Cell-free biogenesis of bacterial division proto-rings that can constrict liposomes

Elisa Godino¹, Jonás Noguera López¹, Ilias Zarguit¹, Anne Doerr¹, Mercedes Jimenez², Germán Rivas² & Christophe Danelon²

A major challenge towards the realization of an autonomous synthetic cell resides in the encoding of a division machinery in a genetic programme. In the bacterial cell cycle, the assembly of cytoskeletal proteins into a ring defines the division site. At the onset of the formation of the *Escherichia coli* divisome, a proto-ring consisting of FtsZ and its membrane-recruiting proteins takes place. Here, we show that FtsA-FtsZ ring-like structures driven by cell-free gene expression can be reconstituted on planar membranes and inside liposome compartments. Such cytoskeletal structures are found to constrict the liposome, generating elongated membrane necks and budding vesicles. Additional expression of the FtsZ cross-linker protein ZapA yields more rigid FtsZ bundles that attach to the membrane but fail to produce budding spots or necks in liposomes. These results demonstrate that gene-directed protein synthesis and assembly of membrane-constricting FtsZ-rings can be combined in a liposome-based artificial cell.
Cell-free biology aims at understanding cellular processes by reconstituting biological functions from their isolated elementary components in vitro model systems. Owing to their openness and easy manipulation, cytoplasmic extracts and systems reconstituted from purified elements are more amenable to customized experimental design and quantitative description compared to living cells. Therefore, the minimal requirements to achieve a particular function can be assessed more reliably. Many complex biological structures and processes taking place in bacterial or eukaryotic cells have already been reconstituted in vitro. Notable achievements include the reconstitution of the minimal translation machinery from *Escherichia coli*, the yeast DNA replication apparatus, filopodial structures, cytokkeleton self-organization and centrosome positioning, egg cytokinesis signaling, DNA segregation with Par5, and clathrin-coated buds. Encouraged by the many cellular pieces that have already been reconstituted in vitro, synthetic biologists have now engaged in the construction of an entire cell.

One of the hallmarks of living systems is their ability to divide. An obvious starting point to conceiving a biology-inspired division mechanism in artificial cells is to consider the canonical pathways taking place in prokaryotes. In most bacteria, symmetrical cell division proceeds by forming a constriction ring that eventually splits the mother cell into equally sized daughter cells. At an earlier stage of cytokinesis, a proto-ring composed of the FtsZ, FtsA, and ZipA proteins, assembles on the inner leaflet of the cytoplasmic membrane at the future division site. The tubulin-related FtsZ is the core constituent of the proto-ring. FtsZ is a GTPase that can polymerize into protofilaments. Anchoring of FtsZ protofilaments to the cytoplasmic membrane is mediated by ZipA and the actin homolog FtsA.

Earlier attempts to divide cell-like liposomal compartments have focused on the reconstitution of the Z-ring from purified proteins. These studies have shown that FtsZ aided by one of its anchoring protein partners—or a chimeric FtsZ bearing a membrane targeting segment—can self-organize into filament patterns on supported lipid membranes. When encapsulated inside vesicles, the elementary cytokellular proteins form ring-like structures that can deform the liposome membrane. Whether FtsZ filaments alone exert a contractile force and contribute to the final stage of division remains a subject of debate and evidence for complete liposome division is still lacking.

A conceptual issue that is inherent to reconstitution assays solely relying on purified proteins, is the impossibility to maintain steady amounts of cytoskeletal proteins from internal mechanisms as the compartment undergoes division. Another problem raised by conventional cell-free assays is the use of oversimplified buffer compositions that have been tailored for a particular set of enzymatic reactions but fail to reproduce the cytoplasmic environment.

Herein, we address these issues by encoding *E. coli* division proteins on DNA templates. Genetic control over protein production offers a general solution to achieve self-replication, as well as self-regulation by establishing feedback loops. In this context, the PURE system, a minimal gene expression system reconstituted primarily from *E. coli* constituents was employed. Different types of proteins and biological functions have already been synthesized de novo with the PURE system, including membrane-associated proteins. Moreover, by containing all relevant factors for gene expression, the PURE system emulates more closely the molecular composition of the bacterial cytoplasm than simple buffers. In the present study we utilized PUREfrex2.0, which provides the best combination of protein yield and expression lifespan.

