Molecular Self-Organization and Pattern Formation during Bacterial Cell Division - A Cell-Free Reconstitution Approach

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Abstract

Spatiotemporal organization and pattern formation of proteins is of pivotal importance for the process of cell division. However, how different cellular processes are precisely coordinated and how additional parameters, such as cell shape and volume, contribute to cell division is not well understood. In this cumulative PhD thesis self-organizing cell division proteins of the model organism *Escherichia coli* have been purified and reconstituted in cell-free systems. The cell-free approach enabled us to study protein self-organization in well-defined environments and helped to understand how individual cellular parameters, such as membrane charge, cell-shape, protein concentrations and molecular interactions contribute to cellular organization during cell division.

This thesis contains three papers and an additional results section and particularly focuses on the cell-free reconstitution of two interacting protein systems. On the one hand side the cell division protein FtsZ, which assembles as one of the first proteins into a ring like structure at the future division site. On the other hand side the Min protein system comprising the proteins MinC, MinD and MinE. The Min proteins oscillate from pole-to-pole in living cells, and form on time-average a bipolar gradient which dictates assembly of the division machinery in *E.coli* to the middle of the cell by interacting with FtsZ.

In the first paper a cell-free reconstitution assay was developed to control the spatial orientation of self-organizing Min protein patterns. Previously, it has been shown that reconstituting a minimal system comprising only the two proteins MinD and MinE, ATP as an energy source and a flat supported lipid membrane, functioning as a substrate for binding and unbinding reactions, results in the formation of randomly oriented protein surface-waves on the lipid bilayer (Loose, Fischer-Friedrich, Ries, Kruse & Schwille 2008). Here, we demonstrated that the orientation of Min protein waves can be controlled over wide areas by using solid membrane supports with parallel microgrooves.

In the second paper we accomplished the reconstitution of pole-to-pole oscillations of the Min proteins for the first time. A novel reconstitution assay based membrane-

clad micro compartments was developed to enclose proteins in picoliter sample volumes of defined shape. In contrast to wave-like Min protein patterns on flat membranes, the confinement into a small compartment resulted in the spatiotemporal oscillatory pattern, also observed in living cells. These experiments provided evidence that compartment shape and volume are important factors for Min protein organization *in vitro* and more generally demonstrated that cell shape is a critical factor for the proliferation of cells.

In the third paper the the biomimetic system for Min protein oscillations was applied to answer three major questions about Min protein mediated cell organization. First, how are different parameters contributing to bipolar gradient formation of the Min protein system? To answer this question, the formation of time-averaged bipolar concentration gradients was first verified *in vitro* and then different parameters, such as membrane charge or compartment width and length were systematically modified. Second, how are Min proteins equally distributed to the two daughter cells after cell division? To address this question, compartments mimicking different stages during cell division were engineered and the modes of Min protein patterns within these compartments analyzed. Our results provided the first direct experimental evidence, that Min proteins can be partitioned to the two daughter cells by a geometry-based mechanism. Third, can downstream targets be positioned by the reconstituted Min protein oscillations? By co-reconstituting the cell division protein FtsZ with the Min protein system *in vitro*, we demonstrated that indeed FtsZ can be positioned to the middle of a synthetic compartment.

In an additional chapter, the reconstitution of Min proteins and FtsZ in water-in-oil droplets is described. Water-in-oil droplets are an alternative system to reconstitute FtsZ-structures and Min protein patterns in small volumes. In contrast to the open microstructures that have an air-water interface on top, membrane-clad droplets are fully enclosed by membranes and thus allow protein self-organization along the complete boundary of the sample volume. Using a droplet system, we demonstrate different oscillation modes of the Min system and the formation of FtsZ-rings and -networks in droplets.

Zusammenfassung

Die raumzeitliche Organisation und Musterbildung von Proteinen ist für den Ablauf der Zellteilung von herausragender Bedeutung. Wie die unterschiedlichen Bestandteile einer Zelle koordiniert werden und wie weitere Parameter, wie z.B. die Zellform und das Zellvolumen, zur Zellteilung beitragen ist dabei allerdings noch nicht vollständig verstanden. In dieser kumulativen Doktorarbeit wurden selbst-organisierende Zellteilungsproteine des Modelorganismus *Escherichia coli* aufgereinigt und in zellfreien Systemen rekonstituiert. Die zellfreie Herangehensweise ermöglichte es uns Proteinselbstorganisation in wohldefinierten Umgebungen zu untersuchen und zu verstehen wie verschiedene Zellparameter, wie z.B. Membranladung, Zellform, Proteinkonzentrationen und molekulare Interaktionen zur Zellorganisation wärend der Zellteilung beitragen.

Diese Arbeit umfasst drei wissenschaftliche Veröffentlichungen sowie ein weiteres Kapitel und beschäftigt sich insbesondere mit zwei unterschiedlichen Proteinsystemen: Zum einen das Zellteilungsprotein FtsZ, welches sich als eines der ersten Proteine in eine ringförmige Struktur an der Zellteilungsebene anordnet. Zum anderen das Min-Proteinsystem, welches aus den Proteinen MinC, MinD und MinE besteht. Die Min Proteine oszillieren in lebenden Zellen von Pol zu Pol und bilden zeitlich gemittelt einen zweipoligen Konzentrationsgradienten, der den Zusammenbau der Zellteilungsmaschinerie in *E. coli* auf die Zellmitte beschränkt, indem es mit FtsZ interagiert.

In der ersten Veröffentlichung wurde ein zellfreies System entwickelt, um die räumliche Orientierung von selbtorganisierenden Min-Proteinmustern zu kontrollieren. Es wurde zuvor bereits gezeit dass die Erstellung eines Minimalsystems bestehend aus den zwei Proteinen MinD und MinE, ATP als Energiequelle und einer flachen festkörperunterstützten Membran zur Entstehung von zufällig orientierten Protein-Oberflächenwellen auf der Membran führen (Loose et al. 2008). Hier zeigen wir, dass die Orientierung der Min-Proteinwellen über weite Flächen kontrolliert werden kann, wenn Membranunterlagen mit parallelen Mikrorillen verwendet werden.

In der zweiten Veröffentlichung haben wir die allererste Rekonstitution der Pol-zu-Pol Oszillationen der Min-Proteine zeigen können. Dazu wurde ein neues Protokoll basierend auf membranausgekleideten Mikrokompartimenten entwickelt, um Proteine in Picoliter-Probenvolumen mit definierter Form einzuschließen. Im Gegensatz zu wellenförmigen Min-Proteinmustern auf flachen Membranen führte die Eingrenzung in kleine Kompartimente zu den physiologisch relevanten Oszillationsmustern. Diese Experimente bewiesen, dass Kompartimentform und -volumen wichtige Parameter für die Min-Proteinorganisation *in vitro* sind und zeigten im Allgemeinen das die Zellform ein wichtiger Faktor für spezifische zelluläre Prozesse ist.

In der dritten Veröffentlichung, wurde das biomimetische Min-Oszillationssystem angewendet um drei zentrale Fragen bezüglich der Min-Protein basierten Zellorganisation zu beantworten. Erstens, wie tragen unterschiedliche Parameter zur Bildung von bipolaren Gradienten des Min-Proteinsystems bei? Um diese Frage zu beantworten, wurde zunächst die Bildung der zeitlich gemittelten bipolaren Gradienten *in vitro* verifiziert und dann verschiedene Parameter, z.B. Membranladung oder Breite und Länge der Kompartimente systematisch variiert. Zweitens, wie werden die Min-Proteine bei der Zellteilung gleichmäßig auf die Tochterzellen aufgeteilt? Um diese Frage zu beantworten, wurden Kompartimente verwendet, welche die unterschiedlichen Stufen der Zellteilung nachahmen und die Moden der entstehenden Min-Proteinmuster analysiert. Unsere Resultate haben den ersten direkten experimentellen Nachweis geliefert, das die Min-Proteine durch einen geometrisch modulierten Mechanismus aufgeteilt werden. Drittens, können Interaktionspartner durch die rekonstituierten Min-Proteinoszillationen lokalisiert werden? Durch gleichzeitiges Rekonstitution des Zellteilungsproteins FtsZ mit dem Min-Proteinsystem *in vitro* konnten wir zeigen, dass FtsZ in der Mitte eines künstlichen Kompartimentes positioniert werden kann.

In einem zusätzlichen Kapitel, wird die Rekonstitution von Min-Proteinen und FtsZ in Wasser-in-Öl-Tröpfchen beschrieben. Wasser-in-Öl-Tröpfchen sind ein alternatives System um FtsZ-Strukturen und Min-Proteinmuster in kleinen Volumina zu rekonstituieren. Membranumhüllte Tröpfchen sind im Vergleich zu Mikrokompartimenten, welche oben offen sind, vollständig von Membranen umschloßen und ermöglichen Proteinen daher sich entlang der kompletten Umgrenzung des Probenvolumens selbst zu organisieren. Unter Verwendung des Tröpfchen-Systems wurden unterschiedliche Oszillationsmoden des Min-Systems und die Assemblierung von FtsZ-Ringen sowie -Netzwerken gezeigt.

Chapter 1

Introduction

Cell division is one of the most fundamental processes of living matter and compromising the process of cell division severely affects the health and viability of organisms. Therefore, cells developed mechanisms and regulatory systems to tightly control cell division in space and time. Although such protein interaction networks for the regulation of cell division in living cells are highly complex, these control mechanisms are ultimately based on fundamental physical and biochemical principles. Thus, it is intriguing to ask what these principles are and how we can tackle the process of cell division on a systems level? Is it feasible to identify the minimal necessary set of interactions that is able to divide a cell and describe cell division in terms of physical laws?

To answer these questions a combination of technical developments and biological expertise is required and the collaborative effort of physicists, biologists, chemists and engineers is of pivotal importance. Along these lines the major focus of this thesis is to study the influence of physical parameters and experimental boundary contitions on specific subcellular self-organization processes during cell division using model membranes, micro fabrication technology and fluorescence microscopy. However a thorough understanding of the biological context and biochemical handling of proteins was required as well. Thus, before introducing the more technical part about *in vitro* reconstitution technologies in chapter 1.2, I will shortly introduce the biological framework of this study in chapter 1.1.

Clearly, the process of cell division and its regulatory mechanisms require out-of-equilibrium dynamics of the molecules involved in this process. Morphological changes, such as cell constriction and positioning of cell division proteins to particular well-defined positions within a cell cannot be achieved by a well-mixed solution of proteins in equilibrium. Thus, dynamic self-assembly processes of proteins under the consumption of chemical

energy in form of ATP or GTP play a major role in the organization of all cells. This thesis focuses in particular on two different self-organizing protein systems involved in cell division of the model bacterium *Escherichia coli*. On the one hand side, FtsZ - a GTP hydrolyzing protein which self-assembles into a ring-like structure (the Z-ring) and localizes as one of the first proteins at the division site, where it functions as a scaffold to recruit further proteins and was suggested to play an active role in membrane deformation and constriction. On the other hand side, the Min system - a protein system which self-organizes into oscillatory protein patterns and regulates the positioning of the cell division site, by inhibiting FtsZ localization at the cell poles. To address the fundamental principles of spatial organization during cell division, we analyzed these self-assembling hallmark proteins of cell-division in well-controlled cell-free environments, where their molecular properties and dependencies on biochemical parameters can systematically and quantitatively be analyzed.

1.1 Bacterial cell division

In order to proliferate many bacteria divide by binary fission, which is a kind of asexual reproduction during which cells divide after doubling their volumes. The rod shaped model bacterium *E. coli* typically elongates to approximately double its size, divides in the middle with respect to its length axis and gives rise to two equally sized daughter cells (Figure 1.1 A). Thereby, the number of bacteria increases exponentially until availability of nutrients or environmental conditions become limiting factors for proliferation. A requirement for binary fission is the successfull duplication and segregation of chromosomes and subsequent splitting of the cytoplasm, a process that involves invagination and fission of the plasma membrane.

A conserved key player during cell division is the tubulin homolog FtsZ. The assembly of FtsZ as a peripheral ring-like structure at the future division site initiates assembly of the divisome, a multimolecular division machine (Figure 1.1 B). Thereby FtsZ has the role of a scaffold and evidence suggests that FtsZ also contributes to cell constriction. Interestingly, the concentration of FtsZ appears to be constant without significant changes during the live cycle of bacteria. Thus, the question arises what determines the temporal and spatial assembly of FtsZ. One possibility would be a mechanism by which the cell length of a growing *E. coli* cell is ultimately used as a cue to determine the time point for FtsZ assembly and cell division. In addition, FtsZ-interacting proteins might affect the timepoint of FtsZ assembly.

Indeed, several FtsZ interacting proteins have been identified, which positively or negatively regulate assembly of FtsZ and contribute to the spatiotemporal assembly of the so called FtsZ ring (Huang, Durand-Heredia & Janakiraman 2013, Lutkenhaus 2007). In particular, in many bacteria the localization of FtsZ to the region of cell division appears to be determined by nucleoid associated FtsZ inhibitors and bipolar gradients of negative regulators. Thereby, regulatory proteins which are associated with the chromosome, such as SlmA in *E. coli* inhibit FtsZ assembly across the chromosomes - a process called nucleoid occlusion (Bernhardt & de Boer 2005). Additional regulatory protein systems generate bipolar gradients in many bacteria and inhibit cell division at the cell poles. In *E. coli* the Min protein system comprising the proteins MinC, MinD and MinE forms dynamic patters which oscillate from pole to pole and result on time-average in bipolar concentration gradients (Raskin & de Boer 1999*b*, Raskin & de Boer 1999*a*, Hale, Meinhardt & de Boer 2001, Fu, Shih, Zhang & Rothfield 2001). In *Bacillus subtilis* static concentration gradients of the protein DivIVA are generated, with the DivIVA gradient being established through the recognition of the negative curvature of the cell poles. Division inhibitors are then recruited to the static DivIVA gradient (Lenarcic, Halbedel, Visser, Shaw, Wu, Errington, Marenduzzo & Hamoen 2009). Other examples of gradient forming protein systems involve the MipZ gradient in the bacterium *Caulobacter crescentus* (Thanbichler & Shapiro 2006) and even specific eukaryotic cells, such as fission yeast in which a Pom1 gradient regulates the onset of mitosis, employ bipolar gradients to define the future cell division side (Celton-Morizur, Racine, Sibarita & Paoletti 2006, Padte, Martin, Howard & Chang 2006, Martin & Berthelot-Grosjean 2009, Moseley, Mayeux, Paoletti & Nurse 2009). Thus studying the mechanisms of FtsZ regulation does not only help to understand the particular regulatory protein system under investigation, but might also be of high general significance for the field of cell division and protein self-organization in general.

1.1.1 FtsZ

FtsZ is an essential cell division protein in *E. coli* and is highly conserved in prokaryotes. In 1991 immunoelectron microscope studies demonstrated for the first time that FtsZ assembles into a ring-like structure at the division site (Bi & Lutkenhaus 1991). Along with this study it has been speculated that the FtsZ-ring might represent a structure through which cell division is regulated. Clearly, for such a function FtsZ needs to be associated with the plasma membrane. Subsequent studies verified the localization of FtsZ at the future division site using GFP-fusions and demonstrated its colocalization with FtsA

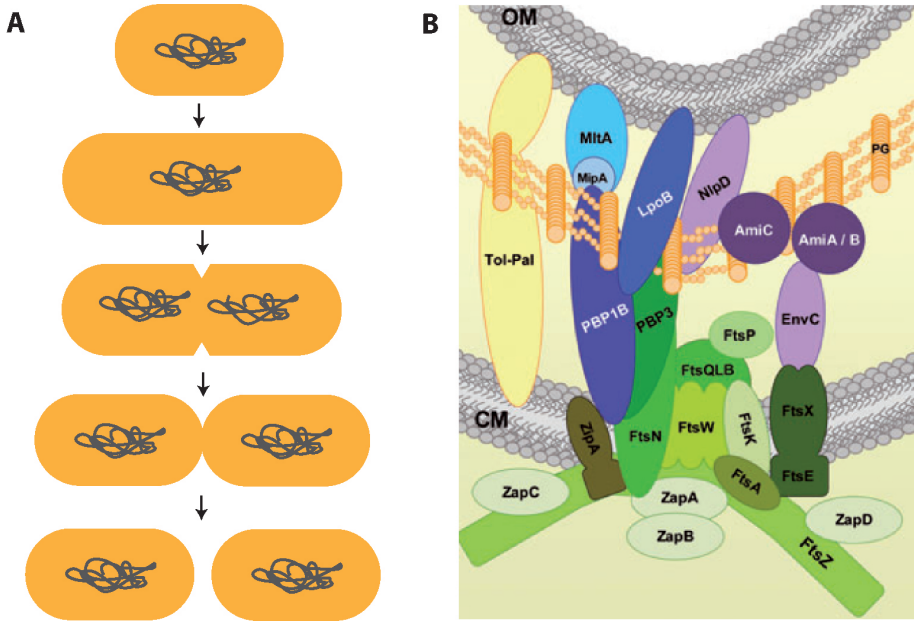

Figure 1.1: Binary cell division of *E. coli*. (A) The rod-shaped bacterium *E.coli* divides in the middle of the cell after chromosome replication. (B) The divisome is a large complex of multiple proteins. CM: cytoplasma membrane, OM: outer membrane (Egan & Vollmer 2013)

and ZipA (Ma, Ehrhardt & Margolin 1996, Hale & de Boer 1997). While FtsZ itself is a cytoplasmic protein without significant affinity for the membrane, the two FtsZ interacting proteins FtsA and ZipA mediate the contact with the plasma membrane and are required for cell division in *E. coli* (Rico, Krupka & Vicente 2013). ZipA is a transmembrane protein that interacts with the C-terminal domain of FtsZ (Liu, Mukherjee & Lutkenhaus 1999). FtsA is a protein which interacts with the membrane via an amphipatic helic and also binds to the conserved C-terminus of FtsZ (Din, Quardokus, Sackett & Brun 1998). The correct ratio of FtsA and FtsZ are critical for cell division, whereby simultaneous overexpression of both proteins results in increased frequency of cell division (Dai & Lutkenhaus 1992, Dewar, Begg & Donachie 1992, Begg, Nikolaichik, Crossland & Donachie 1998). Additional FtsZ associated proteins, such as ZapA are non-essiential, but modulate the assembly of FtsZ-rings (Gueiros-Filho & Losick 2002)

Following the dynamics of fluorescently labeled FtsZ over the time of the bacterial life cycle, it has been shown that new FtsZ rings formed often already at the new future division sites when the old ring was still in its final constriction. Thus, FtsZ can be positioned to its future division site even before the previous division is completed (Sun & Margolin 1998). However, the exact function of FtsZ, apart from being a scaffold for other cell division proteins, is still controversial. In particular, the role of FtsZ during the constriction of the cell is subject to several recent studies. As FtsZ assembles into a large ring-like structure at the division site and is able to hydrolyze GTP, it has been intriguing to hypothesize that FtsZ might provide the force for cell constriction. However, recent evidence suggest, that FtsZ is dissociated from the division site before cell fission, indicating that FtsZ might not be involved in the final constriction and that this process is mediated by unknown factors (Figure 1.2) (Söderström, Skoog, Blom, Weiss, von Heijne & Daley 2014). On the other hand crystal structure analysis of FtsZ protofilaments found hydrolysis depended bending between FtsZ subunits and suggested that this bending might provide sufficient force for cell division(Li, Hsin, Zhao, Cheng, Shang, Huang, Wang & Ye 2013). In addition, *in vitro* experiments suggest that FtsZ is able to constrict the membrane of vesicles (Osawa & Erickson 2013, Osawa, Anderson & Erickson 2008), but quantitative measurements of the generated force in artificial systems is still missing. Thus, to date it is still not fully understood to what extent FtsZ participates in force generation and whether it actively constricts the plasma membrane *in vivo* or whether it facilitates cell division by setting the membrane at the cell division site under tension which is then signaled to further proteins. Alternatively, FtsZ could act mainly as a scaffold and the

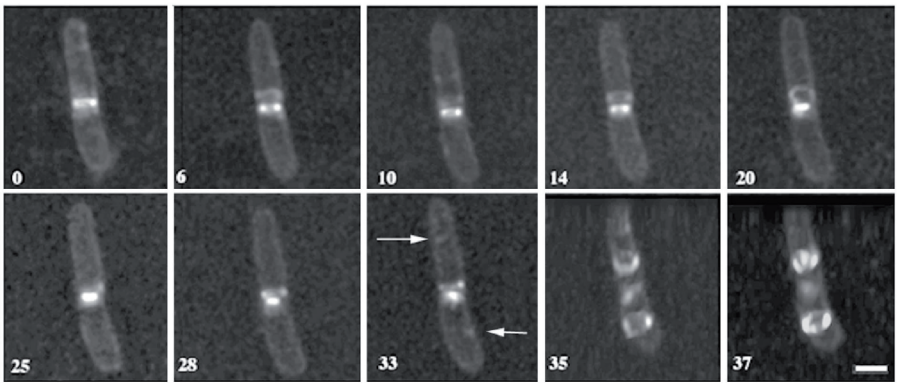

Figure 1.2: FtsZ ring formation during the live cycle of *E.coli*.
Figure adopted from (Sun & Margolin 1998)

constriction is mediated by recruited factors, such as the cell wall synthesis machine. To address how force is generated during cell division and how FtsZ is involved in this process well-defined experimental systems need to be developed to quantitatively measure the forces and identify the parameters and molecules contributing to force generation. One such system, which may be applied to force measurements in the future is presented in chapter 4 of this thesis, in which the achievement of reconstituting FtsZ in membrane-surrounded droplets is described.

