymerization of CtpS is required for feedback inhibition of its enzymatic activity in both eukaryotes and bacteria (Barry et al. 2014; Noree et al. 2014). However, CtpS fulfills an additional, apparently unique, cytoskeletal function in *C. crescentus* where it regulates cell curvature through a crescentin-dependent mechanism (Ingerson-Mahar et al. 2010).

CtpS localization and role in cell curvature

CtpS was first discovered to form filaments in *C. crescentus* through a microscopy-based screen for localized proteins where mCherry-CtpS localized to filament-like structures near the inner curvature of the cell (Fig. 2, 3N) (Werner & Gitai 2010; Ingerson-Mahar et al. 2010). Early ECT images of wild-type *C. crescentus* revealed the presence of filament bundles (ECT filaments) in a similar location that were not composed of any of the then-known polymerizing proteins (Briegleb et al. 2006). These ECT filaments were subsequently confirmed to be formed by CtpS using correlated fluorescence light microscopy and ECT (Fig. 4D) (Ingerson-Mahar et al. 2010).

ctpS is essential, and depletion of CtpS in *C. crescentus* causes slow growth and increased cell curvature (Fig. 3G) (Ingerson-Mahar et al. 2010). Conversely, overproduction of CtpS decreases cell curvature, resulting in nearly straight cells. When CtpS is depleted in a *creS* background however, cells remain straight, indicating that CtpS exerts its influence on cell curvature through crescentin. Consistent with this hypothesis, in *ctpS*-overexpressing cells crescentin localization is aberrant, though crescentin protein levels are unaffected. Instead of assembling into filamentous structures, crescentin forms small foci in the presence of excess CtpS (Ingerson-Mahar et al. 2010). Heterologous expression in *E. coli* and bacterial two hybrid assays indicate that CtpS directly interacts with crescentin (Ingerson-Mahar et al.

2010). Based on these observations, it has been proposed that CtpS negatively regulates crescentin assembly, possibly by causing increased turnover of crescentin and/or fragmentation of polymers. This function of CtpS in regulating cell curvature does not require its ability to synthesize CTP, but rather relies on its glutamine amidotransferase (GAT) domain (Ingerson-Mahar et al. 2010).

Assembly properties of CtpS

CtpS structures observed by ECT in cells typically comprise 4 parallel ribbons that are 10 nm apart (Fig. 4D). The three ribbons closest to the inner membrane are approximately 400 – 500 nm long, 7 nm thick, and 22 nm wide, while the ribbon farthest from the inner membrane is much shorter (Briegel et al. 2006). *C. crescentus* CtpS is refractory to purification, however CtpS from *E. coli* (EcCtpS) assembles into polymers *in vitro* that are similar to those observed for *C. crescentus* CtpS by ECT (Fig. 4E, 5F). They are 200–400 nm long and can assemble into bundles of 3–5 filaments spaced 8–9 nm apart (Ingerson-Mahar et al. 2010). EcCtpS polymerization is induced by its product, CTP, and inhibits the enzymatic activity of CtpS likely by preventing a critical conformational change (Barry et al. 2014).

Interestingly, the assembly and localization of CtpS is dynamic over the cell cycle (Fig. 2, 3N) (Ingerson-Mahar et al. 2010). In new stalked cells, CtpS is present as a focus or short filament in the cytoplasm. The CtpS focus intensifies and elongates in late stalked cells and predivisional cells as it moves from the cytoplasm to the inner cell curvature, where it remains until the cell divides. The CtpS filament is often inherited asymmetrically, with stalked daughters receiving the CtpS filament and swarmer having little to no localized CtpS signal. Although CtpS polymers appear to be absent from swarmer cells, there is no change in the cellular levels of CtpS, suggesting that CtpS filament formation is triggered post-translationally during swarmer to stalk transition (Ingerson-Mahar et al. 2010). Since CTP synthesis feeds into multiple metabolic and cell cycle regulatory pathways, it may be that the assembly of CtpS structures is under the control of cell cycle-dependent metabolic regulation.