We show that cell-free expressed FtsA is able to recruit FtsZ polymers, forming large-scale two-dimensional networks of curved and ring-like structures in the absence of bundling factors. When the entire set of reactions is encapsulated inside liposomes, proto-rings of FtsA–FtsZ filaments are found to confine the vesicle, generating extended membrane necks and budding vesicles, a phenotype that has not been reported before. Co-expression of ZapA, a native stabilizer of FtsZ filaments, yields stiffer FtsZ bundles attached to the membrane that fail to confine into bud necks. FtsZ cytoskeletal structures are also investigated with ZipA membrane-anchor protein. We find that in our low-volume supported lipid bilayer (SLB) assays with ZipA and ≤3 µM FtsZ, the generic crowding agent Ficoll70 is necessary to elicit bundle formation. Cell-free expressed ZapA obviates the need of Ficoll70 and promotes formation of cytoskeletal networks with different, likely more physiological, morphology, and protein monomer dynamics. The prospects of further improvement suggest that the DNA-programmed hierarchical assembly of the Z-ring in liposomes is a promising strategy for dividing synthetic cells. In addition, our approach to reconstituting cellular processes in PURE system provides a generic platform that fills the gap between classical in vitro and in cellulo experiments.

**Results**

**Cell-free synthesized FtsA drives the formation of curved FtsZ filaments.** An essential component of the *E. coli* division proto-rings is FtsA, a homolog of actin that anchors FtsZ filaments to the cytoplasmic membrane. To bypass the difficult purification of FtsA, we directly expressed a sequence-optimized *ftsA* gene on an SLB (Fig. 1a). In the presence of 3 µM purified FtsZ, curved filaments and dynamic ring-like structures formed on the membrane (Fig. 1b, Supplementary Fig. 1, Supplementary Note 1, Movie 1), concurring with previous reports.

To obtain quantitative insights about the concentration of cell-free synthesized FtsA, pre-run PURE system samples were analyzed by liquid chromatography-coupled mass spectrometry (LC-MS) (Supplementary Fig. 2, Supplementary Table 1). Protein abundance was quantified using an internal standard (QconCAT) for the most C-terminal peptide detected. We deduced that, after 3 h of expression, FtsA concentration on the SLB was 2.2 ± 0.2 µM (mean ± SD, three biological repeats) (Fig. 1a-d, Supplementary Tables 2 and 3), corresponding to a protein ratio FtsZ:FtsA ≈ 1.5:1. In vivo, FtsA concentration is ~0.5 µM and the protein ratio FtsZ:FtsA = 3:1–5:1. However, overlapping rings and dynamic filaments on a lipid membrane have also been observed at protein ratios similar as in our cell-free assay. Note that LC-MS data do not report the concentration of active protein, which may differ from the measured concentration of proteolytic peptides.

Promoting lateral interactions of FtsZ protofilaments stimulates the formation of higher-order cytoskeletal structures in vitro. However, little is known about how the nature of these lateral interactions influences the morphology of the FtsZ network. Therefore, we decided to investigate the architecture and dynamics of FtsZ protofilaments in a molecular environment that favors lateral interactions. First, we employed Ficoll70, a generic crowding agent known to elicit FtsZ bundle formation (Fig. 1c). Large SLB areas were covered with curved filaments, rings of different sizes (most having a diameter of 1–2 µm, phenotype 1) and large circular patterns (phenotype 2) (Fig. 1d).

Although Ficoll70 is commonly used as a macromolecular crowder to mimic cytoplasmic conditions, we reasoned that ZapA, an in vivo regulator of FtsZ polymerization, would provide a more targeted and native mechanism to elicit lateral interaction, thus conferring physiologically relevant properties of...
Fig. 1 Cell-free expressed FtsA recruits FtsZ to an SLB and drives the formation of ring-like structures. 

- **a** Schematic representation of the SLB assays with FtsA directly expressed on the membrane. Purified FtsZ-A647 (3 μM) was added. The sequence-optimized construct ftsA<sub>opt</sub> was used. **b** Fluorescence image of FtsZ-A647 forming curved filaments and rings in the presence of in situ synthesized FtsA. The zoom-in image (right) corresponds to the framed region in the left image. **c** As in (a) but the solution was supplemented with Ficoll70. **d** Fluorescence image of FtsZ-A647 forming curved filaments and rings in the presence of in situ synthesized FtsA and Ficoll70. Two representative filament network morphologies observed on the same SLB are shown, phenotype 1 being the most prominent. **e** Schematic illustration of the SLB assays with separately expressed FtsA and ZapA. The constructs ftsA<sub>opt</sub> and zapA were used. Purified FtsZ-A647 (3 μM) was included. **f** Fluorescence images of FtsZ-A647 displaying two representative phenotypes from different regions of the same SLB, phenotype 1 being the most prominent. **g** Schematic illustration of the SLB assays with co-expressed FtsA and ZapA from ftsA<sub>opt</sub> and zapA<sub>opt</sub> constructs. Purified FtsZ-A647 (3 μM) was added. **h** Fluorescence images of FtsZ-A647. The zoom-in image (right) corresponds to the framed area in the left image. Scale bars indicate 10 μm.
cytoskeletal patterns. For this reason, ZapA was produced in a one-pot PURE system reaction led also to reconstitute FtsA-FtsZ cytoskeletal networks inside liposomes (Fig. 3a). The cell-free gene expression solution was supplemented with adenosine triphosphate (ATP, additional 2 mM), guanosine triphosphate (GTP, additional 2 mM) and a mixture of highly purified chaperones (DnaK mix). Although energy regeneration components are present in the PURE system, extra ATP and GTP were provided to compensate for the extra demand from FtsA and FtsZ. Purified FtsZ-A647 was used to visualize protein localization by laser scanning confocal microscopy. FtsZ-A647 was employed at 3 µM concentration, which is similar to that measured in vivo (~3.5 µM)36. Liposomes were formed by natural swelling, with a composition of zwitterionic PC and PE phospholipids, anionic PG and cardiolipin, and a small fraction of TexasRed-conjugated lipid for membrane imaging35. Such a lipid mixture and lysosome preparation method have proved compatible with the cell-free synthesis of membrane-associated enzymes10, DNA replication proteins11 and division proteins12. Liposome size distribution ranges from ~1 µm up to over 15 µm in diameter, which provides a more relevant bacterial cell-size compartment than >20 µm liposomes produced with other methods33,33. In contrast with previous studies33, no crowding agent was included during liposome formation. In fact, we found that Ficoll70 impairs formation of gene-expressing liposomes with our methodology (Supplementary Fig. 12).