Although, to date many question regarding the divisome ring in *E. coli* are still unanswered, FtsZ has been subjected to a number of molecular biological studies, providing insights of its biological importance, its structure and its dynamics. While FtsZ mutants have severe defects in cell division that result in filamentous cells (Lutkenhaus, Wolf-Watz & Donachie 1980, Ricard & Hirota 1973), overexpression of FtsZ results in an increased number of cell division sites and formation of minicells (Ward & Lutkenhaus 1985), indicating that the correct amount of FtsZ is important for division site formation. To determine the structure of FtsZ rings *in vivo*, FtsZ was fluorescently labeled and FRAP experiments as well as super-resolution microscopy were performed. Thereby, FRAP experiments revealed a fast turnover rate of ring associated FtsZ molecules demonstrating that there is a dynamic exchange between FtsZ in the ring and FtsZ in the cytoplasm (Stricker, Maddox, Salmon & Erickson 2002) and photo-activated light microscopy (PALM) experiments supported a model of randomly overlapping FtsZ protofilaments within the FtsZ ring (Fu, Huang, Buss, Coltharp, Hensel & Xiao 2010). For the dynamic turnover of FtsZ molecules a Mg^{2+}-dependent GTPase activity seems to be impor-

tant (Mukherjee & Lutkenhaus 1994, Mukherjee & Lutkenhaus 1998, RayChaudhuri & Park 1992, de Boer, Crossley & Rothfield 1992*b*). Thereby, GTP binding, but not the hydrolysis of GTP is required for FtsZ self-assembly into higher-order structures (Mukherjee & Lutkenhaus 1994, Bramhill & Thompson 1994) and GTP hydrolysis appears to play a role for the FtsZ turnover. Structures into which FtsZ can assemble *in vitro* include sheets, minirings, asters and networks (Erickson, Taylor, Taylor & Bramhill 1996, Yu & Margolin 1997).

Structurally FtsZ consists of two globular domains connected by a helix and its tertiary structure is similar to the structure of tubulin (Erickson 1995, Löwe & Amos 1998). GTP hydrolysis triggers intersubunit bending with FtsZ-GDP prefering a higher bending than FtsZ-GTP, which could on the one hand side generate force and on the other hand facilitate the turnover of FtsZ (Li et al. 2013).

1.1.2 Min protein system

Positioning of the division site at the right place requires the coordinated localization of the division machinery. In *E. coli* division site localization is achieved by two different systems. First, the nucleoid occlusion system prevents cell division across the chromosome. In *E. coli* the protein SlmA binds the chromosomes, as well as interacts with FtsZ and is directly involved in preventing FtsZ assembly accross the chromosomes (Bernhardt & de Boer 2005). Second the Min protein system inhibits cell division at the cell poles. *E. coli* mutants, defective in the Min protein system were first described in 1967. These cells often divided close to one pole, resulting in so called minicells, which were lacking chromosomal DNA (Adler, Fisher, Cohen & Hardigree 1967). The minicell locus *minB* which was mutated in minicelling *E.coli* cells was later described to encode the three proteins MinC, MinD and MinE. Employing GFP-fusions, the Min proteins were shown to perform spectacular pole-to-pole oscillations within the cell with an oscillation period of about 0.5-2 minutes, whereby MinD and MinE are required for the oscillations and MinC follows the oscillations by binding to MinD (Raskin & de Boer 1999*a*, Raskin & de Boer 1999*b*, Hu & Lutkenhaus 1999, Fu et al. 2001, Hale et al. 2001). On time average MinC and MinD thereby form a bipolar gradient with the highest concentrations at the cell poles. Underlying theses dynamic pole-to-pole oscillations is an ATP consuming diffusion-reaction mechanism and the interactions of lipid membranes with the Min protein system.

MinC: MinC is the actual inhibitor of cell division (de Boer, Crossley & Rothfield

1992*a*, de Boer, Crossley & Rothfield 1989, de Boer, Crossley & Rothfield 1990) and has two functional domains (Hu & Lutkenhaus 2000, Cordell, Anderson & Lowe 2001), which are both required for polar inhibition of cell division. The N-terminal domain interferes with FtsZ-FtsZ interactions and shortens FtsZ filaments (Hu & Lutkenhaus 2000, Dajkovic, Lan, Sun, Wirtz & Lutkenhaus 2008). The C-terminal domain is required for dimerization of MinC and binds to MinD (Hu & Lutkenhaus 2000). In the presence of MinD the C-terminal domain also seems to have inhibitory activity, but in contrast to the N-terminal domain, which inhibits FtsZ-FtsZ interactions, the C-terminal domain of MinC interferes with the interaction of FtsZ and its membrane adaptor proteins (Shen & Lutkenhaus 2010, Shen & Lutkenhaus 2009).

MinD and MinE: MinD binds to the membrane as a dimer via an amphipatic helix and MinE is recruited to the membrane by binding to MinD. Then MinE stimulates the ATPase activity of MinD, upon which MinD is released from the membrane (Hu & Lutkenhaus 2001, Raskin & de Boer 1999*a*, Fu et al. 2001, Hale et al. 2001, Hu, Saez & Lutkenhaus 2003). However, it has been suggested that MinE can also interact to the membrane directly (Raskin & de Boer 1999*a*, Hu, Gogol & Lutkenhaus 2002). A recent model suggests a conformational change of MinE releasing a membrane targeting sequence (mts) upon binding to a MinD dimer (Park, Wu, Battaile, Lovell, Holyoak & Lutkenhaus 2011). Following ATP hydrolysis MinD is subsequently released from the membrane and MinE can either dissociate from the membrane or is forwarded to another MinD dimer (Figure 2.2). Thereby, MinE might be transiently associated with the membrane (Park et al. 2011). This 'Tarzan of the Jungle'-model is also in agreement with a longer residence time for MinE than for MinD in travelling Min protein waves on supported lipid membranes (Loose, Fischer-Friedrich, Herold, Kruse & Schwille 2011).

1.1.3 Lipid membranes

The cytosol of cells is surrounded by the plasma membrane, which forms a barrier to separate the inside of a cell from the environment. In addition, gram-negative bacteria such as *E. coli* have a second membrane with the cell-wall sandwiched in between these two membranes. Membrane integrated protein complexes regulate transport of molecules and are involved in numerous other functions, such as signal transduction and positioning of other proteins to the membrane.

Cellular membranes are composed of lipids, which have a polar head group and a hy-

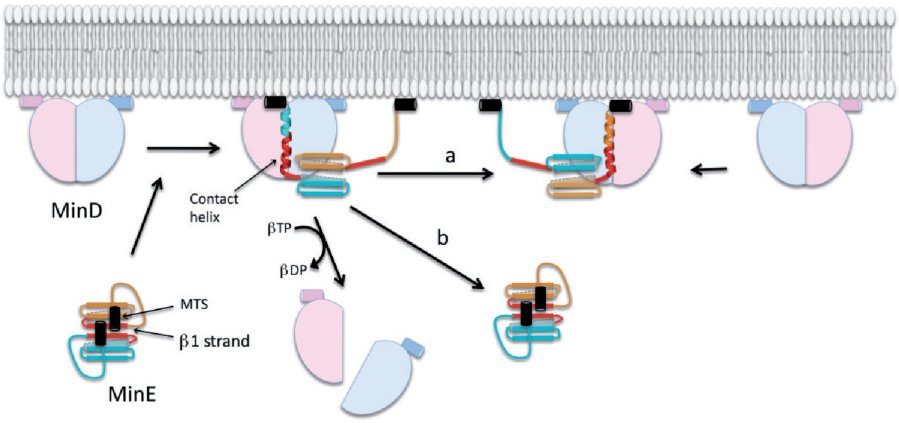

Figure 1.3: The Tarzan of the Jungle model describes the interaction of MinD and MinE on the membrane. MinE dimers bind to MinD dimers and the membrane targeting sequence of MinE is released. After ATP hydrolysis and dissociation of MinD from the membrane, MinE is either released from the membrane (a) or it is handed to another MinD. Figure reproduced from (Park et al. 2011)

drophobic tail. These lipids are assembled in a bilayer structure with a hydrophobic core and hydrophilic surfaces. In the bacterium *E. coli* the major constituents of the plasma membrane are phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CA). Thereby PE has no negative net charge, PG has a single negative charge and CA is double negatively charged. The correct lipid composition of *E. coli* membranes appears to play a critical role for cellular function, because cell membrane mutants of *E. coli* synthesizing only negatively charged lipids display severe defects in the localization and interactions of numerous proteins (Mileykovskaya, Fishov, Fu, Corbin, Margolin & Dowhan 2003, Mileykovskaya, Sun, Margolin & Dowhan 1998). Notably, cardiolipin seems to be mainly localized at the cell poles and the division sites, opening the possibility of lipid domain regulated mechanisms for cell organization (Mileykovskaya & Dowhan 2000). How the Min protein system is affected by different lipid compositions has recently been addressed in the frame of this thesis (Zieske & Schwille 2014) and by the Mizuuchi group (Vecchiarelli, Li, Mizuuchi & Mizuuchi 2014).

1.2 Cell free reconstitution approaches to investigate bacterial cell division

In order to address how cell division is regulated on a systems level, it is reasonable to start investigating cell division of relatively simple cells such as the model bacterium *E. coli*. Many key players of the division process in *E. coli* have been identified and thus, hypotheses addressing the minimal biochemical requirements for particular cellular tasks can be raised and tested. One approach to test whether a minimal set of components and parameters is indeed sufficient to give rise to certain emergent behaviors is by reconstituting the purified components in a minimal synthetic environment. Reconstituting proteins in cell free environments enables the investigation of biological processes under well-defined and simplified conditions.

1.2.1 From the discovery of intracellular oragnization to in vitro reconstitution of complex systems

Visualizing proteins by generating fusions to fluorescent tags is nowadays frequently applied to obtain information on the localization and dynamics of specific proteins *in vivo* and *in vitro*. The technique of labeling proteins fluorescently, is to a large degree, based on the discovery of GFP and only about 20 years ago, when GFP was established as a tool in the field of cell biology, direct visualization of the intracellular dynamics of proteins was rendered possible. Thus, the application of GFP to visualize intracellular proteins represents a major break-through for discovering and visualizing the intriguing dynamics and diverse subcellular localization of proteins.

Among others, the localization of the cell division protein FtsZ in *E. coli*, its membrane anchors as well as spatial regulaters for cell division were investigated using GFP-tags in the 1990s (Ma et al. 1996, Raskin & de Boer 1999*a*, Raskin & de Boer 1999*b*, Hu & Lutkenhaus 1999, Fu et al. 2001, Hale et al. 2001). In subsequent years the biochemical and structural properties of the key players of cell division were further characterized leading to models how they contribute to the process of cell division. In addition, the first evidence for an oscillating Min protein system was presented in 1999 by fusing GFP to MinD. Over the time similar experiments provided more and more insight into the internal organization of bacterial cell and evidence was gathered that bacteria are not simply "bags" with randomly diffusing proteins but are in fact highly organized. Thus, the question arose

how bacteria achieve their dynamic intracellular organization on a systems level.

Biochemical investigation of purified hallmark proteins involved in cellular organization provided important insight into the properties of individual proteins. For instance, the affinity of the Min proteins to phospholipids was discovered by incubating the respective proteins with lipid vesicles (Mileykovskaya et al. 2003). In addition, the ability of specific proteins to hydrolyze ATP or GTP or to assemble into filaments can be addressed *in vitro*. However, as the concerted action of molecules can give rise to much more complicated behavior as the sum of the individual molecules, many cellular processes cannot be explained by only considering the properties of individual proteins

Going one step further and addressing on a systems-level how exactly individual molecules act in concert to give rise to complex cellular behavior is a relatively novel approach to investigate cellular processes. Such an approach requires previous identifications and characterization of key players of cellular processes as well as intensive technical developments. Methods to systematically perturb and analyze individual interactions and assembly of molecular networks are required. In this context, a process in which life science and physics grew together was inevitable to meet the novel requirements to provide answers in quantitative life science. For example a combination of biological sample handling and establishing novel techniques along with theoretical modeling are often required to address biological questions on a systems level. Currently, we are just at the beginning to understand how protein networks act in concert and lead to complex cellular behavior, such as cell division and an exciting time of systems-level characterization of biological processes is lying ahead.

1.2.2 Cell free reconstitution of FtsZ

The cell division protein FtsZ forms filaments, bundles and networks in solution in the presence of GTP, providing evidence that the assembly into higher order structures is an intrinsic property of FtsZ and does not require membrane attachment (Mukherjee & Lutkenhaus 1994, Yu & Margolin 1997). However, in order to study how FtsZ assembly is regulated by FtsZ interacting, membrane associated protein systems or the membrane anchors FtsA and ZipA the reconstitution of FtsZ into membrane environments is required. In addition, lipid membranes might be required to gain insight into how the ring-like structure of FtsZ is established.

Thus, a histidine-tagged ZipA truncation without membrane spanning domain, which can be chemically coupled to lipid membranes, has been used as a membrane anchor

for FtsZ filaments *in vitro* (Lopez-Montero, Lopez-Navajas, Mingorance, Velez, Vicente & Monroy 2013, Lopez-Montero, Mateos-Gil, Sferrazza, Navajas, Rivas, Velez & Monroy 2012). An advantage of such an approach is that the requirement for the more challenging reconstitution of proteins containing transmembrane domains can be avoided. Employing such a reconstitution system atomic force microscope measurements provided evidence that charge of the lipid membrane is a parameter which modulates assembly of FtsZ structures, most likely by interacting with an unstructured domain of ZipA (Mateos-Gil, Marquez, Lopez-Navajas, Jimenez, Vicente, Mingorance, Rivas & Velez 2012).

Intriguingly, although FtsZ is targeted to the cell membrane by other membrane proteins (ZipA and FtsA) *in vivo*, it has been shown that FtsZ assembles into a membrane associated ring without ZipA or FtsA when the C-terminal domain of FtsZ is exchanged with the membrane targeting sequence of MinD (Osawa et al. 2008). These FtsZ rings formed spontaneously when FtsZ-mts and GTP were mixed with multilamellar tubular vesicles and moved laterally along the tubular vesicles, whereby colliding rings were able to coalesce. Intriguingly, the positions of FtsZ-rings often colocalized with vesicle membrane invaginations, indicating that these membrane deformations were generated or stabilized by FtsZ-mts (Osawa et al. 2008). Moreover, fusing a membrane targeting sequence to the C- or N-terminal domain of FtsZ induced concave and convex bulges of membranes, respectively when incubated with giant unilamellar vesicles. These experiments suggested that FtsZ filaments have a defined curvature (Osawa, Anderson & Erickson 2009). In agreement with these studies it has been shown that FtsZ-mts bundles aligned along the curvature of supported lipid membranes on curved solid supports (Arumugam, Chwastek, Fischer-Friedrich, Ehrig, Monch & Schwille 2012).

More recently, a number of studies addressed the membrane assembly of wild type FtsZ by coreconstituting FtsZ with FtsA in lipid environments (Osawa & Erickson 2013, Loose & Mitchison 2014, Szwedziak, Wang, Bharat, Tsim & Lowe 2014). Notably, it has been shown that FtsZ forms dynamic chirally rotating vortices with a diameter of about 1μm on supported lipid membranes when coreconstituted with FtsA. Thereby, FtsA appears to recruit FtsZ to the membrane and simultaneously regulates FtsZ organization negatively, resulting in complex self-organizing patterns of FtsZ (Loose & Mitchison 2014). However although, FtsA and ZipA have been reconstituted *in vitro* and biochemical characterization of the rereconstituted systems lead to important insight into FtsZ assembly, it is not yet fully understood how FtsA and ZipA work in concert to promote assembly of a functional FtsZ ring in bacteria. In addition, the picture of the force generating mechanism for

membrane constriction and fission and how assembly of the cell wall contributes to cell division is still incomplete.

Apart from dissecting self-assembly properties of FtsZ and the interaction with FtsA and ZipA, the interaction of FtsZ with regulatory proteins has been addressed. ZapA and ZapB have been shown to modify FtsZ bundling *in vitro* (Galli & Gerdes 2012) and MinC has been shown to disassemble FtsZ filaments, but much higher concentrations of MinC than in living cells were required for FtsZ disassembly (Hu, Mukherjee, Pichoff & Lutkenhaus 1999). More recently, the reconstitution of FtsZ-mts with Min protein waves on supported lipid membranes demonstrated that the localization patterns of FtsZ-mts are anticorrelated to the MinC protein patterns and that lower concentrations of MinC are required for FtsZ disassembly than in solution due to the enrichment of proteins at the membrane (Arumugam, Petrášek & Schwille 2014). However, the localization of FtsZ to a defined localization site had not been achieved. During this thesis, we therefore tackled the minimal requirements for FtsZ localization and developed a system for FtsZ positioning to the middle of a biomimetic compartment.

1.2.3 Cell free reconstitution of the Min protein system

The cell-free reconstitution of the Min protein system in well-defined environments is of significance for multiple areas of research. On the one hand side, the Min protein system represents a prototype of an active biological system, that self-organizes by a diffusion reaction mechanism and is of high significance in the field of complex system dynamics. On the other hand side its biological relevance in regulating the positioning of FtsZ is of pivotal interest in the field of bacterial cell division. Thus, reconstitution approaches of the Min protein system are aimed at both - the analysis of biophysical parameters and molecular mechanisms for Min protein pattern formation, as well as tackling the role of the Min protein system in regulating the localization of the division site.

Using flat supported membranes and purified Min proteins in ATP containing buffer it has been shown that the two proteins MinD and MinE are sufficient to self-organize into dynamic patterns on the membrane (Loose et al. 2008). The formation of Min protein patterns is thereby robust against small perturbations and persists for hours, which renders this reconstituted system ideal for characterization of biophysical parameters and interaction with other proteins. The division inhibitor MinC has been shown to follow the MinD/E patterns *in vitro* and advanced fluorescence microscopy studies using confocal and single molecule studies provided insight into the order of events during Min pattern formation

(Loose et al. 2011). In addition to reconstituting Min protein pattern on large membrane areas the Min system has been reconstituted on giant unilamellar vesicles, demonstrating that the higher mobility of proteins on freestanding membranes results in protein patterns with larger length scales than on supported lipid membranes (Martos, Petrasek & Schwille 2013). Moreover, patches of supported lipid membranes of defined geometry and a bulk buffer volume on top have been applied to regulate the direction of Min protein waves along the length axis of narrow membrane patches (Schweizer, Loose, Bonny, Kruse, Monch & Schwille 2012). In summary, the self-organizing Min protein patterns have been intensively studied and characterized. However, in contrast to the oscillatory patterns in living cells patterns of traveling waves were observed in all cell-free assays reconstituted so far. Note that, although the membrane systems (large flat bilayers, membrane patches, vesicle) differed in their geometry, the protein and membrane containing buffer solution was always present in large volumes. The reconstitution of a functional Min protein system in small compartments has been achieved in the frame of this study for the first time and experimentally shown to provide the right boundary conditions for triggering pole-to-pole oscillations of the Min system (Zieske & Schwille 2013).