CtpS: unanswered questions

While it is clear that polymerization of CtpS is an ancient adaptation critical to the regulation of CTP metabolism, it is less obvious how and why *C. crescentus* co-opted these polymers for the determination of cell shape. It is interesting to note that EcCtpS can complement loss of *C. crescentus* CtpS both in promoting cell growth and maintaining wild-type cell curvature (Ingerson-Mahar et al. 2010), suggesting that *C. crescentus* CtpS has not acquired an additional activity. Instead, it is likely that crescentin evolved in such a way to allow interaction with and regulation by CtpS. How CtpS, specifically in the polymeric form, regulates the assembly and activity of crescentin, however, is still unknown. Future work to characterize CtpS function and its interaction with crescentin at the molecular level will be required for a mechanistic understanding of CtpS and its role in cell shape. More generally, understanding how a polymerizing metabolic enzyme like CtpS has been co-opted for a cytoskeletal role may lend insight into how similar events occurred, for example in the evolution of the actin-hexokinase-hsp70 superfamily (Kabsch & Holmes 1995).

Bactofilins: scaffolds for stalk morphogenesis

Bactofilins are a class of cytoskeletal proteins unique to bacteria. Representatives are found across all phyla of bacteria with sequenced genomes and function in various cellular processes, including morphogenesis, motility, and development (Kühn et al. 2009; Koch et al. 2011; Lin & Thanbichler 2013). The first bactofilins, BacA and BacB, were discovered in a microscopy-based screen for gene products involved in polar development during the swarmer-to-stalked cell transition in *C. crescentus*. BacA and BacB were demonstrated to function in stalk morphogenesis (Kühn et al. 2009) and are present throughout the cell cycle. However, their localization is cell cycle-regulated, as they are diffuse in swarmer cells, but assemble at the stalked pole at the swarmer-to-stalked cell transition (Fig. 2, 3O, 4E). They then remain at the stalked pole for the duration of the cell cycle and are inherited by the stalked daughter upon division.

Function and localization of bactofilins

Of the two bactofilins, BacA is apparently dominant to BacB, as *bacB* cells have similar but less severe phenotypes than *bacA* or *bacA bacB* cells (Kühn et al. 2009; Hughes et al. 2013). Moreover, BacA is estimated to be in ten-fold excess of BacB (~200 molecules of BacA and only ~20 molecules of BacB per cell) (Kühn et al. 2009). Together they form an ordered sheet-like structure about 5 nm internal to the inner membrane at the base of the stalk in cells visualized by ECT (Fig. 4E) (Kühn et al. 2009). This structure serves as a scaffold for the recruitment of a stalk-specific peptidoglycan synthase, PbpC, that further recruits other factors required for stalk morphogenesis, such as StpX (Hughes et al. 2013). PbpC is a membrane-bound periplasmic enzyme, and it likely interacts with the bactofilin scaffold through its cytoplasmic tail (Kühn et al. 2009; Hughes et al. 2013). Consistent with the role of PbpC in stalk elongation, cells lacking BacA (or both BacA and BacB) fail to elongate their stalks as efficiently as wild-type (Kühn et al. 2009).

It is not clear how bactofilins are recruited to the base of the stalk and how this recruitment is coordinated with the cell cycle. Bactofilins are expressed throughout the cell cycle, however their transcript levels are upregulated during the swarmer to stalk transition (McGrath et al. 2007; Kühn et al. 2009). Although they are expressed in swarmers, bactofilins do not form a scaffold until the swarmer-to-stalked cell transition. In stalked cells, bactofilins can localize in the absence of proteins such as SpmX and DivJ that are known to be specifically recruited to the base of the stalk prior to stalk synthesis (Kühn et al. 2009). Due to their low physiological concentrations, it is possible that bactofilins require a nucleation factor to regulate their assembly temporally. Curiously, overexpression of either bactofilin causes morphological defects with cells becoming increasingly curved over time until they eventually lyse (Fig. 3H). In these experiments, both BacA and BacB were seen to localize to the inner curvature, suggesting a preference for positive curvature for bactofilin structures. This localization mechanism for bactofilins is reminiscent of the curvature-sensing mechanism of the BAR-domains of eukaryotic proteins (Kühn et al. 2009). It is important to note that the high curvature of bactofilin overexpressing cells is independent of the crescentin cytoskeleton that gives wild-type *C. crescentus* its curved morphology (Kühn