FtsZ and internally synthesized FtsA constrict liposomes. The identification of FtsZ and FtsA as the minimal molecular set to obtain membrane-anchored curved filaments and rings in the PURE system prompted us to reconstitute FtsA-FtsZ cytoskeletal networks inside liposomes (Fig. 3a). The cell-free gene expression solution was supplemented with adenosine triphosphate (ATP, additional 2 mM), guanosine triphosphate (GTP, additional 2 mM) and a mixture of highly purified chaperones (DnaK mix). Although energy regeneration components are present in the PURE system, extra ATP and GTP were provided to compensate for the extra demand from FtsA and FtsZ. Purified FtsZ-A647 was used to visualize protein localization by laser scanning confocal microscopy. FtsZ-A647 was employed at 3 µM concentration, which is similar to that measured in vivo (~3.5 µM)36. Liposomes were formed by natural swelling, with a composition of zwitterionic PC and PE phospholipids, anionic PG and cardiolipin, and a small fraction of TexasRed-conjugated lipid for membrane imaging35. Such a lipid mixture and lysosome preparation method have proved compatible with the cell-free synthesis of membrane-associated enzymes10, DNA replication proteins11 and division proteins12. Liposome size distribution ranges from ~1 µm up to over 15 µm in diameter, which provides a more relevant bacterial cell-size compartment than >20 µm liposomes produced with other methods33,33. In contrast with previous studies33, no crowding agent was included during liposome formation. In fact, we found that Ficoll70 impairs formation of gene-expressing liposomes with our methodology (Supplementary Fig. 12).

In control experiments where theftsAoptgene was omitted, FtsZ was exclusively located in the lipidosome lumen (Supplementary Fig. 13). De novo synthesized FtsA successfully recruited FtsZ on the membrane as shown by the colocalization of the FtsZ-A647 and membrane dye signals (Fig. 3, Supplementary Fig. 14). Although homogeneous recruitment of FtsZ to the membrane was commonly found within the liposome population, the majority of the liposomes displayed regions with patches of FtsZ on the inner surface of the membrane (Fig. 3b). Noticeably, the membrane spots with clustered FtsZ coincide with different types of membrane remodeling. In some cases, the recruited FtsZ localizes with outward membrane deformation or short protrusions (Fig. 3c, Supplementary Fig. 15). In other instances, the protrusions developed into vesicles or blebs tethered to the parental liposome through a membrane neck coated with FtsZ (Fig. 3d, Supplementary Fig. 15). Sometimes, the budding neck extends over a few microns in the form of a tubular structure containing one or a few FtsA-FtsZ rings (Fig. 3d, e). Interestingly,
these blebbing structures are dynamic. Events, such as appearance of new constriction sites, growing vesicles and diffusion of protein rings along the tube axis were observed (Fig. 3e, Movie 2). Although membrane recruitment of FtsZ in the form of patches was visible already within 2 h of expression, major liposome-remodeling events, such as budding spots and elongated blebs were observed only after 3–4 h. Moreover, after 6 h expression, small vesicles were found to agglutinate to larger liposomes (Supplementary Fig. 13). FtsA concentration does not significantly increase beyond the first 3 h of expression (Supplementary Fig. 2), in agreement with the kinetic profiles of protein production with the PURE system. Concentration of synthesized FtsA was compared after 3 and 6 h expression, yielding 4.5 ± 0.5 μM and 5.8 ± 1.1 μM, respectively. Therefore, we do not expect that the differences observed at incubation times longer than 3 h can be attributed to an increase in protein concentration. Instead, the time-dependent changes could be due to some delaying factors, such as recruitment of proteins to the membrane, assembly of filaments and bundles, protein clustering into patches and remodeling of the membrane. It is unclear whether the FtsZ-coated membrane necks can close to release mature vesicles. Therefore, we cannot ascertain that the small vesicles observed after 6 h are reminiscent to division events. Yet, these aggregated vesicles were not observed when the ftsAopt gene was omitted (Supplementary Fig. 13), indicating that this global remodeling is dependent on the expression of FtsA.