1.2.4 Boundary conditions in cell-free reconstitution assays

The reconstitution of proteins in cell-free systems is a powerful tool to analyze specific properties of protein systems under defined condidions outside of the complex environment of living cells. However, a simplified model system also goes along with the absence of specific system parameters, which might influence the analyzed protein system in living cells. One aim of cell-free reconstitution investigations is to identify and systematically analyze the influence of such parameters. Thereby, factors which might influence reconstituted proteins are represented by other molecules, such as proteins and other chemical chemical components (e.g. salt, ATP, GTP), which can easily be supplemented to reconstitution systems. Alternatively, non-molecular parameters, such as electrostatic interactions with the sample boundaries or compartment geometry might alter the behavior of reconstituted systems. The latter case often requires the development of novel reconstitution systems, featuring the required parameters.

This thesis particularly focuses on how compartment geometry of cell-free reconstitution systems affects protein self-organization during bacterial cell division. To investigate proteins in micro compartments of defined geometry two major approaches are usually considered. On the one hand side vesicle and droplet assays are being developed to enclose

proteins in micro compartments with cellular volumes. By mechanical deformation these compartments can also be deformed in order to address geometry modulated effects. Thus, FtsZ in tubular vesicles has been shown to self-assemble into rings if the diameter of the vesicles is comparable to the bacterial diameter. On the other hand side micro fabricated well plate chips, channels and compartments of defined geometry are employed to define the geometrical boundaries of reconstituted systems. For example, the distribution of low-copy plasmids, which need to be actively transported to both sides of the cell in order to segregate plasmids to both daughter cells during cell division has been reconstituted using the Par system, a spindle like protein system. Reconstituting this system in micro-fabricated channels, it has been demonstrated that spindles always aligned along the long axis of the channel (Garner, Campbell, Weibel & Mullins 2007). Another study analyzed the positioning of reconstituted microtubule asters using microfabricated barriers to confine the asters in space (Laan, Pavin, Husson, Romet-Lemonne, van Duijn, Lopez, Vale, Julicher, Reck-Peterson & Dogterom 2012).

To analyze the influence of compartment geometry on complex membrane interacting protein systems, such as the Min protein system together with proteins of the divisome, posibilities and challenges of different reconstitution systems need to be considered. Encapsulation of proteins in vesicles is often challenging, since the required salt concentrations for protein stability often compromise the stability of the vesicles. For many proteins the *in vitro* reconstitution of membrane proteins in vesicles has been achieved, but usually the encapsulation required the development of individual protocols, which cannot be easily adopted to other protein systems, the percentage of vesicles containing the reconstituted protein structures is low or it is challenging to encapsulate proteins of well-defined concentration. Thus, in order to reconstitute more complex systems with numerous proteins and to perform experiments at high throughput, more paralleled assays need to be developed. The encapsulation of soluble proteins into the lumen of water-in-oil droplets is well established, and has for instance been used to analyze how droplet volume modulates the size of reconstituted mitotic spindles (Good, Vahey, Skandarajah, Fletcher & Heald 2013). The droplets can also be surrounded by lipids, but since lipids at an oil-water interface form a lipid monolayer, the reconstitution of membrane proteins is limited to peripheral membrane proteins. Nonetheless for subsets of proteins involved in cell division, such as the Min protein system and peripheral proteins of the divisome the reconstitution of proteins into droplets is a promising approach, which is addressed in this thesis to encapsulate the Min system and FtsZ. However, so far the geometry of droplets is limited to simple shapes,

that can be achieved by mechanical deformation.

Taken together, *in vitro* reconstitution is a promising approach in order to investigate the fundamental principles of cell division. Not only has *in vitro* reconstitution been successfully applied to test the minimal requirements for specific biological tasks during cell division, but also provides a promising tool to tackle how the behavior of a system changes if parameters, such as protein concentration, buffer conditions, temperature, etc., are systematically modulated in a well-defined environment. To further investigate the fundamental process of cell division and in particular to address how Min protein oscillations and gradients regulate the localization of the division site, new bottom-up assays were developed during this thesis and applied to address how compartment geometry affects cell organization.

Chapter 2

Results

This cumulative thesis consists of three papers which were published during the PhD work (P1-P3) and a section about reconstitution of cell division proteins in droplets.

1. Zieske K, Schweizer J, Schwille P (2014),Surface topology assisted alignment of Min protein waves. FEBS Lett. 588(15):2545-9.

2. Zieske K and Schwille P (2013),Reconstitution of pole-to-pole oscillations of Min proteins in microengineered polydimethylsiloxane compartments. Angew. Chem. Int. Ed., 52(1): 45962.

3. Zieske K and Schwille P (2014), Reconstitution of self-organizing protein gradients as spatial cues in cell-free systems. eLife 2014;3:e03949

2.1 Surface topology assisted alignment of Min protein waves (P1)

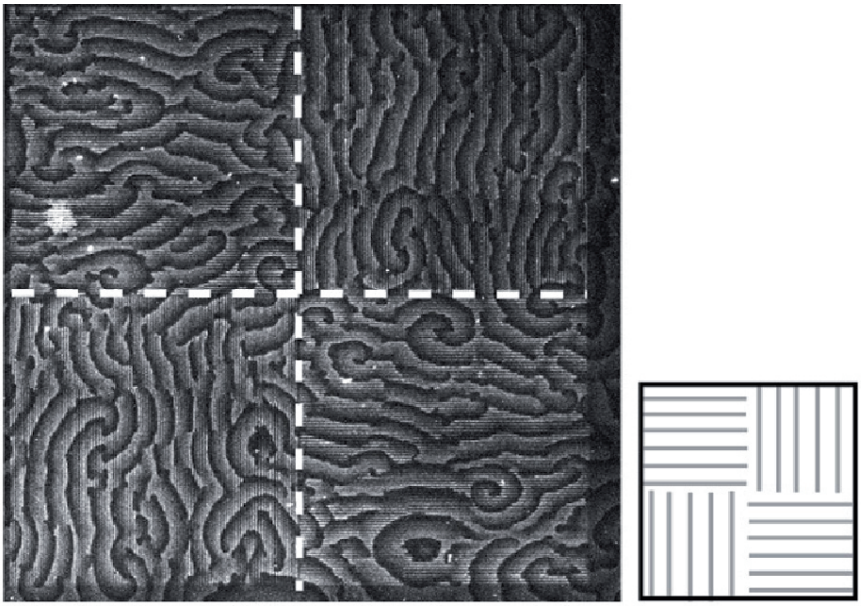

Figure 2.1: Reconstitution of Min protein alignment on micro structured surfaces. Figure reproduced from (Zieske et al. 2014)

2.1.1 Aim and summary

The Min proteins system is a biological example of how pattern formation occurs via a reaction-diffusion mechanism. However, formation of patterns by self-organization is not only a property of living cells, but can also be observed in non-living matter. A famous example for pattern formation is the Belousov-Zhabotinsky reaction a chemical non-equilibrium reaction that results in the formation of complex spatiotemporal patterns. Thus, mechanisms to perturb or control the patterns of reaction-diffusion systems, are of wide significance in the field of pattern formation.

Considering the Min proteins as a prototype for a pattern forming protein system, we addressed the question whether the formation of patterns can be modified by external cues. On flat supported membranes it has been shown that Min proteins self-organize into surface waves without any predefined direction. The most abundant Min protein patterns on flat membranes are spiral waves and parallel waves and on small membrane areas it has

been shown, that the Min protein waves preferentially move along the longest axis of the membranes. However, so far techniques to experimentally control the self-organization of Min protein waves on large membrane surface areas have not been reported.

In this paper, we addressed how topographically structured surfaces influence Min pattern formation. Min proteins were reconstituted on membrane-clad parallel and concentric grooved PDMS surfaces and found to preferentially align parallel to the grooves. Moreover, we found that Min pattern alignment starts early during Min pattern formation and that even millimeter sized areas of protein patterns with well-defined wave orientation can be generated as a result of the membrane topography. Thus, membrane topography was established as a regulatory element to influence Min protein patterns and in particular to control the orientation of the Min protein waves *in vitro*.

2.1.2 Contribution

The project was designed conjointly by Katja Zieske, Jakob Schweizer and Petra Schwille. Experiments were performed and analyzed by Katja Zieske. PIV analysis was performed by Jakob Schweizer. The manuscript was drafted by Katja Zieske. Katja Zieske, Jakob Schweizer and Petra Schwille revised the manuscript.

2.1.3 Publication

This work has been published in the journal FEBS letters.

Zieske K, Schweizer J, Schwille P (2014), FEBS Lett. 588(15):2545-9.

Surface topology assisted alignment of Min protein waves

2.2 Reconstitution of pole-to-pole oscillations of Min proteins in microengineered polydimethylsiloxane compartments (P1)

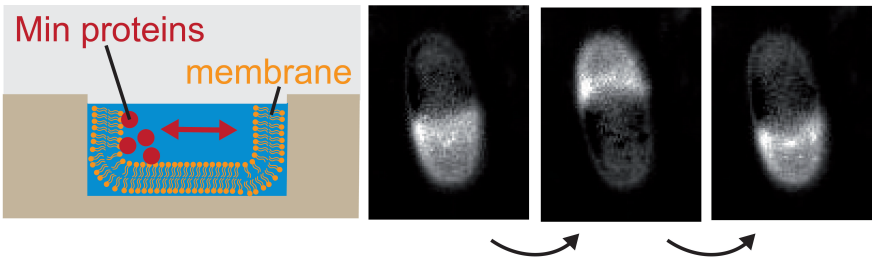

Figure 2.2: Reconstitution of pole-to-pole oscillations of Min proteins. Figure reproduced from (Zieske & Schwille 2013)

2.2.1 Aim and summary

Biological boundary conditions, such as the three-dimensional confinement within a cell can play pivotal roles in many cellular processes. However in bulk reconstitution assays, such as the reconstitution of Min proteins on flat supported membranes the role of boundary conditions cannot be addressed. On flat supported membranes the Min proteins were shown to self-organize in traveling surface waves, however in living ells the Min proteins oscillate from pole to pole. Experiments with shape mutants of *E. coli* had shown, that the Min patterns can me modified by geometry of the cell. Thus we hypothesized, that geometry and confinement might also be deterministic for generating oscillating protein patterns in live cells as compared to parallel waves on supported lipid membranes.

To test this hypothesis, Min proteins were reconstituted in cell-shaped compartments and thereby three major goals achieved. First, a novel synthetic system based on membrane-clad cell-shaped compartments was engineered to reconstitute Min proteins in micro compartments of well-defined geometry. Second, for the first time, pole-to-pole oscillations of Min protein patterns were reconstituted and evidence is provided that confinement into small volumes is a major determinant for oscillatory behavior of the Min proteins as compared to parallel waves in bulk assays. Finally, engineering compartments of different geometries, such as longer compartments and round compartments it was shown that the axis of the compartments plays a role in defining the oscillation axis of Min protein pat-

tern. Moreover, synthetic compartments of cell-shaped, filametous and round geometry supported Min protein patterns which were comparable to the patterns in living cells of the respective geometries. In summary, this study describes the development of a synthetic system for testing how cellular boundaries affect protein networks and its application to Min pattern formation.

2.2.2 Contribution

Project design, experiments and data analysis were performed by Katja Zieske. The manuscript was drafted by Katja Zieske. Petra Schwille contributed by discussing the project design and revising the manuscript.

2.2.3 Publication

This work has been published in the journal Angewandte Chemie.

Zieske K and Schwille P (2013), Angew. Chem. Int. Ed., 52(1): 45962.

Reconstitution of pole-to-pole oscillations of Min proteins in microengineered polydimethylsiloxane compartments

2.3 Reconstitution of self-organizing protein gradients as spatial cues in cell-free systems. (P3)

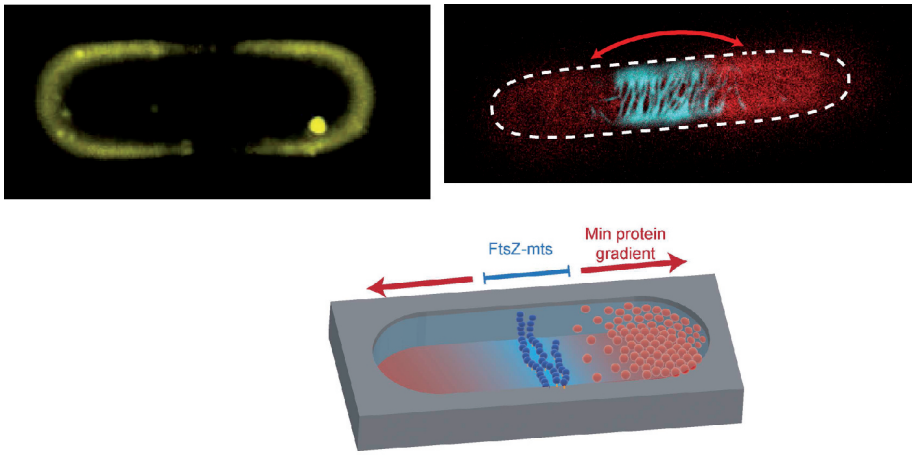

Figure 2.3: Reconstitution of FtsZ localization to the middle of a compartment (right) by Min protein gradients (left). Figure reproduced from (Zieske & Schwille 2014)

2.3.1 Aim and summary

Cell division is one of the most fundamental processes in living matter and crucially depends on the correct subcellular localization of the involved protein machinery (the divisome) in space and time. In *E. coli* a bipolar Min protein gradient plays a major role in defining the middle of the cell. However, due to the large number of biomolecules and complex interaction networks in living cells, it is challenging to dissect the elementary processes to their molecular level in living cells. To elucidate the minimal physical and biochemical requirements leading to bipolar Min gradient establishment and the correct localization of a future division site, a step-by-step *in vitro* reconstitution of protein components is thus a very promising approach. In this paper we applied the Min oscillation assay, that I have developed (Zieske & Schwille 2013) and addressed three major questions. First, what are the minimal requirements to establish bipolar Min protein gradients (a)? Second, can FtsZ be positioned to the middle of a compartment by Min protein gradients in a cell-free system (b)? Finally, how are Min proteins partitioned during cell division (c)?

(a) Analyzing the minimal requirements to establish bipolar protein gradients

To analyze the minimal requirements for Min gradient formation, we first determined the time-averaged profiles of the oscillating MinC/D division inhibitor *in vitro* and observed the *de novo* establishment of bipolar Min protein gradients by self-organization 2.3. We then determined how biophysical parameters, in particular compartment geometry and membrane compositions, affected the pole-to-pole-oscillations of Min proteins. To determine the role of cell shape for Min protein oscillations we analyzed Min protein patterns in compartments with systematically changing length and width. Stable Min pole-to-pole oscillations formed in compartments with a narrow width and a defined length interval. Thereby the minimal and maximum value of the oscillation supporting length interval differed by a factor of more than two which should be relevant for living cells as they typically double their length before they divide. In contrast, the oscillation mode switched or the oscillation axis became unstable in very short, long or wide compartments.

To test whether the membrane composition is relevant for pattern formation of the Min system we and, in a different study, the Mizuuchi-group reconstituted the Min proteins on flat supported bilayers with systematically varying amounts of the negatively charged membrane component cardiolipin, which has been suggested to be involved in Min pattern formation (Vecchiarelli et al. 2014, Zieske & Schwille 2014). Although different amounts of cardiolipin modified the patterns of the Min proteins we found that charge rather than the specific chemical composition or polar cardiolipin domains plays a role in Min pattern formation.

In addition to membrane geometry and membrane composition structural features of Min proteins might play a role in bipolar gradient formation. Reconstituting a specific truncation mutant of MinE we observed irregular patterns which did not oscillate along the length axis of the compartments. These findings indicated that specific functional domains of the Min proteins are nonessential for pattern-formation, but have a regulatory function and are required for bipolar gradient establishment.

(b) Reconstituting FtsZ localization in a cell-free system

The cell division protein FtsZ is anchored to the membrane by ZipA and FtsA in *E.coli*. However, it has been shown that FtsZ fused to the membrane targeting sequence of MinD (FtsZ-mts) self-assembles into rings when reconstituted in vesicles (Osawa et al. 2008). To investigate whether FtsZ can be localized to the middle of a compartment FtsZ-mts and Min proteins were co-reconstituted in micro compartments. We observed that FtsZ-mts

localization was limited to the middle of the compartments 2.3 and thus, we conclude that a minimal module of only a membrane compartment comprising FtsZ-mts, the Min system and GTP and ATP as energy sources are sufficient for division site localization.

(c) Elucidating a mechanism for partitioning of Min proteins during cell division

To answer the question how a cell distributes the Min proteins equally to the two daughter cells during cell division microcompartments mimicking the geometry of a bacterium with a closing septum were engineered. Reconstituting the Min system in such compartments, a symmetric double oscillation pattern was observed in compartments with a narrow septum. These results provided the first direct experimental evidence for a geometry based mechanism for distribution of Min proteins to the two daughter cells during cytokinesis.

2.3.2 Contribution

Katja Zieske designed the project with help of Petra Schwille. Katja Zieske performed experiments, analyzed data and drafted the manuscript. Petra Schwille revised the manuscript.

2.3.3 Publication

This work has been published in the journal Elife.

Zieske K and Schwille P (2014), eLife 2014;3:e03949

Reconstitution of self-organizing protein gradients as spatial cues in cell free systems.

2.4 Reconstituting cell division proteins in droplets

In living cells the cytosol is completely surrounded by the plasma membrane that provides a boundary and assembly platform for the enclosed proteins. In particular, the division machinery assembles into a membrane associated ring-like structure. Therefore, the encapsulation of cell division proteins in closed compartments is highly relevant to further study the structure and regulation of the cell division apparatus *in vitro*.

2.4.1 Reconstituting the Min protein system in droplets

We first aimed at encapsulating a functional Min protein system and focused on water-in-oil droplets as an encapsulation system. The simplest method to enclose proteins in droplets is by mixing a protein solution with oil. To generate a lipid monolayer around the droplets, a lipid mixture containing neutrally charged DOPC and negatively charged cardiolipin was dissolved in mineral oil. Then, a macroscopic droplet of a master-mix buffer including MinD, eGFP-MinD, MinE and ATP was added to the oil-lipid mixture (Figure 2.4 A). Since the water and oil phase are not mixable, the water droplet was visible as a sphere within the oil. To break the macroscopic droplet into many microdroplets the solution was repeatedly pipetted up and down and finally pipetted on a PDMS coated glass coverslip (Figure 2.4 B,C). The lipids in the oil solution thereby assembled at the water-oil interface to generate a lipid monolayer with the hydrophilic head groups in the direction of the water phase and the hydrophobic tails directed to the oil phase. When the droplets were imaged with confocal microscopy, protein patterns were observed in up to about 50% of the droplets (Figure 2.4 D). This was the first experiment resulting in encapsulation of the Min proteins into a freestanding and completely closed membrane system. A potential reason for the droplets without self-organizing Min patterns are defect membranes due to protein aggregation at the water-oil interface before the lipid membrane has formed correctly.