et al. 2009). Whether the bactofilin structure assembles by sensing positively curved surfaces or if it induces positive curvature is yet to be determined.

Assembly properties of bactofilins

Bactofilins are spontaneously polymerizing proteins that can form linear polymers, bundles, sheets and crystalline arrays *in vitro* (Fig. 5G) (Kühn et al. 2009; Koch et al. 2011; Zuckerman et al. 2015). Based on sequence similarities and electron microscopy, BacA and BacB appear to behave similarly. BacA is easier to purify than BacB and has a lower critical concentration (250 nM), allowing for a more thorough characterization of the former. BacA assembles into polymers without a requirement for nucleotide and over a large range of pH and salt concentrations (Kühn et al. 2009). In this regard, bactofilins are similar to intermediate filaments. However, unlike intermediate filaments, bactofilins lack any coiled-coil regions. Like all bactofilins, BacA and BacB have a rigid core made of a DUF583 domain flanked by N- and C-terminal tails (Kühn et al. 2009; Vasa et al. 2015). Solid-state NMR and quantitative electron microscopy data led to a structural model in which each monomer of BacA adopts a β -helical structure comprising 6 turns made of 3 β -sheets each, thus making a triangular hydrophobic core. Head-to-tail assembly of DUF583 domains yields filaments that are 3 nm in diameter, which form loosely packed bundles at low salt concentrations. At physiological salt concentrations, these filaments assemble as ribbons and/or two-dimensional sheets. The physiological relevance of the different assemblies of bactofilins observed *in vitro* is currently unknown. The N- and C-terminal tails of BacA are flexible, and might serve as flanks that help position the structure relative to the inner membrane *in vivo* (Vasa et al. 2015). It is not clear whether the tails also function in interactions between bactofilins and other proteins such as PbpC.

C. crescentus cytoskeletons: Future perspectives

The past decade has seen the identification and characterization of numerous unique cytoskeletal elements in bacteria, and specifically in *C. crescentus*, owing largely to advances in microscopy techniques. However, a major question still remains for most of these factors – how do the structures observed *in vitro* correlate with the localization patterns and functions observed *in vivo*? Barring a few proteins such as PopZ and bactofilins, it is not clear if the filamentous structures formed by purified proteins *in vitro* are physiologically relevant for the function of these cytoskeletal proteins. In some cases such as ParA and MreB, it has not been definitively demonstrated that these proteins form filaments at all *in vivo* (Briegleb et al. 2006; Swulius et al. 2011; Lim et al. 2014). While ECT and super-resolution microscopy have helped discern structures that had previously been resolution-limited, these techniques have their own challenges, making it difficult to identify non-canonical structures, dynamic assemblies, membrane-associated polymers, and/or heteropolymers.

Unlike eukaryotes, a change in cytoskeletal organization alone does not mean a change in bacterial cell shape; instead, cytoskeleton-driven cell shape changes require coordinated cell wall remodeling (Pichoff & Lutkenhaus 2007). It is not obvious how the differential assembly of cytoskeletal proteins affects cell wall remodeling enzymes to cause cell shape

changes. Are MreB and FtsZ, for example, purely scaffolds that regulate assembly of cell wall synthetic complexes or do they more actively contribute to shape maintenance through mechanical means, as has been proposed for crescentin? A fascinating problem unique to *C. crescentus* and other stalked organisms is the development of a slender protrusion from a cylindrical cell of a much larger diameter (Wagner & Brun 2007). While it is apparent that many of the players involved in cell elongation are important for stalk biogenesis, it is not clear how these proteins are able to direct formation of a structure of predetermined dimensions, whether through as yet unknown forms of regulation of known players or *via* an unknown structural element.