We then decided to investigate how the presence of cell-free expressed ZapA would modulate the properties of the cytoskeletal filament networks. Different cytoskeletal network phenotypes were observed when ZapA concentration was increased upon expression of the optimized zapAopt construct (Supplementary Fig. 9). Scale bars indicate 10 μm.

**Fig. 2** Purified sZipA and FtsZ form co-filament networks in the PURE system. a Schematic representation of the SLB assays. Purified sZipA-A488 was first incubated on an SLB. The solution on top of the SLB was replaced by a minimal reaction buffer containing 3 μM purified FtsZ-A647 and 2 mM GTP. b Fluorescence images of sZipA-A488 (left) and FtsZ-A647 (right) in the minimal reaction buffer without Ficoll70. c Schematic representation of the SLB assays. Purified sZipA-A488 was first incubated on an SLB. The solution on top of the SLB was replaced by PUREfrex2.0 supplemented with 3 μM purified FtsZ-A647, 2 mM GTP and 50 g L⁻¹ Ficoll70. d Fluorescence images of sZipA-A488 (left) and FtsZ-A647 (right) in PUREfrex2.0 with Ficoll70. Large-scale filaments with colocalizing FtsZ-A647 and sZipA-A488 are exclusively observed in the presence of Ficoll70. This conclusion is valid in both the minimal reaction buffer and in PUREfrex2.0 background. More fields of view are displayed in Supplementary Fig. 7. e Schematic illustration of the SLB assays with purified FtsZ-A647 (3 μM) and cell-free synthesized ZapA incubated on top of an sZipA-A488-bound SLB. ZapA was expressed from the native gene zapA. f Fluorescence images of sZipA-A488 (left) and FtsZ-A647 (right) in a sample containing cell-free synthesized ZapA and additional 2 mM GTP.
patterns in liposomes (Fig. 4a). Co-expression of *ftsA*<sub>opt</sub> and *zapA*<sub>opt</sub> DNA constructs induced formation of FtsZ-A647 clusters on the inner surface of the membrane (Fig. 4b). Liposomes with different cytoskeletal protein phenotypes were observed, such as homogeneous coating to the membrane, patches or filaments, and large ring-like structures. Bundles of FtsZ polymers adopting apparent ring-like structures predominantly localize at the interface of two liposomes (Fig. 4b), coinciding with a membrane septum (i.e., a membrane separating two adjacent vesicles; it could be a single bilayer or two bilayers). However, ZapA abolishes the formation of membrane protrusions, vesicle budding and clustering of FtsZ on tubular membrane structures (Fig. 4b). We have seen that, in the presence of ZapA, the small ring-like structures do not form on SLB, where longer, curved filaments dominate (Fig. 1). We observed a decrease of the filament curvature in the presence of ZapA, especially during co-expression of FtsA and ZapA (Supplementary Fig. 3b), which correlates with a higher concentration of ZapA in the assay (Supplementary Table 3). The straighter cytoskeletal filaments are likely not able to develop into contractile rings. Instead, they accommodate to the large compartment and are unable to deform the membrane into narrow necks (Fig. 4b). These results indicate that the mechanical properties of FtsZ-ZapA bundles impede the formation of membrane-constricting, high-curvature cytoskeletal filaments, which suggests that temporal

![Figure 3](https://example.com/figure3.png)

**Fig. 3** In-liposome synthesized FtsA assembles with FtsZ into ring-like structures that drive vesicle budding. a Schematic illustration of liposome reconstitution assays. The *ftsA*<sub>opt</sub> DNA template was expressed within phospholipid vesicles in the presence of 3 µM purified FtsZ-A647. b-d Confocal fluorescence images of liposomes exhibiting different morphologies of FtsZ-FtsA cytoskeletal structures and membrane remodeling: recruitment of proteins to the membrane in the form of clusters with no visible membrane deformation, budding spots induced by local accumulation of FtsZ-FtsA, and budding vesicles from a parental liposome with a clear FtsA-FtsZ-coated membrane neck. d, e Time series images showing that a ring-forming protein cluster localized at a constriction site can split, which induces multiple necks separated by blebbing vesicles (see Movie 2). Timespan is 120 s between the first and second row of images, and 96 s between the second and third row. Fluorescence from the membrane dye is colored in green and FtsZ-A647 signal is in magenta. The composite image is the overlay of the two channels. Asterisks indicate budding spots or constriction sites. Scale bars represent 10 µm. More examples of liposomes are shown in Supplementary Figs. 14 and 15.
vejicles. In these earlier studies, membrane-tethered proto-rings in particular.