To analyze the modes of Min pattern formation, time lapse confocal movies were acquired. The majority of droplets had diameters of several tens of micrometers. Bigger droplets contained complex protein patterns of higher order modes. However, the smaller droplets with diameters between 10μm to 50μm showed mainly two distinct protein patterns. First, patterns that oscillated repeatedly from one side of the droplet to the other side and second, protein patterns, that moved in circles around the periphery of the droplets (Figure 2.5 A,B). These two patterns are comparable to the patterns observed in spherical

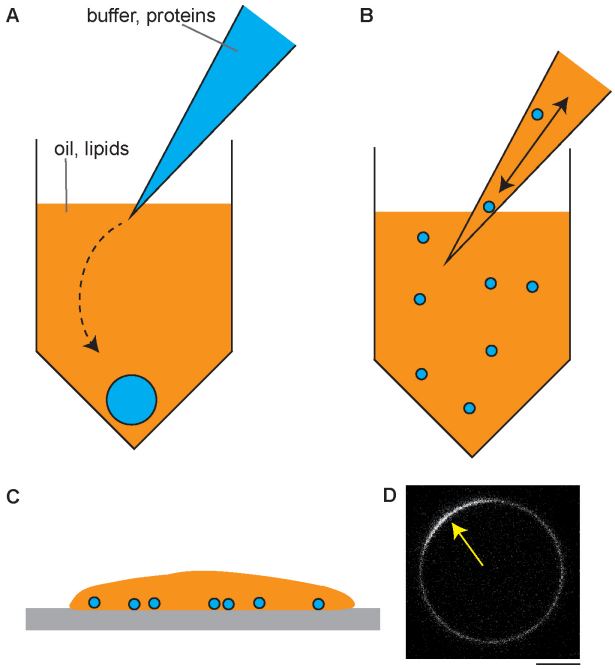

Figure 2.4: Encapsulation of Min proteins in droplets. A) Buffer (blue) with Min proteins and ATP is added to an oil-lipid solution (orange). B) The mixture is pipetted up and down several times to generate many small water-in-oil droplets. C) The droplet solution is pipetted on a passivated cover slip. D) Imaging of labeled Min proteins withing droplets reveals pattern formation of the Min proteins within the droplets. Scale bar: 10μm

living cells and round microstructures (Corbin, Yu & Margolin 2002). Notably, the end-to-end oscillation patterns were observed in droplets with larger diameter than the maximal length of microcompartments that supported pole-to-pole oscillations. This observation is in agreement with the finding that Min protein patterns on freestanding membranes have a larger wavelength than on supported lipid membranes due to the increased mobility of lipids and proteins on such membranes (Martos et al. 2013).

Interestingly, the end-to-end oscillations occurred along with temporal MinD protein oscillation within the lumen of the droplets, while only weak fluctuations were observed in droplets with patterns that moved in circles (Figure 2.5 C-E). This result demonstrated that a large fraction of available proteins participated in the formation of pole-to-pole oscillations. Comparing the signal oscilations in the lumen of the droplets with the background signal it can be estimated that at least 50% of all available MinD molecules are involved in every oscillation period. From these data, the diameter of the droplets and the approximation that half the area of a droplet is occupied at maximum membrane occupation, also an upper boundary of the average intermolecular distance of MinD molecules on the membrane can be estimated to be in the order of 10nm.

Next, the onset of formation of Min protein oscillations was investigated. Therefore we analyzed droplets directly after protein encapsulation. In contrast to the reconstitution of Min protein oscillations in microcompartments, proteins and membranes on the droplet system are not in contact before proteins are confined into small volumes. Thus, the droplet systems allowed us to investigate *de novo* protein pattern formation in confined volumes. Interestingly, we observed first a temporal oscillation of the Min proteins at the lipid membrane before the symmetry was broken and we finally observed spatiotemporal oscillations (Figure 2.6 A,B). In other words, immediately after the encapsulation of Min proteins within droplets the proteins repeatedly associated and disassociated homogeneously at the whole membrane, resulting in anticorrelated blinking of the membrane and the droplet lumen (Figure 2.6 C). Since such a protein blinking has not been observed in very large droplets and on flat supported membranes using the same concentrations of proteins, we infer that one parameter that triggers the effect of Min protein blinking is the confinement of proteins into small volumes.

To further analyze this effect of homogeneous protein attachment and detachment from the membrane, we addressed, whether Min protein blinking can be triggered on supported lipid membranes by using different parameters. Thus, the ratios of MinD and MinE on supported lipid membranes were systematically varied. Interestingly, blinking of Min pro-

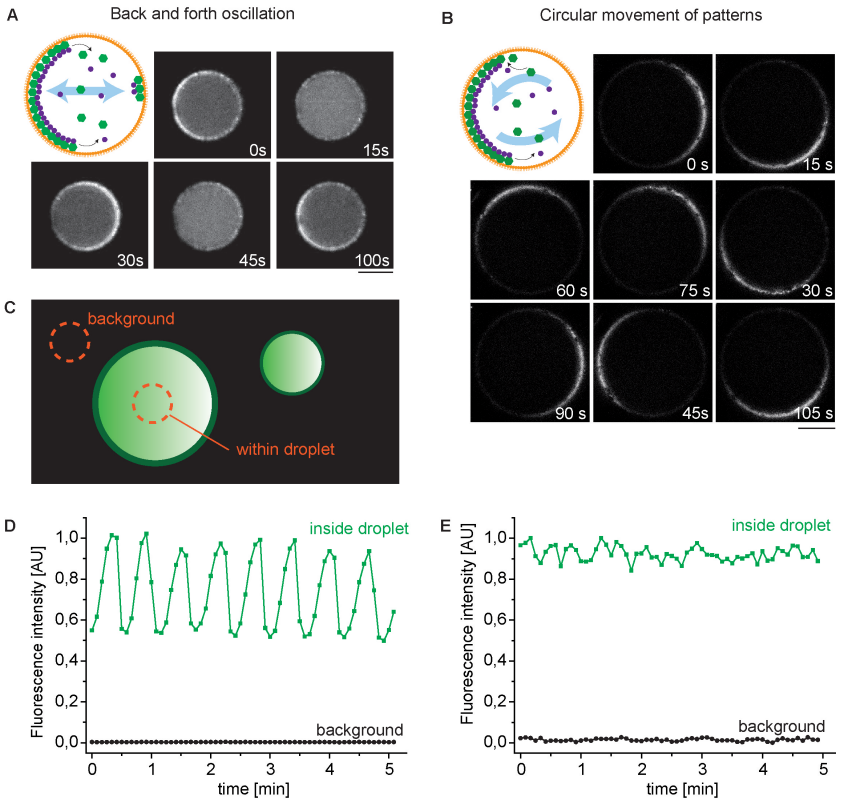

Figure 2.5: Min proteins in droplets assemble in two distinct patterns. A) Min patterns can oscillate back and forth between opposite sides of the droplet. 1μM MinD (supplemented with 10% eGFP-MinD) and 1μM MinE. Scale bar: 10μm B) Alternatively Min proteins move in circles around the boundary of the droplet. Scale bar: 10μm C) For determining the time-resolved fluorescent signal in the lumen of droplets, the fluorescence signal within the droplets and the background signal outside the droplets was recorded. The Schematic graph depicts that datapoints were acquired by averaging the signal of an area with the same size inside and outside of a droplet. D) The fluorescent signal of eGFP-MinD within the lumen of droplets oscillates in droplets with oscillating patterns. The amplitute of the lumen oscillations is about 50% of the maximal fluorescent signal. E) In droplets with circular moving patterns the fluorescent intensity within the droplets did not change significantly.

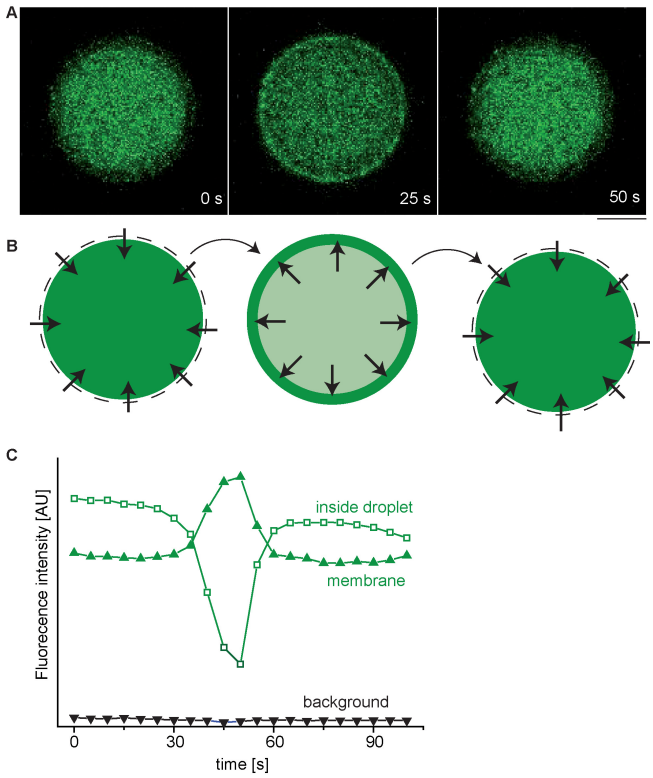

Figure 2.6: Min protein blinking at the beginning of pattern formation. Scale bar: 10μm A) After encapsulation, Min proteins produce blinking patterns until the symmetry breaks and travelling or oscillating patterns are formed. Alternating Min protein localization within the lumen of the droplet and homogeneous membrane attachment are typical for the onset of pattern formation in droplets. 1μM MinD (supplemented with 10% eGFP-MinD) and 1μM MinE B) Schrematic graph of Min Protein blinking in droplets. C) The time resolved fluorescent signals demonstrate that the blinking signal along the boundary of the droplet and within the droplet lumen are anticorrelated.

tein patterns was indeed observed when the MinE concentration was very high (3.7uM) and the membrane was very clean without small defects (Figure 2.7 A). Under these conditions the Min proteins associated and disassembled homogeneously at the membranes and these temporal oscillations persisted for more than half an hour (Figure 2.7 B). Our results demonstrate, that reconstituting Min proteins in droplets results in protein blinking at the onset of pattern formation, an oscillation mode, which had not been observed previously and which can be achieved on flat membranes by high MinE concentrations.

2.4.2 Reconstituting FtsZ-mts in droplets

FtsZ assembles into a peripheral ring structure during cell division and is attached to the membrane by the two proteins FtsA and ZipA. While the cell-free microcompartment assay, which has been developed during this PhD project (Zieske & Schwille 2013), is an ideal system to study Min protein mediated localization of FtsZ as well as the effects of the cell shape on the MinCDE/FtsZ system under well-defined conditions, alternative assays based on freestanding and closed membrane compartments are required to study formation of the full FtsZ-ring and the hypothesized Z-ring mediated force on lipid membranes. In contrast to the previous chapter, which focused on the localization of FtsZ with respect to the length axis of a compartment, we therefore aimed at reconstituting FtsZ into freestanding membrane surrounding droplets. Previously, the Erickson group accomplished the first cell-free reconstitution of a membrane attached Z-ring by encapsulating FtsZ-mts in tubular liposomes (Osawa et al. 2008). Later, the same group encapsulated a more natural ring forming protein system comprising the wild type FtsZ and the membrane anchor FtsA in vesicles and demonstrated that these vesicles constrict at the site of ring formation(Osawa & Erickson 2013). More recently, the Löwe group accomplished to reconstitute FtsZ and FtsA proteins from *Thermotoga maritima* into several hundred nanometer large vesicles and analyzed the FtsZ structures with electron cryotomography. This study confirmed that vesicles constricted in the presence of FtsZ rings (Szwedziak et al. 2014).

However, while FtsZ-rings have already been successfully reconstituted in vesicles, the high thoughput generation of vesicular compartments with well-defined size and with defined protein concentrations is challenging. A simple alternative approach to encapsulate proteins in small volumes is the application of micro droplet systems. In the simplest case, this can be achieved by manually mixing a protein solution in oil containing a defined lipid mixture. Moreover, a major advantage of reconstituting division ring proteins in membrane surrounding droplets is the potential to integrate droplet reconstitution assays on

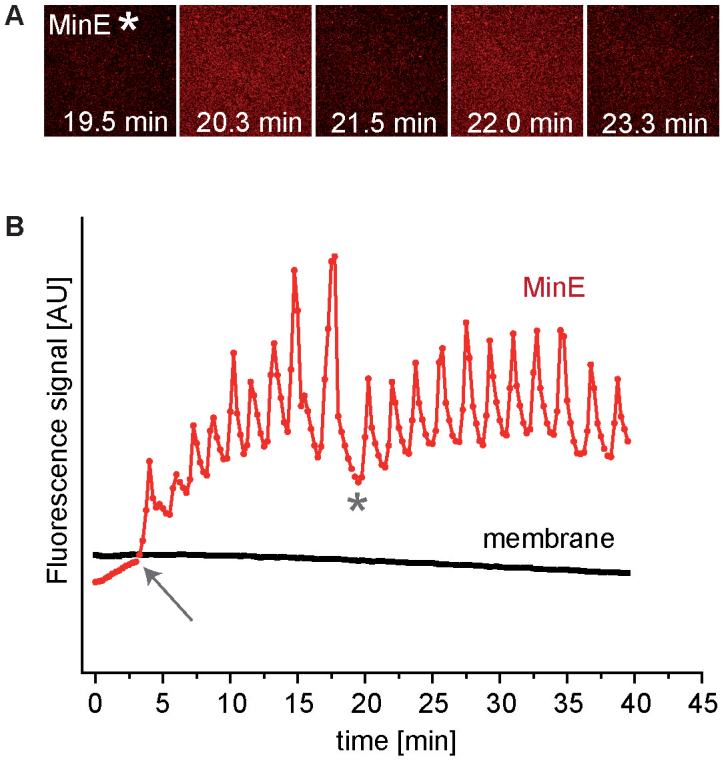

Figure 2.7: Min protein blinking at high MinE concentrations. A) Confocal images of MinE at the membrane demonstrate that the fluorescence signal is oscillating homogeneously when high concentrations of MinE are used. 0.8μM MinD and 3.7μM MinE(supplemented with 1% MinE.Atto655). Areas of 300μmx300μm are shown, which is larger than the typical wavelength of the Min protein patterns. B) The averaged fluorescent signal of a 300μmx300μm large area in dependence of the time oscillates. Before starting to oscillate the Min protein density at the membrane first increases slowly and has a steeper slope when the density starts to oscillate (arrow) The start indicates the time of the first frame in (A). As a control, the membrane was labeled with DiI and demonstarted to have a constant signal (black line).

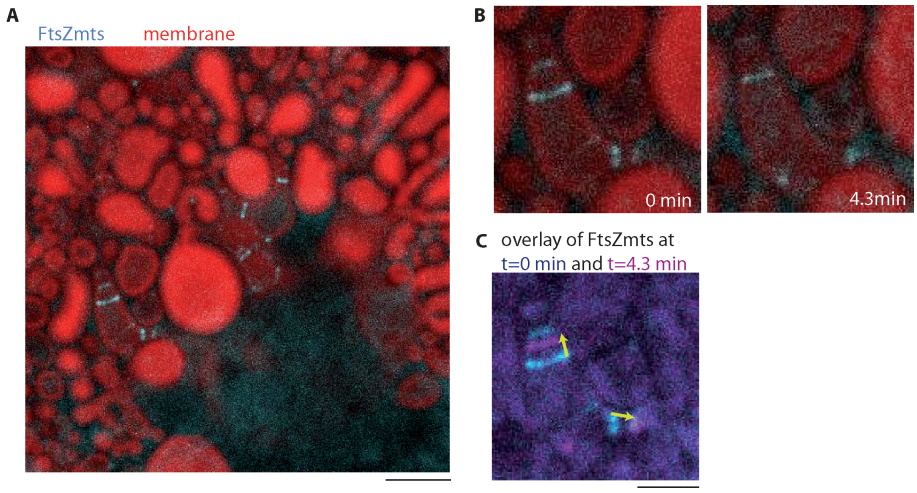

Figure 2.8: Assembly of FtsZ-mts rings in vesicles. A) FtsZ-rings (blue) are formed in a small fraction of multilamellar vesicles (red). Scale bar: 10μm B) At conical areas of the vesicles FtsZ-mts rings slide towards the narrower diameter of the vesicle. Scale bar: 5μm C) An overlay of the pictures in(B) is represented. Scale bar: 5μm

microfluidic platforms for high throughput sample generation.

To establish a system for studying division ring assembly and compare our results with previous studies, FtsZ-mts was in a first step reconstituted in vesicles by a protocol established in the Erickson-lab (Osawa et al. 2008). Comparable to the results reported previously (Osawa et al. 2008), we observed the formation of FtsZ rings in multilamellar, tubular vesicles with a diameter of about 1μm (Figure 2.8A). The concentration of FtsZ-mts within these vesicles is not known since FtsZ-mts was encapsulated by leaking into the vesicles from a protein containing solution at the outside of vesicles during the reconstitution process and only a small fraction of vesicles contained FtsZ-rings. We were able to confirm that these FtsZ-rings were highly dynamic and sliped along the walls of the vesicles. In agreement with previous studies we also observed that FtsZ-rings in conically narrowing tubular droplets slipped towards the side with the smaller diameter, indicating that the FtsZ-rings were under tension (Figure 2.8B, C) (Osawa et al. 2008, Osawa & Erickson 2013).

In a second step we aimed at reconstituting the same protein in droplets by pipetting a defined volume of FtsZ-mts and GTP containing buffer in an oil-lipid mixture and breaking the buffer solution into numerous small droplets, whereby the dissolved lipids in the oil phase self-assembled as a monolayer at the boundary of the droplets. Interestingly,

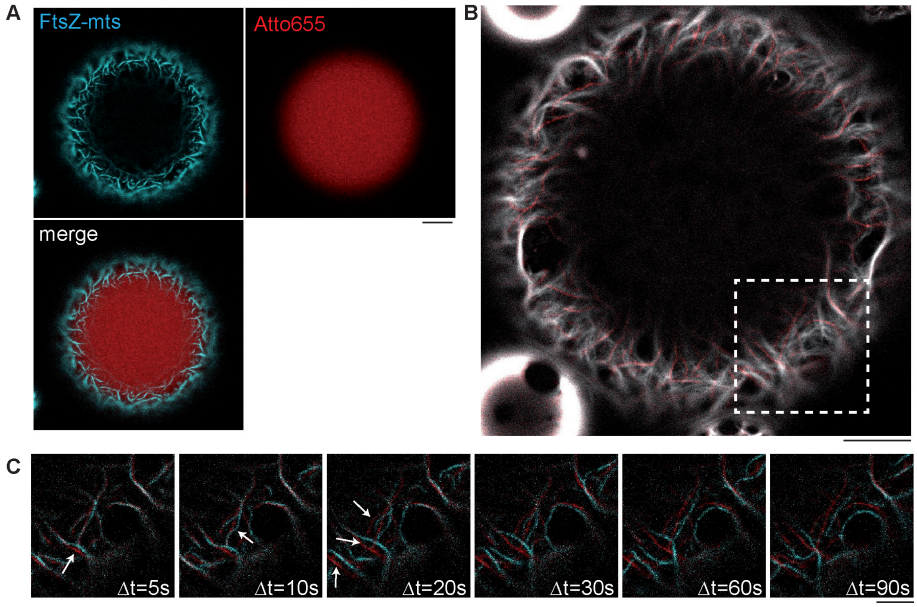

Figure 2.9: Encapsulation of FtsZ-mts in droplets. A) FtsZ-mts within droplets self-assembles into networks at the membrane interface. The soluble reference dye Atto655 (red) is homogeneously distributed within the lumen of the droplet. Scale bar: 10μm B) Overlay of FtsZ-mts fluorescence signal within droplets at various timepoints. Scale bar: 10μm C) Magnified area of (B) with an overlay of the FtsZ-mts fluorescence signal at two different timepoints. Arrowheads point to exemplary locations where the FtsZ bundles are not localized. Scale bar: 5μm

we also observed membrane attached FtsZ-structures in large droplets of several tens of micrometers. In large droplets, FtsZ-mts assembled into membrane attached networks of FtsZ bundles (Figure 2.9A). In contrast to FtsZ-mts networks on supported lipid membranes, the FtsZ bundles on freestanding droplet membranes were mobile and slipped on the droplet surface under the constraint of being integrated into a network of FtsZ bundles (Figure 2.9B, C). The ability to slip along freestanding membranes is in agreement with the results in vesicles. It is not known, why no FtsZ-mts structures were observed in large vesicles using the previous encapsulation assay, but potentially these vesicles were not leaky enough or the period for leaking into the vesicles was not long enough to encapsulate sufficient protein for the formation of FtsZ structures.

Thus, obvious advantages of the droplet assay are on the one hand side the simple and fast encapsulation protocol and on the other hand the encapsulation of defined concentrations of proteins. Although not all droplets contained an FtsZ network, the amount of droplets with FtsZ structures was higher than in the previous assay. Depending on the

sample we observed FtsZ structures in about one third of the droplets. Moreover, future integration of the droplet assay on microfluidic chips might render the droplet generation step more defined which might result in an even higher percentage of FtsZ structure containing droplets.