Another question that deserves attention is, how do these cytoskeletal elements interact with each other? It is evident from individual characterizations of these proteins that there is considerable amount of crosstalk between cytoskeletons, directly and indirectly. For example, MreB interacts with FtsZ, and most probably with crescentin and affects PopZ localization as well. Tight spatiotemporal coordination between cytoskeletal structures is central to cell cycle progression while maintaining cell shape. A major goal for the future is to develop a holistic view of cytoskeletal regulation in the larger context of the cell cycle and developmental program of *C. crescentus*.

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Figure 1. Cytoskeletal elements involved in cell cycle progression

Swarmer cells (left, with polar flagellum) and newly divided stalked cells have a unipolar PopZ matrix and a single ParB focus (bound to *parS*) close to the old pole. ParA and MipZ bind DNA (gray line) non-specifically and localize as diffuse clouds enriched at the pole(s). In newborn cells, MipZ is concentrated at the old pole by ParB bound to PopZ whereas ParA is concentrated at the new pole by TipN. Following initiation of chromosome replication, a new PopZ matrix is assembled at the new pole. At the same time, PopZ at the old pole recruits SpmX to drive stalk biogenesis. As chromosome replication progresses, one of the ParB foci moves up the ParA concentration gradient towards the new pole, leading to an *ori-ter-ori* orientation of the chromosome. MipZ is segregated to the new pole with ParB-*parS*, leading to a MipZ concentration gradient with the minimum at approximately midcell. See text for more details.

Figure 2. Cytoskeletal regulation of cell shape and morphogenesis

In swarmer cells, dynamic clusters of MreB are distributed along the length of the cell and direct cell elongation. FtsZ is localized to the new cell pole in swarmers, but due to the dynamic relocation of the MipZ concentration maxima to the poles upon centromere segregation, FtsZ is displaced from the new pole and reassembled at midcell to form the Z-ring. Following Z-ring assembly, MreB is recruited to the midcell where it drives midcell elongation along with FtsZ. Finally, following the arrival of late division proteins, MreB is displaced from the midcell into dispersed clusters. Crescentin and CTP synthase work synergistically to direct cell curvature. Crescentin forms short structures at the inner cell curvature in swarmer cells, which elongate as the cell grows until these crescentin filaments reach the cell poles. CTP synthase forms shorter, predominantly cytoplasmic filaments in swarmer cells and longer, membrane-associated structures in stalked cells. Bactofilins assemble into a scaffold in early stalked cells at the base of the stalk and regulate pole morphogenesis.

Figure 3. Function and localization of *C. crescentus* cytoskeleton

(A) Phase contrast image of wild-type *C. crescentus* cells (Unpublished results). Figures (B) – (H) represent morphological changes upon deletion, depletion or overexpression of individual cytoskeletal elements. Scale bars = 2 μ m. (B) DIC image of *popZ* cells. Arrows highlight minicell formation (Ebersbach et al. 2008). (C) Phase contrast image of cells overexpressing *parA* (Mohl & Gober 1997). (D) Phase contrast image of cells depleted of FtsZ for 4.5 hours (Sundararajan et al. 2015). (E) Phase contrast image of cells depleted of MreB for 10 hours (Figge et al. 2004). (F) DIC image of *creS* cells (Ausmees et al. 2003). (G) Phase contrast image of cells depleted of CTP synthase (Ingerson-Mahar et al. 2010).