compartmentalization of the Z-ring constituents could be provided by regulating the expression of individual genes through transcriptional circuits, such as cascade or feedback motifs. Mindful of the limitations to apprehend the PURE system and to rationally design liposomes harboring desirable properties encoded in genes, we believe that in vitro evolutionary optimization, by exploring a wide genetic diversity, provides additional opportunities to build cellular functions, and FtsZ proto-rings in particular.

**Methods**

**DNA constructs.** FtsZ and FtsA gene fragments were amplified by standard polymerase chain reaction (PCR) from the chromosomal E. coli BL21 DNA with primers 509 and 374 (ftsZ), and 508 and 376 (ftsA) (Supplementary Table 4). These primers contain overhangs for Gibson assembly with the pET11-a plasmid. PCR products were checked on a 1% agarose gel stained with EtBr or SYBR safe, imaged with a ChemiDocTM Imaging System (BioRad Laboratories), and purified with the Wizard SV Gel kit. The purified DNA was incubated with DpnI (New England BioLabs®, Inc.) to remove residual plasmid and the linear DNA was purified again with Wizard SV Gel kit. DNA concentration and purity were measured using a ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies). Gibson assembly (Gibson Assembly® Master Mix of New England BioLabs®, Inc.) was performed at equimolar concentrations of linearized plasmid (pET11-a) and DNA fragments for 1 h at 50°C. E. coli TOP10 competent cells (ThermoFisher Scientific, USA, catalog number C4040-10) were transformed with the Gibson assembly products by heat shock. Cells were centrifuged, resuspended in 50 µL of fusogenic membranes with a size <15 µm. The FtsA-dependent recruitment of FtsZ on the membrane frequently induces the formation of FtsZ clusters that constrict the liposome membrane into bud necks. It is clear from our data that FtsZ, assisted by FtsA, does not preferentially accommodate to pre-existing membrane areas with a high curvature. Conversely, membrane constriction and extended neck-like regions connecting the mother and budding vesicles are the product of localized FtsZ-FtsA pattern assembly. Noteworthily, these types of membrane remodeling, cytoskeletal protein organization, and dynamic blebbing structures were not observed in previous reports.

**Fig. 4 Co-expressed FtsA and ZapA organize FtsZ into long membrane-tethered bundles within liposomes.** a Schematic illustration of liposome reconstitution assays with 3 µM purified FtsZ-A647 and co-expression of the ftsAopt and zapAopt DNA constructs. b Confocal fluorescence images of liposomes exhibiting membrane recruitment of FtsZ-A647 after 3 h incubation. Fluorescence from the membrane dye is colored in green and FtsZ-A647 signal is in magenta. Only the composite images are displayed. Arrows indicate membrane septa with co-localized FtsZ. Scale bars represent 5 µm.

regulation of the local concentration of ZapA might play a role in the constriction of the FtsZ ring.

Collectively, the results demonstrate that gene-based reconstitution of membrane-constricting cytoskeletal protein filaments within liposomes is feasible. Moreover, FtsA and FtsZ form the minimal architecture to establish E. coli cell division proto-rings from native constituents in vitro.

**Discussion**

Compartmentalization of PURE system and of the Z-ring constituents inside liposomes provides a realistic cellular environment. Purified FtsZ and FtsA proteins have already been enclosed within small (diameter < 200 nm) liposomes or giant vesicles. In these earlier studies, membrane-tethered protofilaments of FtsZ could assemble with FtsA*, a mutant of FtsA that cannot polymerize. In another report, the FtsZ-sfGFP fusion protein was recruited to the membrane of giant liposomes (diameter 15–100 µm) by FtsA in the presence of Ficoll. In their work, Furusato et al. reported a homogenous recruitment of FtsZ to the membrane in the presence of FtsA but no membrane deformation. Local reshaping of liposomes was exclusively observed with ZipA as the FtsZ membrane anchor, but no constriction sites nor protein ring-like structures were observed. Here, we show that wild-type FtsA and FtsZ are capable to deform the membrane in PURE system-loaded liposomes with a size <15 µm. The FtsA-dependent recruitment of FtsZ on the membrane frequently induces the formation of FtsZ clusters that constrict the liposome membrane into bud necks. It is clear from our data that FtsZ, assisted by FtsA, does not preferentially accommodate to pre-existing membrane areas with a high curvature. Conversely, membrane constriction and extended neck-like regions connecting the mother and budding vesicles are the product of localized FtsZ-FtsA pattern assembly. Noteworthily, these types of membrane remodeling, cytoskeletal protein organization, and dynamic blebbing structures were not observed in previous reports.