Note, that the size distribution of the droplets generated by manual pipetting was usually very large with droplets ranging from about 1μm to 100μm. When small droplets were observed, motile bright patches of FtsZ-mts were observed (Figure 2.10 A). As confocal microscopy images only provide a two-dimensional image, these patches could be part of FtsZ bundles or independent patches. To distinguish between these two possibilities, Z-Stack images were acquired and three-dimensional images reconstructed from the acquired images at different planes of small droplets. Interestingly the structures in small vesicles often corresponded to ring structures within the droplets (Figure 2.10 B, C). These FtsZ-mts rings were motile and often rotated along the periphery of droplets without a stable localization axis. This difference compared to tubular liposomes is likely due to the spherical symmetry of droplets as compared to tubules with a defined length axis. In summary, our experiments establish membrane surrounded droplets as an alternative system to reconstitute FtsZ structures in closed compartments. While FtsZ tends to self-assemble into branched networks within large droplets it can assemble into rings within small droplets.

2.4.3 Methods

Reconstitution of Min proteins and FtsZ-mts in droplets by pipetting. Cardiolipin (15%) and DOPC(85%) are mixed in chloroform and dried under a nitrogen stream. The lipids are dissolved in oil at a concentration of 2.5mg/ml. 5μl Buffer (25 mM TrisHCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$) containing 0.5mM ATP, 1μM MinD (supplemented with 10% eGFP-MinD and 1μM MinE was pipetted into 100μl oil-lipid mixture. The solution was repeatedly pipetted up and down. When FtsZ-mts was reconstituted in droplets, the buffer was supplemented with 0.5 μM FtsZ-mts and with 0.5mM GTP instead of ATP.

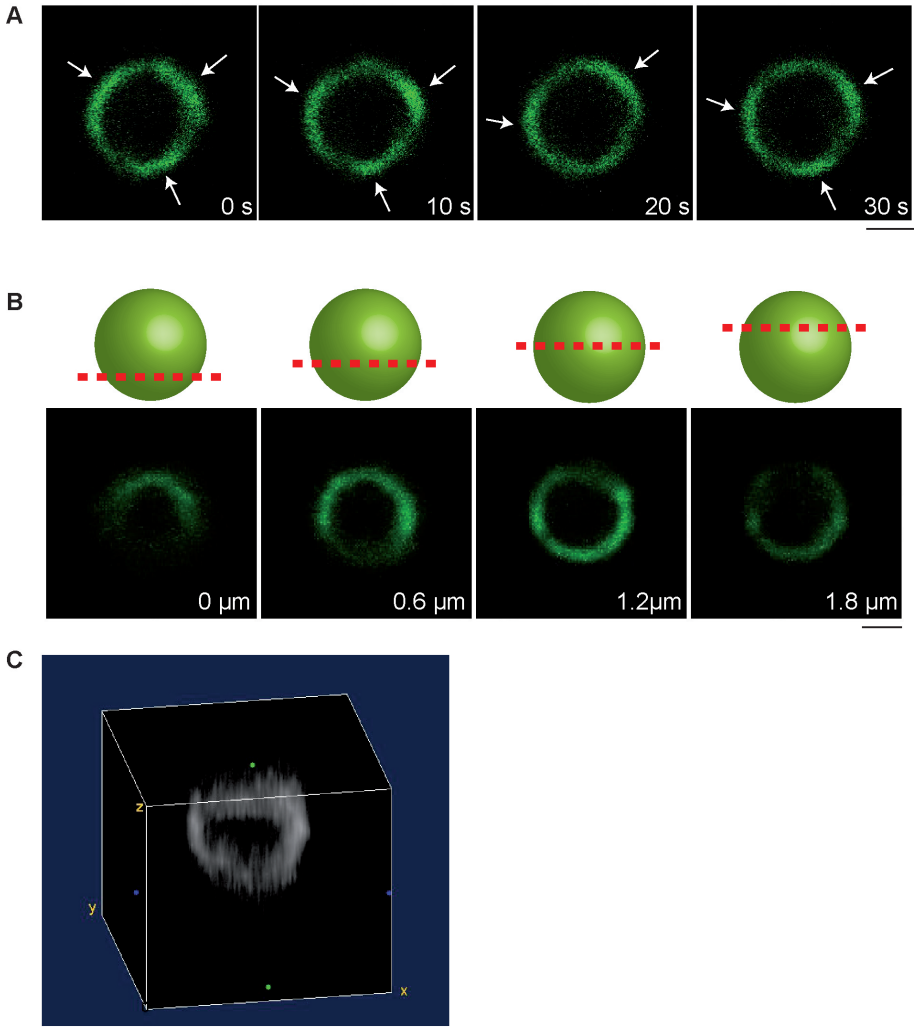

Figure 2.10: FtsZ-mts (green) in small droplets self-assembles into rings. A) Confocal time lapse images of FtsZ-mts in small droplets demonstrates that the protein self-assembles into dynamic higher order structures. Scale bar: 1μm B) Z-stack aquisition of the FtsZ-mts signal demonstrated that several small droplets contained ring-shaped structures of FtsZ-mts. Scale bar: 1μm C) Three-dimensional reconstruction of the data in (B). Image Processing was performed with ImageJ.

Chapter 3

Concluding remarks and future work

Despite the simple geometry of *E.coli*, proteins have evolved to participate in intracellular biochemical reactions within the rod-shaped, confined volume of this bacterium and the structure and function of proteins is thus well adapted to their small natural reaction compartments. Using well-defined cell-free systems we demonstrated that the self-organizing patterns of Min proteins and the positioning of FtsZ is coupled to compartment geometry, but we are just beginning to understand how the geometry of a cell influences intracellular reactions. In the future, similar cell-free systems could help to investigate further protein systems in confined volumes and within compartments of well-defined geometries.

In addition, despite the progress we made in understanding the minimal requirements to position FtsZ many questions about divisome assembly remain unanswered. For example what is the role of additional divisome associated proteins and how do these proteins regulate cell division? The mature septal ring contains more than two dozen different proteins and *in vivo* ten of these proteins are required for cell constriction (de Boer 2010). Increasing the complexity of present cell-free FtsZ reconstitution assays by adding additional purified components of the division machinery could help to understand the mechanisms for regulating and coordinating the cell division machinery in *E. coli*.

Furthermore, the mechanism of force generation during cell division is not yet fully understood. Although FtsZ rings within vesicles has been shown to result in the constriction of vesicles (Osawa & Erickson 2013, Osawa & Erickson 2011, Szwedziak et al. 2014), quantitative measurements of force generation of FtsZ-rings *in vitro* and its correlation with the required force to divide a living cell are still missing. It is also not fully understood to what extent the protein machinery in *E.coli* is dividing the cell and what the role of the cell wall generating apparatus has during septum formation in *E. coli*.

Moreover, in order to fully understand the mechanism of Min protein self-organization the biochemical mechanism and the function of individual protein domains need to be studied in more detail. Single molecule studies and analysis of Min protein profiles of known Min protein mutants might help to better understand Min protein pattern formation.

Chapter 4

Abbreviations

ATP	adenosine triphosphate
ADP	adenosine diphosphate
GTP	guanosine triphosphate
GDP	guanosine diphosphate
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
CA	cardiolipin
GFP	green fluorescent protein
FRAP	fluorescence recovery after photobleaching
PALM	photo-activated light microscopy
mts	membrane targeting sequence

Chapter 5

Enclosed papers

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Review

Surface topology assisted alignment of Min protein waves

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ABSTRACT

Self-organization of proteins into large-scale structures is of pivotal importance for the organization of cells. The Min protein system of the bacterium *Escherichia coli* is a prime example of how pattern formation occurs via reaction–diffusion. We have previously demonstrated how Min protein patterns are influenced by compartment geometry. Here we probe the influence of membrane surface topology, as an additional regulatory element. Using microstructured membrane-clad soft polymer substrates, Min protein patterns can be aligned. We demonstrate that Min pattern alignment starts early during pattern formation and show that macroscopic millimeter-sized areas of protein patterns of well-defined orientation can be generated.

1. Introduction

Pattern formation by self-organization of biomolecules is of key significance for cellular organization. During the process of self-organization, global order arises from an initially disordered system and results in organization of living systems on subcellular, as well as on multicellular level. Already in 1952, Alan Turing suggested a diffusion–reaction system as one mechanism to generate patterns of molecular concentrations [1].

An example for such a pattern forming reaction–diffusion driven protein network is the Min system of the bacterium *Escherichia coli*. This system consists of the three proteins MinC, the ATPase MinD, and MinE, which oscillate from pole to pole in the bacterium to spatially regulate cell division [2–5]. MinD and MinE dynamically attach and detach from the membrane by a reaction–diffusion driven process, which is powered by ATP. MinC is a negative regulator for division site placement, which binds to MinD [6,7]. On time-average, the Min protein oscillations result in a non-homogeneous concentration gradient of the division inhibitor MinC with the lowest concentration in the middle of the cell. Thus, misplaced cell division near the cell poles is inhibited [7].

Earlier computational models suggested that MinD and MinE constitute a minimal system for pattern formation and dynamic pole-to-pole oscillations [8–10]. In line with the theoretical simulations, more recent reconstitution experiments of MinD and MinE in vitro clearly demonstrated that the two proteins MinD and MinE alone are indeed capable of forming dynamic surface patterns on supported membranes, and oscillate from pole-to-pole when they are enclosed in cell-shaped membrane compartments [11,12]. However while Min-oscillations have a predefined propagation axis on small elongated membrane patches and in cell-shaped compartments [12,13], the orientation of wave and pattern propagation in bigger systems, such as large areas of planar membranes, have no globally preferred orientation.

Here we report a novel in vitro assay to achieve orientation of Min protein patterns also in large-scale systems. In other words, we show that dynamic Min protein patterns can be aligned in predefined directions on large membrane areas using micro-fabricated devices of specific surface topology. In particular, we investigate reconstituted Min pattern on supports with two different arrays of membrane clad micro grooves. Interestingly, our data show that reaction–diffusion induced patterns can indeed be modulated by surface topology. Thus, although protein patterns are mainly regulated by biochemical properties of participating proteins, our data emphasize the potential of surface geometry as a superimposed regulatory cue to alter pattern formation.

2. Material and methods

2.1. Min proteins

eGFP-MinC, MinD and MinE were purified as his-tagged fusions. All proteins were expressed in BL21 cells grown in 2xYT medium

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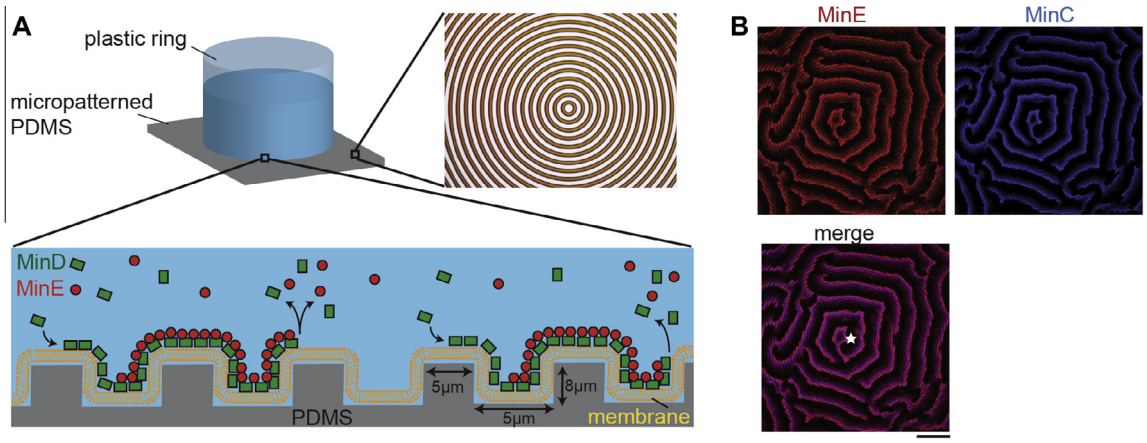

Fig. 1. Reconstitution of Min protein pattern on topologically structured supports (A) Experimental setup: A reaction chamber was placed on top of microstructured PDMS supports with concentric rings. The microstructured support was clad with membrane and the buffer volume on top of the membrane was supplemented with MinC, MinD, MinE and ATP. Inset: Light microscopy image of PDMS structures. (B) Confocal microscopy image of MinC/D/E protein pattern on PDMS structures. 0,1 µM eGFP-MinC, 1 µM MinD, 1 µM MinE. The star marks the midpoint of the concentrical rings. Height of structures: 8 µm. Scale bar: 100 µm.

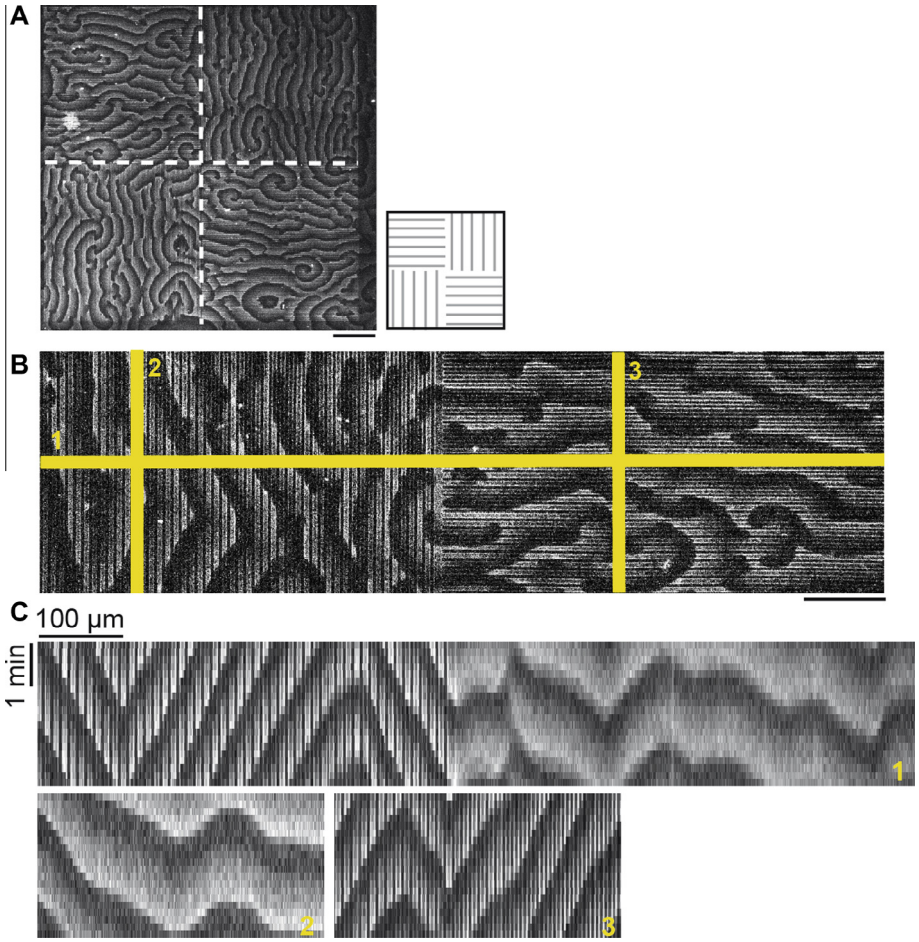

Fig. 2. Min protein waves propagate perpendicular to arrays of Microfabricated parallel grooves. (A) MinD/E patterns on fields of parallel, membrane clad micro walls. The orientation of the walls is depicted in a schematic graph. Scale bar: 200 µm. (B) Image of MinD/E patterns on vertically (left) and horizontally (right) patterned surface. Scale bar: 100 µm (C) Kymographs (time vs. space plots) along the lines depicted in (B).

and purified as described previously [11,14]. MinE was labeled with AlexaFluor 488 C5 maleimide (Molecular Probes) according to the manufacturer's instructions.

An overexpression vector encoding His6-eGFP-MinD was generated by amplifying MinD from a pET28a-MinD [11] and ligating it into a pET28a-eGFP vector using the restriction sites for HindIII and

EcoRI. The resulting ORF encoded a MinD fusion with an N-terminal hexahistidine-tag and eGFP. The construct was transformed in B21 cells, which were grown in LB medium and His6-eGFP-MinD was purified according to the protocol for MinD.

To reconstitute Min protein waves 1 μM MinD, 1 μM MinE and 2.5 mM ATP were incubated in reaction buffer (25 mM Tris–HCl pH7.5, 150 mM KCl, 5 mM MgCl) on top of supported bilayers. eGFP-MinC was used at a concentration of 0.1 μM.

2.2. Micro structured supports

Photoresist patterns of 8 μm height on silicon wafers were produced using the resist ma-P 1275 (micro resist technology GmbH). Afterwards the wafer was coated with chlorotrimethylsilane (Sigma–Aldrich).

PDMS monomer and crosslinker solution (Sylgard184, Dow Corning) was mixed at a ratio of 9:1, degased in a vacuum, and poured on top of the wafer. A glass cover slip was pressed on top of the si-waver to sandwich a thin layer of PDMS between the glass cover slip and the waver. Then the PDMS was cured for 3 h at 80 °C. The cured PDMS layer together with the glass cover slip was carefully peeled off. Before the microstructured PDMS was used as a membrane support, it was sonicated for 5 min in ethanol, washed with water, air dried, and hydrophylized using an air plasma.

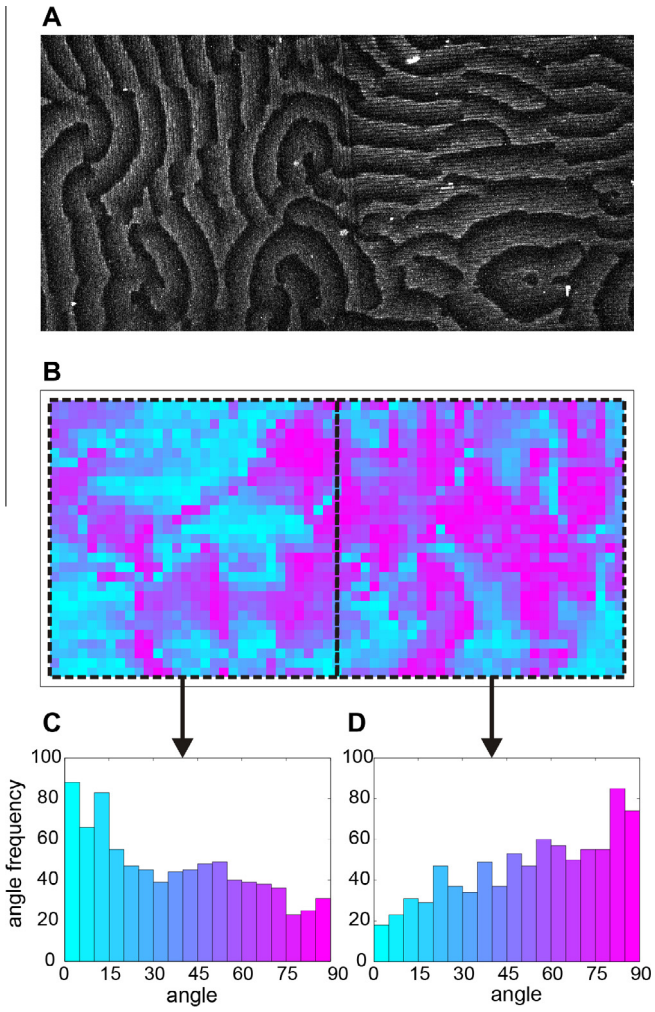

Fig. 3. Min waves on supports with vertical (left) and horizontal (right) grooves. As the PIV-analysis reveals, waves propagate perpendicularly to the orientation of the trenches. (A) Source image (B) Angle map. (C and D) Histogram of angle frequency. For vertical trenches, lower angles (C) dominate whereas for horizontal, angles close to 90° dominate (D).

2.3. Supported lipid membranes

Supported lipid membranes were produced by vesicle fusion. We prepared small unilamellar vesicles from *E. coli* lipid extract (Avanti polar lipids) by sonication in reaction buffer (25 mM Tris–HCl pH7.5, 150 mM KCl, 5 mM MgCl). 0.5 mg/ml vesicles were filled into a reaction champer on top of plasma cleaned, micro structured surfaces. $CaCl_2$ was added and the sample incubated for 15 min at 37 °C. Finally the formed lipid membrane was washed with reaction buffer to remove residual vesicles.