(H) Phase contrast image of cells overexpressing *bacA-venus* (Kühn et al. 2009). Figures (I) – (O) represent localization pattern of individual cytoskeletal proteins. (I) Epifluorescence microscopy showing polar localization of PopZ-YFP (in red overlaid on DIC images) in synchronized cells; I1 – early swarmer with unipolar PopZ localization, I2 – Pre-divisional cell with bipolar PopZ localization, I3 – Bipolar PopZ localization in cell undergoing cytokinesis. Scale bar = 2 μ m (Ebersbach et al. 2008). (J) PALM image of ParA-Dendra2 expressed from native locus (J1) or under the control of P_{xyI} promoter (J2). Scale bar = 1 μ m (Lim et al. 2014). (K) HT-PALM image of FtsZ-Dendra2 in a predivisional cell; (K1) and (K2) represent longitudinal and cross-sectional views of the Z-ring respectively. Scale bar = 500 nm (Holden et al. 2014). (L) PALM image of eYFP-MreB. Scale bar = 300 nm (Biteen et al. 2008). (M) Immunofluorescence microscopy image showing CreS-FLAG (red) and the nucleoid stained with DAPI (blue). Scale bar = 2 μ m (Ausmees et al. 2003). (N) Epifluorescence microscopy of cells expressing mChy-CtpS (red overlaid on phase contrast image). Scale bar = 2 μ m (Ingerson-Mahar et al. 2010). (O) Merged image showing localization of BacA-eCFP and BacB-Venus. Scale bar = 2 μ m (Kühn et al. 2009).

Figure 4. Diverse cytoskeletal structures observed *in vivo*

A subset of the cytoskeletal elements described in *C. crescentus* has been imaged at high resolution *in vivo* using electron cryotomography (ECT). ECT images of *C. crescentus* cells showing (A) Ribosome-free zone associated with the PopZ matrix at the old cell pole (highlighted by dotted line) (Bowman et al. 2010), (B) FtsZ protofilaments (red) assembled into a ring proximal to the inner membrane (blue) at the site of division (Z. Li et al. 2007), (C) Filaments formed on overproduction of crescentin_{N27} (scale bar = 50 nm) (Cabeen et al. 2010), (D) CTP synthase ribbons assembled at the inner curvature of the cell proximal to the inner membrane (scale bar = 200 nm) (Briegel et al. 2006; Ingerson-Mahar et al. 2010) (E) Bactofilin scaffold assembled at the base of the stalk (scale bar = 50 nm) (Kühn et al. 2009).

Figure 5. Filaments and higher order structures formed by cytoskeletal proteins *in vitro*
 Transmission electron microscopy (TEM) images of purified proteins reported to have been assembled into higher order structures *in vitro*. (A) PopZ filaments, scale bar = 50 nm (Bowman et al. 2008). (B) ParA filaments assembled in the presence of ATP, scale bar = 100 nm. (B1), (B2), (B3) show enlarged images of filament bundles, scale bar = 20 nm (Ptacin et al. 2010). (C) FtsZ protofilaments assembled in the presence of GTP, scale bar = 100 nm (Unpublished results). (D) Double protofilaments of MreB assembled on a lipid monolayer in the presence of ATP, scale bar = 100 nm (van den Ent et al. 2014). (E) Crescentin filaments (arrowheads) and bundles (arrows and asterisks) assembled at pH 6.5 in the

absence of Mg^{2+} , scale bar = 250 nm (Cabeen et al. 2010). (F) Filaments formed by *E. coli* CTP synthase, scale bar = 100 nm (Ingerson-Mahar et al. 2010). (G) Filaments (arrows) and 2D-crystalline sheets (asterisks) formed by BacA, scale bar = 100 nm (Vasa et al. 2015). Note that the identities of structures seen in some TEM images published in papers on cytoskeletal filaments have been questioned (Ghosal et al, 2014, supplementary file; Griffith & Bonner, 1973), since contaminating cellulose fibers and thin uranyl acetate crystals may each be mistaken for protein polymers; here, the FtsZ and MreB filaments in C and D appear white, as expected for protein embedded in negative stain, and are also unmistakably identifiable from longitudinal repeats corresponding to the sizes of the monomers, but the EM evidence for filament formation by other proteins is weaker.