Not every liposome exhibits the same phenotype with regard to FtsZ recruitment and membrane deformation. This disparity is presumably the manifestation of the probabilistic encapsulation of all PURE system components and DNA, which leads to a large heterogeneity in FtsA expression levels, as recently quantified with a fluorescence reporter gene. It is therefore difficult to know the precise concentration of synthesized FtsA in individual liposomes and to correlate it with a particular phenotype.

Further investigations will be necessary for directing the assembly of an all-gene-based contractile FtsZ proto-ring that can divide liposomes. Although we do not exclude that assisting proteins, such as the Min system and the FtsZ-interacting partners SlmA and ZapB, might have to be introduced to complete membrane scission and release budding vesicles, the present results suggest that expression of FtsA and FtsZ might suffice to generate daughter vesicles of a few microns in size. The precise timing of protein interaction is essential for the hierarchical assembly of the proto-ring. This represents a major issue that is inherent to in-liposome compartmentalization of purified cytoskeletal proteins or with temporally unregulated expression of multiple genes. An additional level of temporal control that might be decisive for sequential assembly of the Z-ring constituents could be provided by regulating the expression of individual genes through transcriptional circuits, such as cascade or feedback motifs. Mindful of the limitations to apprehend the PURE system and to rationally design liposomes harboring desirable properties encoded in genes, we believe that in vitro evolutionary optimization, by exploring a wide genetic diversity, provides additional opportunities to build cellular functions, and FtsZ proto-rings in particular.
fresh prefiltered liquid lysogeny broth (LB) medium and incubated for 1 h at 37 °C and 250 rpm. The cultures were plated on solid LB medium with ampicillin and grew overnight at 37 °C. Colonies were picked up and cultured in 1 mL of liquid LB medium with 50 µg mL⁻¹ of ampicillin in 1.5-mL Eppendorf tubes for 6 h at 37 °C and 250 rpm. Plasmid purification was performed using the PureYield™ Plasmid Miniprep System (column method, Promega). Plasmid concentration and purity were checked on a Nanodrop. Linear templates for PURE system reactions were prepared by PCR using the plasmids as substrates with primers 194 and 709 (Supplementary Table 4). Amplification products were checked on a 1% agarose gel and were purified using the Wizard SV Gel kit. DNA concentration and purity were measured using a ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies).

The DNA fragment containing the zapA gene (original sequence from E. coli K12 strain) was inserted in a pIDTSMART-AMP plasmid (Integrated DNA Technologies).

The cultures were plated on solid LB medium with ampicillin and 250 rpm. Plasmid purification (Supplementary Table 4). Amplification products were checked on a 1% agarose gel and were purified using the Wizard SV Gel kit. DNA concentration and purity were measured using a ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies).

The DNA fragment containing the zapA gene (original sequence from E. coli K12 strain) was inserted in a pIDTSMART-AMP plasmid (Integrated DNA Technologies).

**LC-MS/MS analysis.** LC-MS/MS analysis was performed on a 6460 Triple Quad LC/MS system (Agilent Technologies, USA) using Skyline software. A, in all, 7 µL of sample was injected per run to an ACQUITY UPLC® PepMap CSH® C18 Column (Waters Corporation, USA). The peptides were separated in a gradient of buffer A (25 mM formic acid in MilliQ water) and buffer B (50 mM formic acid in acetic- one) at a flow rate of 500 µL per minute and at a column temperature of 40 °C. The column was equilibrated with 98% buffer A. After injection, the gradient was changed linearly over 20 min to 70% buffer A over the next 4 min to 60% buffer A, and over the next 30 s to 20% buffer A. This ratio was held for another 3 min and the column was finally flushed with 98% buffer A to equilibrate for the next run. Selected peptides were measured by multiple reaction monitoring. For both ZapA and FtsA two peptides were present in the QconCAT. In addition, two peptides from ribosomal proteins were also measured as control.

**Labeling of in vitro synthesized proteins and gel analysis.** PUREflex2.0 reaction mixtures were supplemented with 1 µL of Greenfluorotect™ (GlycoLight, Promega) gene expression was performed in a test tube as described above. Samples were treated with RNase (RNaseA Solution, Promega) for 30 min and proteins were denatured for 10 min at 90 °C in 2x SDS loading buffer with 10 mM DTT. Samples were loaded on a 18% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gel. Visualization of the fluorescently labeled translation products was performed as a fluorescence gel imager (Typhoon, Amersham Biosciences) using a 488-nm laser and a band pass emission filter of 520 nm.