To test the mobility of membranes *E. coli* polar lipids were supplemented with 0.1 mol% fast-DiI (Invitrogen) and motility of the membrane was verified with FRAP experiments.

2.4. PIV analysis

Direction of wave propagation was analyzed by particle image velocimetry (PIV) using a custom made MATLAB code based on the MatPIV-function developed by J Kristian Sveen [15,16]. The routine detects spatial shifts of signals in x and y on a local level between two subsequent frames of a xyt-stack. For this, each xy-plane is subdivided into two-dimensional elements (here of 32×32 pixels) and for each element, a vector is calculated representing the direction of local shifts. The ensemble of vectors can be plotted in a vector map. For an image stack of n frames, $(n-1)$ of such vector maps are calculated. Since the direction of wave propagation remains unchanged throughout the acquisition of the respective time-lapses, all vector maps corresponding to one image stack could be averaged to one single vector map (Fig. 3B and Supplementary Fig. 4B and C). From this mean angle map, angles were calculated by the arctan of the respective vectors and color-coded for an angular spectrum between 0° and 90°. An angle of 0° (blue) represents horizontal direction of the travelling wave, whereas an angle of 90° (pink) corresponds to a vertical propagation. The PIV-elements at the border of a frame produces artifacts, therefore they were omitted for the analysis (white frame in the color-coded images of Fig. 3).

3. Results

3.1. Topologically structured membranes guide Min patterns in predefined directions

To examine the effect of membrane topology on the spatiotemporal organization of Min protein patterns, we used grooved membrane supports with groove sizes of 5 micrometers and a distance of 5 micrometers, corresponding to about the tenth fraction of Min protein wavelength. PDMS layers with arrays of microfabricated PDMS grooves were clad with *E. coli* polar membranes using a vesicle fusion technique. The fluidity, i.e., lipid mobility within the membranes, which adopt the topology of the underlying PDMS grooves, was confirmed by labeling membranes with 0.1% DiI and subsequent FRAP experiments (data not shown).

At first we engineered PDMS grooves that were arranged in concentric rings (Fig. 1a). The ring-like grooves were 8 μm high, 5 μm wide and had a distance to the next groove of 5 μm. To reconstitute Min protein pattern on top of the membrane clad structures, we added 1 μM MinD, 0.9 μM MinE, 0.1 μM MinE-Atto655 and 2.5 mM ATP. After the incubation time of 30 min the Min proteins were imaged and shown to form dynamic protein patterns on the topological structured membranes.

Previous experiments demonstrated that the Min system self-organizes into similar dynamic surface waves on flat supported membranes. However, on top of planar membranes, Min proteins form patterns without globally preferred orientation, such as

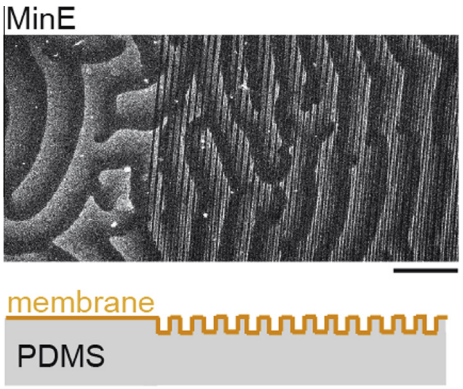

Fig. 4. Min patterns on microstructured membranes and flat membranes have similar wavelength. Confocal image of reconstituted MinD/E waved doped with MinE.Atto655 on parallel walls (right) next to a flat membrane region and schematic image of the corresponding membrane topology. Scale bar: 100 μm.

spirals and parallel, randomly oriented waves. In contrast, a significant preferential orientation of the Min wave fronts was observed on membrane supports with concentrically aligned grooves: The wave fronts predominately oriented themselves parallel to the grooves. In other words, the waves mainly propagated towards or away from the center of the surface structures (Fig. 1b, Supplementary Figs. 1 and 2). While on long term the pattern on structured supports resembled those on flat bilayers, a distinct alignment of protein patterns persisted for an observation time of about 2 h.

3.2. Min protein patterns follow discontinuous groove alignments

To address the question whether the alignment of Min waves by micro structured membrane supports is prominent enough to follow discontinuous orientation, we designed rectangular fields of parallel straight-lined PDMS grooves, oriented with 90° to each other like on a chessboard (Fig. 2).

Interestingly, the discontinuity in the orientation of pattern did not disturb the effect of Min wave alignment. In agreement with experiments on concentrically structured membrane supports, the Min wave fronts again aligned preferentially parallel to the PDMS grooves over almost the full range of the rectangular patches. Thus, although these highly organized protein patterns ultimately originate from specific properties of nanometer-sized proteins, macroscopic alignment pattern on square-millimeter big areas were observed.

To investigate the alignment of propagating Min protein patterns on microstructured supports more quantitatively, we performed a particle image velocimetry (PIV) analysis and examined the exact angle of wave propagation (Fig. 3). The distribution of propagation angles was plotted in histograms using a bin width of 5° (Fig. 3C,D and Supplementary Fig. 4). In line with our qualitative observation and kymograph analysis, the PIV data demonstrate that Min waves preferentially propagated perpendicular to the orientation of the groves. Although velocity vectors of all orientations were detected on microscopic scale, the occurrence of vectors perpendicular to the walls was about three to four times larger than the frequency for vectors parallel to the walls.

Notably, the wavelength of Min protein patterns on flat membranes was similar to the wavelength on topologically structured structures when measured by beeline (Fig. 4), although the patterns traveled though the grooves and were also observed on the bottom of grooves (Supplementary Fig. 3). This observation seems to be counterintuitive, because the actual distance along the surface of the membrane is, compared to beeline, more than two times larger. However, we have previously shown that Min waves can couple across flat membranes patches when the patches were separated by smaller than 10 μm wide gold barriers [13]. Note that the distance between two adjacent microfabricated grooves is 5 μm and that this distance is small compared to the Min wavelength of about 50–100 μm. The observation of a contained beeline wavelength is therefore consistent with the previous experiments and originated from the three-dimensional reaction–diffusion principles of the Min system.

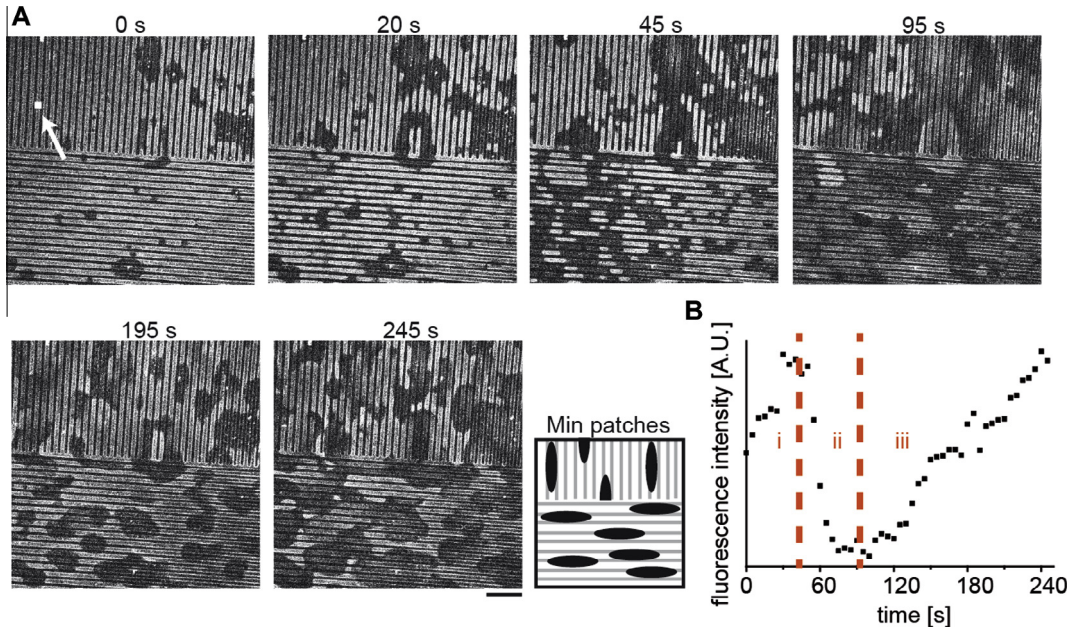

Fig. 5. Initiation of Min pattern alignment. (A) Confocal time-lapse images of first oscillation cycle. Pattern of 1 μM MinD and 1 μM MinE (supplemented with 10% MinE.Atto655) were oriented within the attachment-detachment cycle. Scale bar: 50 μm (B) Development of fluorescence intensity of a square area with 10 μm width (depicted in A, first frame). (i) Increase of intensity due to increasing protein attachment, (ii) detachment of the Min protein from the membrane, (iii) reattachment of Min proteins to free membrane.

3.3. Min pattern alignment originates early during pattern formation

To investigate how the alignment of Min protein patterns parallel to the underlying grooves is initiated, we analyzed the onset of Min pattern formation. On top of flat supported bilayers it has been shown that dynamic protein patterns originate radially from several "hot spots" at arbitrary locations, due to stochastic variations in the protein concentrations on the membrane, and only later converge towards parallel wave fronts [11]. Thus, the intrinsic mechanism of the proteins to cooperatively attach and detach from the membrane then results in the fusion of patch-like pattern and finally in the formation of protein surface waves with no globally preferred orientation. Typically, a flat membrane is covered with areas of spirally and parallel, randomly oriented wave-like patters, when the patterns are equilibrated.

In comparison, the beginning of pattern formation on micro structured PDMS supports initially resembles Min pattern formation on flat supported membranes. First, when the proteins are incubated on top of the membrane, MinD proteins start to attach to the membrane and the protein concentration on the membrane increases. Then the proteins detach at individual localizations due to the inhibitory action of the antagonist MinE, which results in patch-like patterns. The patterns propagate and in repeating cycles, new MinD protein reattaches at free membrane areas, and thus generate dynamic surface waves after several oscillation cycles. However, in contrast to flat supported membranes the formation of Min protein patterns on micro structured supports preferentially started at the edges of microstructures. Thus, when the Min patches increase in size and merge into patterns with intrinsic wavelengths, the wave fronts tend to be oriented parallel to the micro walls. Already after the first cycle of membrane attachment and detachment, an orientation of the Min patches was detectable (Fig. 5).

4. Discussion

Using topologically micro-structured devices as membrane supports, we have established a novel lab-on-chip assay for aligning self-organizing Min protein patterns in predefined orientations. In particular, we employed two different arrays of membrane clad micro grooves and demonstrated that the patterns preferentially propagated perpendicularly to the walls. Thus, in addition to planar bilayers with specific shapes, large-scale orientation of Min patterns can also be achieved on topologically structured membranes. Notably, while the biological function of the Min system is to organize the space of a few micrometer-sized cells, we exploited the combination of intrinsic properties of the Min system with external cues to generate millimeter-sized areas with patterns of predefined orientation. Furthermore we demonstrated that pattern alignment occurs early during pattern formation. Taken together, our results show how nanometer sized protein machineries can be reconstituted to generate macroscopic patterns of well-defined orientation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.06.026.

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Erratum

Erratum to "Surface topology assisted alignment of Min protein waves" [FEBS Lett. 588 (2014) 2545–2549]

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Contrary to the document header 'Review' this paper should be classified as a Research Letter.
The Publisher apologizes for the inconvenience caused.

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Synthetic Biology

Reconstitution of Pole-to-Pole Oscillations of Min Proteins in Microengineered Polydimethylsiloxane Compartments**

*Katja Zieske and Petra Schwille**

Cell division is a highly regulated process, and even "simple" organisms such as the bacterium *Escherichia coli* use complex mechanisms to control their temporal and spatial organization during cytokinesis. *E. coli* typically divides by binary fission, a process that is mediated by the divisome, a protein complex that assembles into a ringlike structure in the middle of the cell. The placement of the divisome is spatially regulated by the Min system, which consists of the three proteins MinC, MinD, and MinE. A particularly interesting feature of these proteins is their ability to self-organize and dynamically oscillate from pole to pole,[1–6] thereby generating, on time average, a non-uniform concentration profile of Min proteins with the highest concentration at the cell poles and the lowest concentration in the middle of the cell.[3,5–7] During the last three decades, the roles of MinC, MinD, and MinE in spatially regulating cell division in *E. coli* have been studied intensively. Experiments with deletion mutants and fluorescently labeled proteins revealed that MinE and MinD account for the mechanism of the dynamic pole-to-pole oscillations, while MinC is a division inhibitor which follows the movement of the other Min proteins by binding to MinD, and thereby prevents cell division near the cell poles.[2,4,8–10] The biochemical basis of the Min system was elucidated by in vitro studies with purified Min proteins.[11–14] It was shown that MinD is an ATPase and that MinD.ATP binds to membranes through an amphipathic helix.[12,15,16] The association of MinE with MinD activates the ATPase activity of MinD, thereby resulting in a dissociation of the Min proteins from the membrane.[13,14] Subsequently, MinD.ADP is converted into MinD.ATP, and in repeated circles of cooperative membrane attachment and detachment the Min proteins self-organize and form patterns.[3,5,6] Recently, Loose et al. demonstrated that purified Min proteins are capable of self-organizing in a synthetic environment on supported lipid membranes.[17] The Min proteins were found to form dynamic wavelike patterns along the lipid membranes, thus suggesting that the mechanism responsible for the protein waves in vitro is the same as for the Min oscillations in vivo.[17] But what are the mechanistic requirements for the appearance of regular, pace-making oscillations? Studies with aberrantly shaped *E. coli* cells as well as theoretical models indicated that cell shape plays a role in Min pattern formation.[18–22] To date, however, oscillations of Min proteins have not been reconstituted in minimal systems and, thus, a well-defined model system to study the relationship between compartment geometry and the ability of oscillations to act as a spatial cue for cell (i.e., compartment) division is still missing.

Here we show for the first time that Min protein oscillations can be generated in a synthetic system. We use a device composed of model membranes and micrometer-sized reaction compartments and demonstrate that Min oscillations occur in restricted sample volumes with bacteria-like shape. Furthermore, we investigate the influence of geometry on Min pattern formation in synthetic systems and compare our results to live cell studies that showed the influence of aberrantly shaped bacteria on the dynamics of Min proteins. Our findings demonstrate that pattern formation in round and filamentous cells can be reproduced in vitro, and provide further evidence that cell geometry plays a pivotal role in biological pattern formation and pace-making.

Min pattern formation in spherical and filamentous *E. coli* cells is modified compared to that in wild-type cells.[3,18,23] Therefore, we hypothesized that oscillatory Min patterns in *E. coli* require a specific size and shape of the bacterium, and that the same is true for an in vitro system. To test this hypothesis, we produced a biomimetic device with restricted reaction volumes of well-defined geometries, in which dynamic Min pattern formation could be investigated. To restrict the sample volume to micrometer-sized dimensions, we produced microfabricated polydimethylsiloxane (PDMS) chambers. Their dimensions were scaled, according to the approximately ten times longer Min wavelengths in vitro than in live bacteria. Resist micropatterns with rodlike shapes and 10 μm height were produced by standard photolithographic techniques on Si wafers, against which PDMS was molded. After curing the set-up at 80 °C for three hours, the PDMS (Figure 1a) was peeled off, which resulted in a solid PDMS layer containing microcavities that was utilized as the support for lipid membranes. *E. coli* lipid bilayers that adopt the topography of the underlying PDMS structures were produced by vesicle fusion, and the two-dimensional mobility of the membrane was verified by fluorescence recovery after photobleaching (FRAP) experiments (data not shown). The formation of self-organizing surface waves, similar to the patterns reported by Loose et al.,[17] were observed when purified MinD (1 μM), MinE (1 μM), and Alexa488-labeled MinE (0.1 μM) were added to the buffer reservoir on top of

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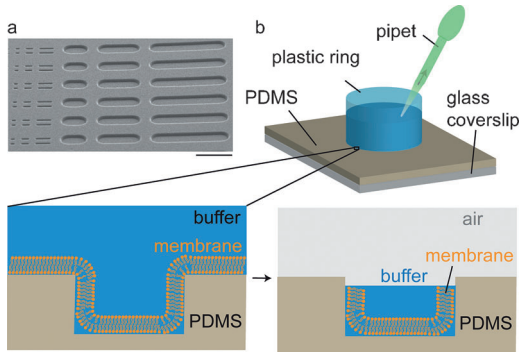

Figure 1. Experimental setup of a synthetic model system for Min protein oscillations. a) Electron micrograph of a microstructured PDMS membrane support. Scale bar: 100 μm b) Membrane bilayers were produced on PDMS microstructures by vesicle fusion techniques. MinD and MinE were added to the buffer and subsequently the buffer level was reduced below the upper rim of the microcavities. The image is not to scale.

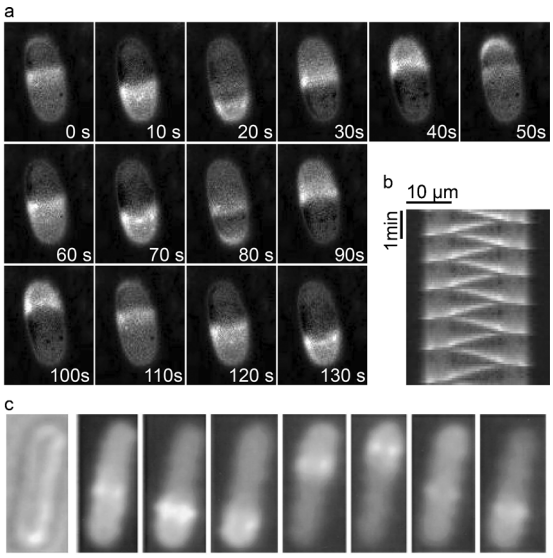

Figure 2. Synthetic Min protein oscillations are comparable to pattern formation in vivo. a) Confocal time-lapse images of purified Min proteins on a lipid membrane in a PDMS microcompartment. The PDMS structure is 10 μm wide, 25 μm long, and 10 μm high. 1 μM MinD, 1 μM MinE doped with 10% Alexa488-labeled MinE. Images were modified by subtracting the average intensity of time-lapse images, thereby removing nondynamic fluorescent background signals. b) Kymograph for MinE-Alexa488 along the long axis of the compartment in (a). c) Min protein oscillations in vivo. Images reproduced from Ref. [5] (Copyright 2001, The National Academy of Sciences, U.S.A).

the bilayer (see movie 1 in the Supporting Information). After formation of the Min protein waves, the buffer level was decreased to a level below the upper rim of the PDMS to produce picoliter sample volumes (Figure 1 b).

To investigate Min pattern formation in restricted volumes with well-defined geometry we observed the fluorescent signal from MinE-Alexa488 in rod-shaped microcavities of 10 μm height, 10 μm width, and 25 μm lengths by using confocal time-lapse microscopy. Strikingly, the confinement to small volumes and a rod-shaped geometry indeed led to regular oscillations of the fluorescent signal. In other words, MinE-Alexa488 rapidly oscillated between the two poles of the PDMS cavity in a manner strongly resembling Min oscillations in vivo (Figure 2). In repeating cycles, the Min proteins disassembled from the membrane when they reached one pole of the microcompartment, then reassembled near the middle of the compartment and moved towards the opposite pole. Oscillatory Min patterns were formed by repeating cycles of cooperative membrane association, wave propagation towards the pole, and membrane dissociation. To better compare the oscillations in vitro with oscillations in *E. coli* we determined the average oscillation period in the synthetic system. The oscillation period in *E. coli* is 0.5 to 2 min, which is well below its generation time of about 20 min under optimal growth conditions.[3,5,6] This short oscillation period allows the Min proteins to perform several oscillations between two successive cell divisions and thus enables the cell within its generation time to build a time-averaged protein concentration profile with the lowest protein concentration in the middle of the cell. In accordance with the literature values of 0.5 to 2 min for the oscillation period in *E. coli*, the average oscillation period of $n = 6$ synthetic samples was 70 s with a standard deviation of 13 s. Taking into account that Min proteins form surface waves before the sample volume is reduced and the proteins start to oscillate, our findings provide evidence that wavelike patterns indeed underlie the same mechanism as the oscillatory pattern, and that the size and geometry of the reaction volume is indeed constitutional for oscillations to occur. It should be noted that in contrast to a three-dimensional cell, the reaction volume is not completely enclosed by a membrane, but has the upper side exposed to air. Thus, although the lipid membrane and a restricted sample volume are required for the occurrence of Min oscillations, the encapsulation of Min proteins in closed membrane compartments is not absolutely necessary.