**Fabrication and cleaning of the imaging chambers.** Home-made glass chambers were used in both SLB and liposome experiments. Three microscopy glass slides (1-mm thick) were glued on top of each other with NOA 61 glue (Norland Products) and holes with a diameter of 2.5 mm were drilled. A 150 µm-thick coverslip (Menzel-Gläser, Germany) was glued with NOA 61 to cover the apertures, creating the bottom of glass chambers. Cleaning was performed by successive washing steps of 10 min each in a bath sonicator (Sonorex digitec, Bandelin), as follows: chloroform and methanol (1:1 volume ratio), 2% Hellmanex, 1 M KOH, 100% ethanol and finally MilliQ water. For SLB experiments the glass chambers were further treated every two to three experiments with Acid Piranha.

**Preparation of small unilamellar vesicles.** Small unilamellar vesicles (SUVs) were used as precursors for the formation of SLBs. Lipids DOPC (4 µmol), DOPG (1 µmol) and DGS-NTA (0.25 µmol), all dissolved in chloroform (Avanti Polar Lipids), were mixed in a glass vial. A lipid film was deposited on the wall of the vial upon solvent evaporation by applying a gentle flow of argon and was further desiccated for 30 min at room temperature. The lipid film was reconstituted with 400 µL of SLB buffer (50 mM Tris, 300 mM KCl, 5 mM MgCl₂, pH 7.5) and the solution was vortexed for a few minutes. The final lipid concentration was 1.25 mg mL⁻¹. A two-step extrusion (each of 11 passages) was carried out using the Avanti mini extruder (Avanti Polar Lipids) equipped with 250 µL Hamilton syringes. Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE-TexasRed) was from Invitrogen.

**Formation of SLBs.** The imaging chamber was treated with oxygen plasma (Harrick Plasma basic plasma cleaner) for 30 min to activate the glass surface. Immediately after plasma cleaning the SUV solution was added to the sample reservoir at a final lipid concentration of 0.94 mg mL⁻¹ together with 3 mM CaCl₂. The chamber was closed by sticking a coverslip using a double-sided adhesive silicone sheet (Life Technologies) and the sample was incubated for 30 min at 37 °C. Next, the chamber was opened and the SLB was carefully washed six times with SLB buffer. Under these conditions, the SLB contains 4.8 mol % of 18:1 DGS-NTA (N₁⁺) lipids, which is within the range studied by ref. 25 (0.5–10 mol %). Similar results were also found in ref. 25 (0.02–0.08 mol % of full-length ZipA, DGS-NTA lipid was not used in this study) and lower than in ref. 25 (10 mol %).
Activity assays on supported membranes. In the experiments involving 
\( \text{ZipA-A488} \), \( 1 \mu \text{L} \) of the purified protein was first incubated on top of an SLB for 10 min 
at room temperature. The SLB was washed with \( 10 \mu \text{L} \) reaction buffer (50 mM Tris-
HCl, 150 mM KC1, 5 mM MgCl\( _2 \), pH 7.5). Then, \( 20 \mu \text{L} \) of sample (composition is 
specified where relevant) was added on top of the SLB and the chamber was sealed 
by sticking a \( 20 \times 20 \text{ mm} \) coverslip with a double-sided adhesive silicone sheet. In 
the experiments with FtsA, the \( \text{FtsA} \) or \( \text{FtsA} \) was either directly expressed on 
top of an SLB and subsequently added onto an SLB as part of the 
sample. In the earlier configuration, a \( 20 \mu \text{L} \) PUREflex2.0 reaction was carried out 
on top of an SLB and 10 \( \mu \text{L} \) were removed and replaced by the activity assay 
mixture. The exact composition of the sample varies for the different experiments 
and is where appropriate. In all cases, samples contained 2 \( \mu \text{M} \) GTP, 
supplemented with 2 \( \mu \text{M} \) ATP in FtsA experiments. In all assays without ZapA, 
Ficoll70 was added to a final concentration of 50 g L\(^{-1}\). No oxygen-scavenging 
system was used, unlike in ref. 28 but like in ref. 38.

Spinning disk microscopy. SLBs were imaged with an Olympus IX81 inverted 
fluorescence microscope equipped with a \( \times 100 \) oil immersion objective (Olympus), 
an IXon3 EMCCD-camera (Andor Technology) and a Nipkow spinning disk (CSU-
XL, Yokogawa). FtsZ-A647 and ZipA-A488 were imaged using a 640 nm and 491 
nm laser line, respectively, and appropriate emission filters (685/40 nm or 525/50 
nm). The software Andor iQ3 (Andor Technology Ltd.) was used for image 
acquisition and identical settings were used for all experiments. 
Conducting at room temperature.