As mentioned above, the wavelength of Min patterns on flat membranes is typically an order of magnitude larger than in vivo (3.0–4.0 μm).[24] Thus, PDMS compartments significantly larger than bacterial cells could be used to observe the same patterns. Although the oscillations can now be controlled in vitro, it is still an open question as to how the size of Min patterns is determined exactly. The patterns were suggested to be influenced by viscosity,[17] but other regulating factors, such as increased biochemical competition for the Min proteins in the cellular environment, cannot be excluded. Currently, experimental evidence for a key parameter that modulates the size of Min patterns is still missing and should be addressed in future studies. Nevertheless, the possibility to reproduce Min protein patterns in larger departments than a bacterial cell is highly convenient, because cavities of tens of micrometers can be easily produced by photolithographic techniques, and the observation of the dynamic processes is much simpler, well above the optical resolution limit.

Having observed oscillating Min patterns in rod-shaped cavities, we rationalized whether our approach could be applied to mimic Min patterns in filamentous cells that disclose multiple dynamic MinE rings (Figure 3 a).[5,6,24] To study the dependency of pattern formation on the compartment length the Min pattern in 45 μm long compartments,

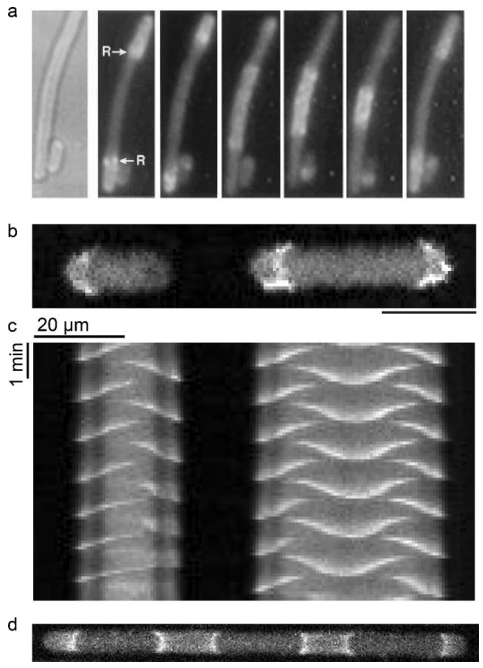

Figure 3. Min pattern formation is modulated by the length of the reaction compartment. a) Min protein pattern in filamentous *E. coli* cells. Reproduced from Ref. [5] (Copyright 2001, The National Academy of Sciences, U.S.A). b) Confocal image of Min protein patterns in a short and long microcavity. Both structures are 10 μm high and 10 μm wide. The cavity length is 25 μm and 45 μm, respectively c) Kymograph for MinE-Alexa488 along the long axis of the cavities in (b). Confocal image of Min protein patterns in a microcavity with a length of 195 μm. The structure is 10 μm high and 10 μm wide.

instead of the aforementioned 25 μm long compartments, were investigated. Pole-to-pole oscillations of MinE is still observed in some of the long compartments (see movie 2 in the Supporting Information). Intriguingly, however, the MinE ring patterns in many of the 45 μm long compartments indeed mimicked the patterns of filamentous cells: similar to short bacterial filaments, two distinct MinE regions were detected in the synthetic system (Figure 3b) and they repeatedly passed through the following sequence of steps. The Min regions moved towards the cavity poles and subsequently disassembled from the membrane and reassociated between the poles and the center. Then the fluorescent MinE regions moved in the opposite direction towards the cavity center, disassembled from the membrane, reassembled again between the center and the poles, and moved towards the poles of the cavity. The movement of MinE patterns thereby appears to be highly coordinated, with one MinE structure mirroring the other with respect to a symmetry plane in the center, perpendicular to the long axis of the cell. When compartments even longer than 45 μm were investigated, the percentage of patterns with one bright oscillating area was reduced, in favor of patterns with multiple bright areas. In extremely long cavities of 195 μm, we even observed dynamic patterns with up to four bright areas (Figure 3d). It seems clear from the different patterns obtained in short and long synthetic cavities (but otherwise under the same conditions) that the compartment length can modulate Min patterns. In line with studies in filamentous cells,[5,6,24] our data suggest that multiple Min rings in filamentous bacteria arise as a consequence of the length of the cells, and only geometrical cues are needed to account for the formation of multiple versus single mobile MinE rings.

To further elucidate the influence of the sample geometry on pattern formation we investigated Min patterns in round, that is, cylindrical PDMS cavities with a height of 10 μm and a diameter of 40 μm. If the directionality of the Min patterns is determined by the length axis of the reaction volume, no preferential direction of the Min protein movement should be detected in cylindrical cavities. As expected, no unique directionality of traveling Min patterns could be observed in such reaction volumes. We mainly observed three kinds of Min patterns in cylindrical cavities. First, oscillations with switching oscillation axes were detected (Figure 4). Second,

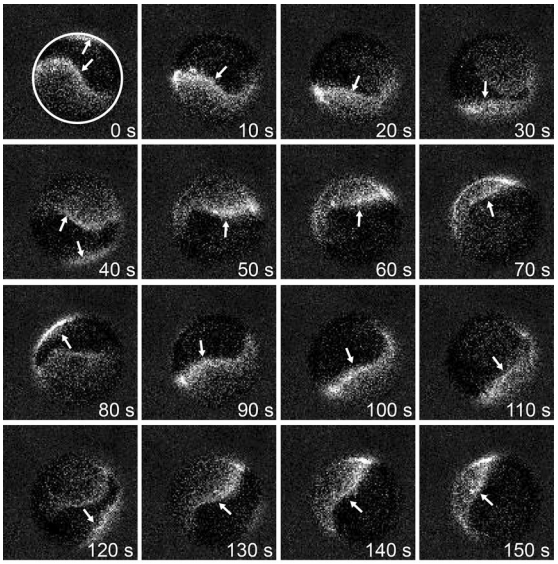

Figure 4. Self-organization of purified Min proteins in round cavities. 1 μM MinD and 1 μM MinE doped with 10% Alexa488-labeled MinE self-organize in cavities of 10 μm height and a diameter of 40 μm. The images were modified by subtracting the average intensity of time-lapse images, thereby removing nondynamic fluorescent background signals.

patterns moving around a circle were detected (see movie 3 in the Supporting Information). Finally, we observed oscillations along a fixed axis (see movie 4 in the Supporting Information); however, the angle distribution of cavities with a fixed axis seemed to be random, and we cannot exclude a switching of the axes after the acquisition times of time-lapse series. The findings in cylindrical artificial systems are in accordance with studies in spherical cells, in which Min proteins were reported to move along drifting or fixed axes or to perform circular sweeping motions.[18] We observed movements along one axis exclusively in all of the aforementioned elongated cavities. Therefore, our investigations of Min patterns in round and elongated cavities demonstrate that Min patterns can be produced in geometries without a long axis, but that elongated geometries result in high fidelity in movements along a fixed axis.

In summary, we have shown that dynamic Min protein patterns can be modulated by geometrical cues in synthetic minimal systems composed of microstructured membrane supports, lipid bilayers, and purified Min proteins. The emergence of Min oscillations similar to those in wild-type, filamentous, and spherical cells was observed by adapting the size and geometry of the reaction volume. Our experimental results suggest that the cell shape of wild-type *E. coli* is optimal to give rise to oscillatory behavior, and provide evidence that variations in cell shape lead to variations in Min protein patterns and the occurrence of pace-making oscillations. Thus, although the geometry of *E. coli* membranes is rather simple compared with many other cells, cell shape seems to play a pivotal role in Min pattern formation and, therefore, in spatial and temporal regulation of cell division. We believe that our approach to investigating the role of cellular shape on Min pattern formation provides an attractive assay for studying spatial regulation during cell division and might open up new channels in the field of bottom-up synthetic biology.

Experimental Section

Protein purification and labeling: His-MinD and His-MinE were purified with an N-terminal His tag, as previously described in Ref. [17]. MinE was labeled with AlexaFluor488 C_5 maleimide (Molecular Probes) according to the manufacturer's instructions.

Microfabrication of PDMS devices: Photoresist patterns (ma-P 1275, micro resist technology GmbH) on top of Si wafers (Si-Mat, Kaufering) were produced by photolithography by using a chrome mask (Compugraphics Jena GmbH). The wafer with the resist structures was coated with chlorotrimethylsilane (Sigma–Aldrich). PDMS (Sylgard184, Dow Corning) was mixed at a monomer/cross-linker ratio of 9:1, degased in a vacuum, and cured on top of the wafer for three hours at 80 °C. The cured PDMS was carefully peeled off and stored at room temperature until further use. Before the microstructured PDMS was used as a membrane support, it was sonicated for 5 min in ethanol, washed with water, air dried, and treated with an oxygen plasma.

Supported lipid membranes (SLBs): SLBs were produced by using standard vesicle fusion techniques. *E. coli* polar lipid extract in chloroform was purchased from Avanti Polar Lipids. The lipids were dried under a nitrogen flow and placed in a vacuum for 30 min. Subsequently, the dried lipids were dissolved in buffer A (25 mM Tris-HCl pH 7.5, 150 mM KCl, 5 mM $MgCl_2$), incubated at 37 °C for 30 min, sonicated for 15 min, and applied to the PDMS support at a concentration of 0.5 mg mL^{-1}. 2.5 μM $CaCl_2$ was added to facilitate vesicle rupture. The vesicles were incubated for 20 min at 37 °C to form lipids membranes, which were subsequently washed with buffer A. For the experiments with Min proteins, 1 μM MinD, 1 mM MinE doped with 10% Alexa488-labeled MinE, and 2.5 mM ATP were added.

Microscopy: Image acquisition was performed on a ZEISS (Jena, Germany) LSM780 confocal laser scanning microscope equipped with a ZEISS Plan-APO 25x/NA 0.8 objective.

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RESEARCH ARTICLE

Reconstitution of self-organizing protein gradients as spatial cues in cell-free systems

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Abstract Intracellular protein gradients are significant determinants of spatial organization. However, little is known about how protein patterns are established, and how their positional information directs downstream processes. We have accomplished the reconstitution of a protein concentration gradient that directs the assembly of the cell division machinery in *E.coli* from the bottom-up. Reconstituting self-organized oscillations of MinCDE proteins in membrane-clad soft-polymer compartments, we demonstrate that distinct time-averaged protein concentration gradients are established. Our minimal system allows to study complex organizational principles, such as spatial control of division site placement by intracellular protein gradients, under simplified conditions. In particular, we demonstrate that FtsZ, which marks the cell division site in many bacteria, can be targeted to the middle of a cell-like compartment. Moreover, we show that compartment geometry plays a major role in Min gradient establishment, and provide evidence for a geometry-mediated mechanism to partition Min proteins during bacterial development.
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Introduction

Intracellular concentration gradients of proteins in micrometer-sized cells have long been thought to be unsustainable due to diffusion. However, the past decade revealed the existence of multiple intracellular gradients in eukaryotes and prokaryotes and their significance in providing positional information within the cellular compartment (***Kiekebusch and Thanbichler, 2014***). Although these protein gradients are now emerging as significant general motifs to spatially organize cells, little is known about how protein patterns are established by local unmixing, and maintained on a molecular level. What are the ultimate cues and mechanisms to localize gradient forming proteins? Are protein gradients modulated by boundary conditions of the cell and how are downstream proteins directed by the positional information of protein gradients?

Cellular reconstitution methods enable us to study biological processes under defined conditions, and have significantly contributed to our understanding of molecular interactions and kinetics of proteins in simplified environments. To systematically investigate the mutual dependence between biochemical networks and three-dimensional cellular organization several techniques to confine proteins in micro compartments have recently been devised. One approach to achieve three-dimensional confinement of proteins and cytoplasmic extracts is by their encapsulation in water-oil droplets or vesicles. (***Pinot et al., 2009***, ***2012***; ***Good et al., 2013***; ***Hazel et al., 2013***) These systems add tremendously to our understanding of how a constrained reaction space, and varying surface-to-volume ratios affect (bio) chemical reactions. Applying subtle mechanical forces to these systems, they may be also deformed into non-spherical shapes. However, if more complex geometries need to be realized, or if the compartments need to be mechanically stabilized, biochemical systems may also be reconstituted on and in spatially tailored microfabricated environments (***Garner et al., 2007***; ***Laan et al., 2012***). In particular, combinations of two- and three-dimensionally engineered substrates with lipid membranes

eLife digest When a cell divides, it is important that its contents are separated in the right place to make sure that both daughter cells have everything that they need to survive. To do this, the molecular 'machinery' that physically divides the cell needs to know where to assemble.

In the bacterium *E. coli*, the location of cell division depends on a group of proteins called the Min proteins. These proteins are not evenly distributed over the cell. Instead, they oscillate back and forth to set up concentration gradients, with the concentration of Min proteins being lowest in the middle of the cell and highest at the ends. The machinery that divides the cell assembles at the point where the concentration of Min proteins is lowest. However, it is not clear exactly how the protein gradients are set up in the cell, and whether these gradients are indeed sufficient to position the cell division machinery.

To explore this process, Zieske et al. engineered artificial cells that mimicked some of the basic properties of living cells. In these artificial cells, the Min proteins organized themselves into gradients that were similar to those found in living cells. This gradient then caused another protein called FtsZ—which is involved in cell division—to accumulate in the middle of the artificial cell. Zieske et al. also showed that the shape of the artificial cell influenced the shape of the protein gradient.

This research shows that the interplay between the shape of a bacterial cell and a defined set of proteins could control the position of cell division. The simplified system that Zieske et al. have developed could also be used to study other aspects of cell organization and cell division.

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have been applied to study the role of simple membrane geometries on membrane interacting protein networks in vitro (***Schweizer et al., 2012***; ***Zieske and Schwille, 2013***). However, although such in vitro experiments are now being successfully applied to identify the minimal components and interactions of protein networks, we are still far from understanding the mutual interdependence of protein functionalities and physical parameters in generating nonhomogeneous protein distributions for providing positional information within the cellular compartment. Therefore, further development of bottom-up approaches are required to tackle the minimal requirements for nonhomogeneous protein localization. Studying gradient formation in a simplified and controlled environment, detached from the complexity of living cells, should shed light on the role of individual biochemical and physical parameters for regulated protein localization in space and time.

Among the most fundamental and significant gradients in cells are those that set the spatial cues for cytokinesis. In many bacteria, and also in eukaryotes that undergo symmetric division, protein gradients with the lowest concentration in the middle of the cell and the highest concentration at the cell poles have been identified. These gradients target the division site through polar inhibition of the division machinery assembly. For instance, in fission yeast, a Pom1 gradient regulates the onset of mitosis (***Celton-Morizur et al., 2006***; ***Padte et al., 2006***; ***Martin and Berthelot-Grosjean, 2009***; ***Moseley et al., 2009***). Similarly, in many bacteria, the assembly of the conserved ring-forming cell division protein FtsZ is inhibited at the cell poles by protein gradients of FtsZ inhibitors. In *Caulobacter crescentus*, a gradient of the cytokinesis inhibiting protein MipZ is established, (***Thanbichler and Shapiro, 2006***) whereas in *Bacillus subtilis*, an inhibitory complex of MinC and MinD is attached to the cell poles by MinJ and DivIVA. (***Edwards and Errington, 1997***; ***Bramkamp et al., 2008***; ***Patrick and Kearns, 2008***) Comparably, *Escherichia coli* employs the membrane-targeted MinC/MinD complex to inhibit FtsZ assembly at the cell poles. (***Lutkenhaus, 2007***) Interestingly however, MinC and MinD in *E.coli* do not form a static gradient, but the Min protein patterns oscillate from pole to pole. (***Raskin and de Boer, 1999b***; ***Raskin and de Boer, 1999a***) These dynamic oscillations require ATP as an energy source for the ATPase MinD and MinE, which accelerates hydrolysis of ATP by MinD. (***de Boer et al., 1991***; ***Hu and Lutkenhaus, 2001***) On time-average, the oscillations result in an effective concentration gradient of the MinC/MinD complex.

While MinD and MinE have been identified as a minimal set of proteins that is able to self-organize into dynamic pattern on flat supported membranes, (***Loose et al., 2008***) the minimal requirements for gradient formation, and the mechanisms of forwarding positional information to downstream processes are still controversial and only begun to be elucidated. Labeling MinE and co-reconstituting MinE with MinD in cell-shaped compartments, we demonstrated that pole-to-pole oscillations of the

Min system can be reconstituted in vitro (***Zieske and Schwille, 2013***). However, time-averaged Min protein gradients in vitro have not been reported to date and thus, direct evidence for the establishment of steady protein gradients by self-organizing oscillations of MinD and MinE without additional proteins is still missing.

Moreover, it is still ambiguous how cell geometry affects gradient formation. On the one hand, live cell experiments with cell shape mutants, as well as in vitro reconstitutions, have shown that compartment geometry affects pattern formation of the Min proteins (***Corbin et al., 2002***; ***Varma et al., 2008***; ***Raskin and de Boer, 1999b***; ***Zieske and Schwille, 2013***). On the other hand, protein gradients should be robust against morphological changes during cell development and growth. Living bacteria typically double their length during one live cycle and gradually constrict during cell division. Thus, they undergo significant changes in cell geometry, which should not disturb the establishment and functional role of Min protein gradients. Whether cell shape affects pattern formation only if the geometry of a cell is artificially perturbed, or whether a cell might use its own geometry as a readout and control parameter to shape protein gradients, still has to be determined. Thus, the identification of principal cues for gradient establishment, and their modification by spatial parameters, such as the three-dimensional geometry of a cell, remains an outstanding challenge.

In this study, we were able to establish, from a system of soluble and initially well-mixed proteins, an effective steady protein concentration gradient, potent of targeting the assembly of cell division proteins to the middle of artificial membrane-clad compartments. In particular, FtsZ, which marks the cell division site and represents the cytoskeletal backbone for the divisome machinery in many bacteria, can be targeted to the middle of a cell-like compartment by a gradient of Min proteins. Moreover, we demonstrate that orientation and partitioning of Min protein gradients is controlled by compartment geometry. Notably, our synthetic system opens a new way to study complex organization principles in a simplified environment and provides novel insights into the basic biophysical and biochemical requirements for gradient formation.

Results

Reconstitution of protein gradients in cell-shaped compartments

To determine the minimal requirements for gradient formation, we investigated the spatially self-organizing Min system as a prototype for gradient formation in a synthetic environment (***Figure 1A***). We first enclosed purified MinD and MinE proteins from the bacterium *E. coli* and ATP in membrane clad soft polymer compartments of picoliter sample volume and cell-shaped geometry (***Figure 1—figure supplement 1***, ***Figure 1—figure supplement 2***). Consistent with previous results and live-cell studies, MinE and MinD dynamically oscillate from pole-to-pole. Previous controls with purified Min proteins on flat bilayers and the non-hydrolysable ATP analog ATPγS confirmed that the dynamic protein patterns were thereby powered by ATP (***Loose et al., 2008***).