Preparation of lipid-coated beads. Lipid-coated beads were prepared according 
to our published protocol\(^{35}\) with the following lipid composition: DOPE (50 mol 
%), DOPE (36 mol %), DOPG (12 mol %), 1,811 Cl (2 mol %), DSPE-PEG-biotin (1 
mass%) and DHPE-TexasRed (0.5 mass%) for a total mass of 2 mg. Lipids dis-
solved in chloroform were mixed in a round-bottom glass 

Preparation of lipid-coated beads

Production and immobilization of gene-expressing liposomes. A PUREflex2.0 
reaction mixture was assembled as described above. Either or both \( \text{ftsA} \) and 
\( \text{zipA} \) DNA constructs were added at a final concentration of 5 \( \text{nM} \) and 10 \( \text{nM} \), 
respectively. The solution was supplemented with (final concentrations indicated): 
2 \( \mu \text{M} \) GTP, 2 \( \mu \text{M} \) ATP, 3 \( \mu \text{M} \) FtsZ-A647 and MilliQ to reach a final volume of 20 
\( \mu \text{L} \). About 20 mg of lipid-coated beads was added to the solution and liposomes 
were formed by natural swelling of the lipid film for 2 h on ice, protected from 
light. During incubation, the tube was gently rotated manually a few times. Four 
freeze-thaw cycles were then applied by dipping the sample in liquid nitrogen and 

Production of lipid-coated beads

Conflacal microscopy. A Nikon A1R Laser scanning confocal microscope 

Conflacal microscopy

Fluorescence recovery after photobleaching. FRAP experiments were performed 
on an Olympus IX81 spinning disk microscope. Images were acquired using the 
following protocol: 10 frames every s, 10 frames every 250 ms, 10 frames every 2 s, 
10 frames every 4 s. Analysis of the FRAP images was performed with ImageJ\(^{35}\) using 
the FRAP profiler plug-in. The intensity of a bleached region of interest (ROI, 
29 \( \times 29 \) pixels) was measured over time and normalized to the intensity of the 

Fluorescence recovery after photobleaching

Structured illumination microscopy (SIM). 3D SIM images have been acquired 
with a Nikon SIM microscope equipped with a Nikon \( \times 100 \) and 1.49 NA Apo TIRF 
SR objective and a 640 nm laser line. The acquisition and reconstruction of the SIM 
images have been performed using the Nikon NIS element software. SIM raw 
data and their corresponding reconstructed images were quality-checked using the 
Fiji plug-in SIMcheck.\(^{55}\)

Total internal reflection fluorescence microscopy. FtsA-FtsZ ring dynamic was 
investigated using a Nikon TiE inverted fluorescence microscope equipped with an 
ILAS2 illumination system, a Plan Apo \( \times 100 \) oil immersion objective and a 2× 
Photometrics EMCCD Evolve Camera. The 640 nm laser line was used in com-
bination with appropriate emission filters to image FtsZ-A647. MetaMorph\(^*\) 
Microscopy Automation Software was used for image acquisition.

Quantitative image analysis. Image analysis was performed using Mathematica 
(Wolfram Research, version 11.3). All images were corrected for uneven illumina-
tion by applying a Gaussian filter with radius 70 pixels to each image, fitting a 
third-degree polynomial to the filtered image, and dividing the original image 
pixel-by-pixel with the fitted polynomial. To segment the filaments a ridge filter 
with sigma = 1 was applied and the resulting image was binarized using mor-
phological binarization with the default parameters. When needed this image was 
convolved with a Laplacian-of-Gaussian filter with radius two pixels and inverted 
(necessary for images with thin filaments). Filament thicknesses were calculated 
from this image as the distance of the centroid of filaments to the edge using the 
distance transform function. Branch point density and filament curvature were 
calculated after the thinning operation was applied to the segmented image. 

Quantitative image analysis

Statistics and reproducibility. All experiments reported in this study have been 
reproduced and similar results have been obtained. Microscopy images displayed in 
the main text figures are representative of the sample properties as analyzed from 
larger fields of view in at least three independent biological repeats.

Reporting summary. Further information on research design is available in the Nature 
Research Reporting Summary linked to this article.

Data availability

All data and custom codes generated during the current study are available from 
the corresponding author on reasonable request. Source data underlying the plots shown 
in Supplementary Figures are provided in Supplementary Data 1.

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Author contributions
C.D. conceived and supervised the research. E.G., J.N., and C.D. designed the experiments. E.G., J.N., A.D., and L.Z. performed the experiments. E.G., J.N., L.Z., A.D., and C.D. analyzed the data. C.D. and E.G. wrote the paper. M.I. and G.R. provided the purified FtsZ and ZipA proteins. All authors discussed the results and gave inputs on the manuscript.

Competing interests
The authors declare no competing interests.

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