Compared to living cells, the temporal scale for the oscillation period is conserved in the synthetic system. However, both the compartments and the spatial scales of the protein waves are about ten times bigger as compared to living cells. Why the Min patterns in vivo are about ten times larger than in vitro is quantitatively not fully understood. Different protein ratios, ionic strength of the buffer and membrane composition have been shown to affect the spatial scales (***Loose et al., 2008***; ***Vecchiarelli et al., 2014***). Furthermore the membrane potential (***Strahl and Hamoen, 2010***) and molecular crowding in a living cell could influence Min protein patterns. An advantage of the larger spatial scales in vitro is the possibility to study with much greater detail how the concentration profiles of MinD and MinE, which result in the formation of MinD gradients, are modulated during an oscillation period. Following the MinD and MinE concentration profiles along the compartment length over time, we found that the dynamic profiles are characterized by the following succession of events: First, attachment and increase in concentration of MinD at one site of the compartment, then delayed attachment of MinE to MinD and increase in MinE intensity at the trail of MinD (***Figure 1—figure supplement 3***). Although the size of bacterial cells and photo bleaching is limiting for characterizing the Min profiles in vivo in such detail as it is possible in vitro, the intensity peak of MinE at the trail of the Min pattern was also observed in *E. coli* and is generally referred to as 'MinE ring'. (***Raskin and de Boer, 1997***; ***Fu et al., 2001***) After formation of the MinE peak MinD/E were disassembled from the membrane at the trail of the MinD zone, which was accompanied by assembly of a new MinD/E zone at the other pole of the compartment (***Figure 1—figure supplement 3***). This disassembly of Min protein patterns

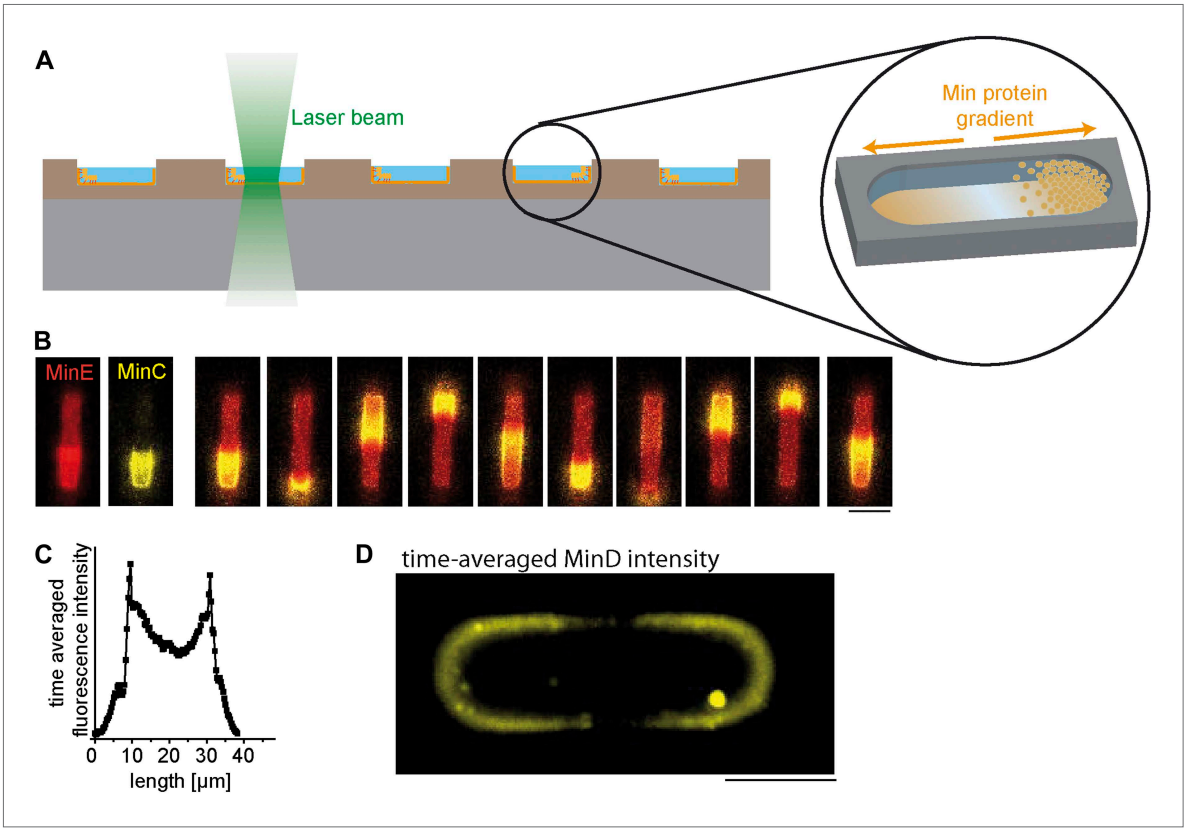

Figure 1. Protein gradients self-organize in soft-polymer containers. (**A**) Experimental setup. Purified proteins of the MinC/D/E system were reconstituted in membrane-clad soft-polymer compartments and imaged by confocal microscopy. Profiles and electron micrographs of the compartments are shown in ***Figure 1—figure supplement 1*** and a more detailed description of the assay to reconstitute Min protein oscillations is presented in ***Figure 1—figure supplement 2***. ***Figure 1—figure supplement 3*** demonstrates how the intensity profiles of MinD and MinE along the length axis of the compartments are modulated with time. To mimic a bacterial membrane we used *E. coli* polar lipids to generate supported lipid membranes. A more detailed description of how lipid composition influences pattern formation of Min proteins is presented in ***Figure 1—figure supplement 4***. (**B**) In cell-shaped compartments, eGFP-MinC (yellow) follows the oscillations of MinD and MinE (red). Comparable results were obtained in more than a hundred soft-polymer compartments. Confocal time-lapse images with the image plane at the bottom of the compartment. Protein concentrations: 1 µM MinD, 0.9 µM MinE, 0.1 µM MinE.Atto655 (red), 0.05 µM eGFP-MinC (yellow). Time between individual frames: 30 s. Scale bar: 5 µm. (**C**) The time-averaged concentration profile of MinC along the long axis of a compartment has a distinct concentration minimum in the middle of the compartment. The time averaged distribution of protein concentrations was calculated by acquiring time-lapse-images with the focal plane at the middle of the compartment and averaging the intensity of the acquired frames. (**D**) Time-averaged fluorescent signal of eGFP-MinD with image plane in the middle of a compartment. An intensity offset is subtracted to better visualize the gradient along the boundary of the compartment. 0.9 µM MinD, 0.1 µM eGFP-MinD, 1 µM MinE. Scale bar: 5 µm. Stable pole-to-pole oscillations which result in the time-averaged gradient of MinD are severely affected if the membrane targeting sequence of MinE is deleted (***Figure 1—figure supplement 5***).

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The following figure supplements are available for figure 1:

Figure supplement 1. Micro compartments in a soft-polymer chip.

DOI: 10.7554/eLife.03949.004

Figure supplement 2. Experimental setup.

DOI: 10.7554/eLife.03949.005

Figure supplement 3. Protein gradients in vitro are established by dynamic redistribution of proteins.

DOI: 10.7554/eLife.03949.006

Figure supplement 4. Lipid composition dependent formation of protein gradients.

DOI: 10.7554/eLife.03949.007

Figure supplement 5. Deletion of the membrane targeting sequence of MinE affects pattern formation in micro compartments.

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at the trailing zone during each oscillation is comparable to the disassembly at the trailing edge of travelling Min patterns on flat membranes. The main difference of Min proteins profiles on flat membranes and in microcompartments is that the concentration profiles on flat membranes only change their localization while traveling across the membrane. In contrast, during oscillations in compartments new membrane attached Min protein patterns need to be repeatedly established and disassembled every half oscillation period, which results in a remarkable remodelling of the concentration profiles during the oscillation cycle and an asymmetry in their rise and decay phases at the poles.

To determine the mean spatial distribution of MinD, we averaged the fluorescence signal of MinD within the cell-shaped compartments over time using confocal time-lapse movies. Remarkably, MinD formed indeed a clear nonhomogeneous concentration profile, with the lowest concentration in the middle of the compartment (***Figure 1D***). Thus, we conclude that using the Min system, the establishment of an effective protein gradient indeed requires only a lipid membrane, an appropriate reaction space, and two proteins that self-organize under the consumption of energy, in the form of ATP.

While the requirements for Min gradient formation are now understood to a point, at which these gradients can be reconstituted in vitro, less is known about the importance of the structural properties of the gradient forming proteins. Although MinD has been shown to interact with membranes in vitro and MinE interacts with MinD (***Hu et al., 2002***), MinE also harbours a short membrane targeting sequence at its N-terminus (***Park et al., 2011***). Mutations in the membrane targeting domain of MinE result in defects in cell division and in distinct phenotypes, such as filamentous cells or a minicelling phenotype, respectively (***Park et al., 2011***). However how the membrane targeting of MinE contributes to a normally functioning Min system is not fully understood. To gain a deeper insight in how the membrane targeting sequence of MinE contributes to gradient formation of the Min system we purified and labelled a MinE mutant without its membrane targeting sequence (MinE without amino acids three to eight, herein referred to as MinE(Δ3–8)) and reconstituted it with MinD in microcompartments. Interestingly, MinE(Δ3–8) resulted in a severe phenotype in the bottom-up system. The Min system was still able to self-organize into dynamic pattern, but instead of a stable pole-to-pole oscillation, the Min system formed dynamic patches with irregular shape and without a constant axis of movement (***Figure 1—figure supplement 5***). Thus, although MinE(Δ3–8) still counteracted MinD, no stable MinD gradients were generated. Depending on the degree of affecting the membrane targeting sequence of MinE, the effective generation of a gradient and its localization along the long axis of the cell might therefore be altered to different degrees, which might account for the distinct phenotypes in vivo, such as filamentous cells or mini cells.

While the structural properties and the diffusion-reaction driven self-organization of MinD and MinE determine the non-equilibrium distribution of the Min system, a third protein, MinC, is proposed to be the actual mediator to forward positional information of the Min gradient to downstream targets. In particular, MinC is proposed to inhibit FtsZ–the cytoskeletal framework of divisome assembly (***Hu et al., 1999***; ***Hu and Lutkenhaus, 2000***; ***Shen and Lutkenhaus, 2009***, ***2010***). To investigate the spatial distribution of eGFP-MinC in our minimal system, we co-reconstituted MinD and MinE oscillations with MinC in micro compartments. We found that eGFP-MinC oscillated from pole-to-pole by coupling to MinD/E patterns, which strongly resembles the dynamics of MinC in vivo (***Figure 1B***). Based on time-lapse movies, we then analyzed the time-averaged distribution of eGFP-MinC and found the concentration profile of MinC also exhibit a pronounced minimum in the middle of the compartment (***Figure 1C***). This experiment revealed that the effective non-equilibrium distribution of the complete Min system, with the highest concentration of MinC at the cell poles, is fully recapitulated,in our simple cell-free setting.

Note that the *E. coli* lipid extract for generating membranes in these experiments is a mixture of mainly three different lipids, (phosphatidylethanolamine [PE, neutral], phosphatidylglycerol [PG, negatively charged] and cardiolipin [CA, negatively charged]) and that the membranes have a net negative charge. Recently it has been shown that a minimal lipid bilayer for pattern Min formation on flat supports only requires two different lipids. Moreover, cardiolipin, which has previously been proposed as a structural polar cue, which might help in triggering the pole to-pole oscillations of Min proteins, was not required for Min pattern formation on planar supports. Instead, the negative charge has been suggested to play a primary role in formation of surface waves on planar membranes. (***Vecchiarelli et al., 2014***) To address whether membrane charge and not the chemical membrane composition is also sufficient for gradient forming pole-to-pole oscillations, we established the minimal membrane composition for Min protein oscillations in our synthetic system. First, we confirmed the aforementioned

results that membrane charge is a strong determinant for Min pattern formation on flat supported membranes (***Figure 1—figure supplement 4A–D***). Then we reconstituted the Min proteins in compartments with two different minimal membrane mixtures: First, with 70% neutrally charged DOPC and 30% negatively charged cardiolipin, and then with 70% DOPC and 30% negatively charged PG. In both cases, the proteins retained their ability of pattern formation and pole-to-pole oscillations (***Figure 1—figure supplement 4E***). Thus, we conclude that Min gradients can be established without any structural cues in the membrane. In contrast, simple physical parameters, such as electrostatic interactions between the membrane and the proteins, are strong determinants for gradient formation. For high amounts of negatively charged lipids, the length scales of the protein patterns decrease (***Figure 1—figure supplement 4B*** and [***Vecchiarelli et al., 2014***]). Thus, the spatial scales of the Min proteins on highly negatively charged membranes would be too small to form stable pole-to-pole oscillations in compartments, which were engineered for protein length scales on *E.coli* membranes. The implication that Min protein oscillations are perturbed if the membrane charge increases is in agreement with live cell studies of GFP-MinD in PE-lacking *E.coli* cells, which demonstrate that Min protein pattern are affected if the membrane charge is increased (***Mileykovskaya et al., 2003***) and provides further evidence that a balanced ratio of charged and non-charged lipids is of critical importance for the live cycle of *E.coli*.

Localization of downstream targets by reconstituted protein gradients

Intracellular protein gradients play a pivotal role in cellular organization through providing positional information for downstream proteins. However, although protein gradients are now emerging as general motives to organize cells, tools to study gradient-mediated organization of three-dimensional space are limited. To directly investigate whether the established Min gradient indeed provides an efficient cue to position downstream targets, we attempted the assembly and positioning of FtsZ to the middle of a synthetic compartment in a co-reconstitution assay. To keep the system as minimal as possible, we avoided the native membrane adaptors of FtsZ, FtsA and ZipA, and used a FtsZ hybrid protein, fused to YFP and a membrane targeting sequence (FtsZ-mts) (***Osawa et al., 2008***; ***Arumugam et al., 2014***). In spite of this simplification, we found that highly FtsZ-mts enriched regions were clearly visible in the center of the compartments, while FtsZ-mts localization was strongly reduced at the polar regions (***Figure 2***). Negative controls with FtsZ-mts bundles on flat supported bilayers confirmed that all thee Min proteins are required to deplete FtsZ-mts from the membrane (***Figure 2—figure supplement 1***) (***Arumugam et al., 2014***). This unequivocally demonstrates that an effective time-averaged protein gradient can indeed regulate the localization of down-stream targets to predefined localizations, such as the middle of a compartment.

In contrast to the living cell, the FtsZ-mts distribution in the middle of synthetic compartments appears to be rather broad. The major reason for the wide FtsZ-mts distribution is most likely the ten times larger Min protein pattern in vitro, as compared to living cells. The wide MinC minimum allows multiple FtsZ-mts bundles to localize in a wide region in the middle of the compartment.

Note that FtsZ-mts bundles in the central region aligned perpendicular to the long axis of the compartments (***Figure 2A,B,D***). This finding is in agreement with previous results that demonstrated a preferential alignment of FtsZ-mts along negatively curved membranes on grooved glass as membrane support. (***Arumugam et al., 2012***).

The MinC-G10D mutant interacts with the MinD/E system, but does not inhibit FtsZ-mts at compartment poles

Positional information of the MinD gradient in vivo and in our synthetic system is mediated by the FtsZ inhibitor MinC which directly interacts with FtsZ (***Hu et al., 1999***). MinC has a C-terminal and an N-terminal domain, (***Hu and Lutkenhaus, 2000***; ***Cordell et al., 2001***) which are both involved in FtsZ inhibition. The C-terminal domain of MinC is responsible for binding to MinD and dimerization of MinC. Furthermore, it binds to the conserved C-terminus of FtsZ and was suggested to compete with FtsA and ZipA to inhibit FtsZ ring formation. (***Hu and Lutkenhaus, 2000***; ***Shen and Lutkenhaus, 2009***). Note, that FtsZ-mts comprises amino acids 1–366 of *E. coli* FtsZ, but instead of the C-terminally conserved tail, it comprises YFP and a membrane targeting sequence. The proposed interference of the C-terminal domain with FtsZ is therefore not involved in the system described above, which suggest that the inhibitory activity of the C-terminal domain is not required in a minimal system to inhibit FtsZ at compartment poles. In the context of a living cell, the inhibitory activity of the C-terminal

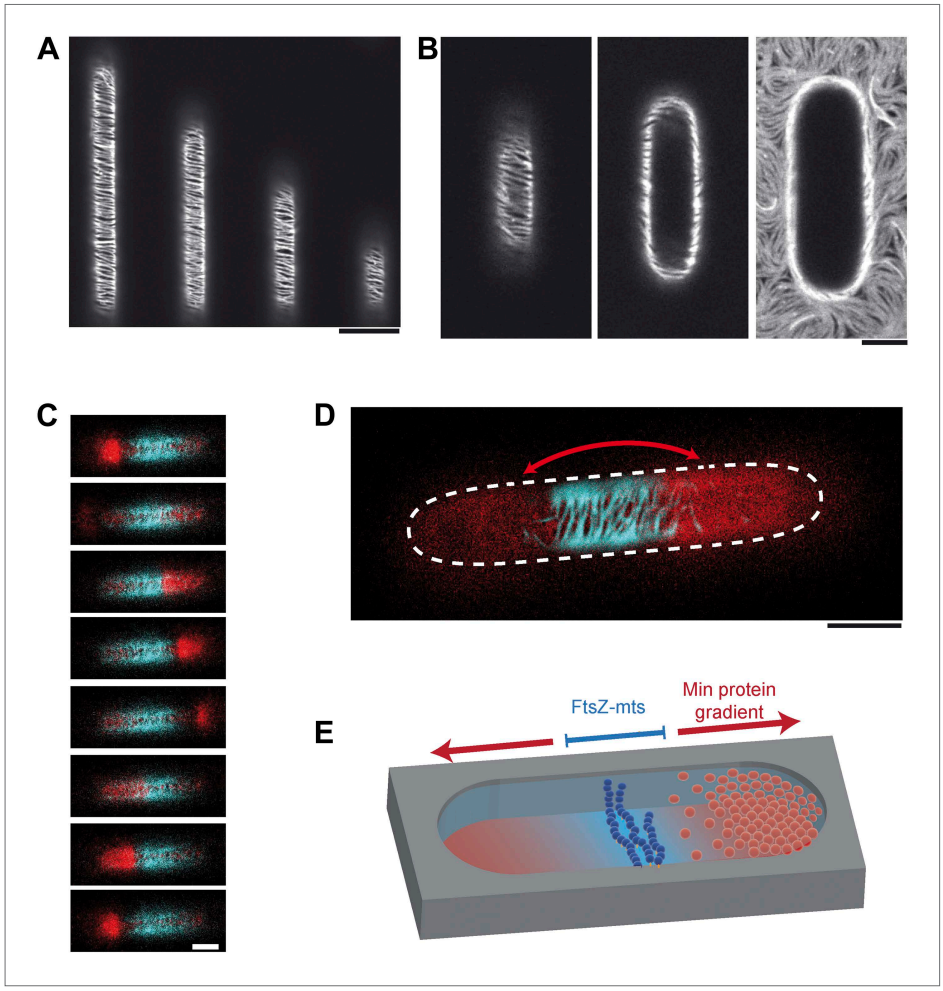

Figure 2. Reconstituted protein gradients coordinate localization of downstream targets in cell-shaped compartments. (**A**) Confocal image of the tubulin homolog FtsZ-mts on the bottom of membrane-clad compartments. FtsZ-mts assembles into bundles which are aligned perpendicular to the length axis of the compartments. Scale bar: 10 μm. (**B**) Images with the focal plane at the bottom, middle and top of the compartment, respectively. The buffer was not yet reduced to a level below the upper level of the chip. Therefore membrane and FtsZ-mts network are still intact on the upper level of the chip. Scale bar: 5 μm. (**C** and **D**) When the gradient forming Min system (red: MinE.Atto655) and FtsZ-mts (blue) are reconstituted together, the Min gradient coordinates the localization of FtsZ-mts to the middle of the compartments. Scale bar: 5 μm. (**C**) Time between frames: 20 s. Negative controls in ***Figure 2—figure supplement 1*** confirm that MinD and MinE are required to form dynamic patterns and that MinC is needed in addition to deplete FtsZ-mts from the membrane. (**E**) Schematic image of reconstituted FtsZ-mts positioning.

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The following figure supplement is available for figure 2:

Figure supplement 1. MinD MinE and ATP are required to form dynamic patterns and MinC is needed to deplete FtsZ from the membrane.

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domain of MinC might provide an additional mechanism for disrupting FtsZ, rendering the Min system more robust.

The N-terminal domain of MinC inhibits FtsZ polymerization by binding to an alpha-helix of FtsZ at the interface of FtsZ subunits in a filament (***Hu et al., 1999***; ***Shen and Lutkenhaus, 2010***). Since the FtsZ-binding region for the C-terminal domain of MinC is not present in FtsZ-mts, the major inhibitory activity contributing to FtsZ-inhibition in our synthetic system should originate from the N-terminal domain of MinC. To test this hypothesis, we used a MinC mutant (MinC-G10D) with lower inhibitory

activity of its N-terminal domain (***Hu et al., 1999***; ***Shen and Lutkenhaus, 2009***, ***2010***). The MinC-G10D mutant has reduced ability to inhibit FtsZ in vivo and in vitro, but interacts with MinD (***Hu et al., 1999***). We first characterized MinC-G10D by co-reconstituting it at different concentrations with FtsZ-mts, MinD and fluorescently labeled MinE on flat supported membranes. Consistent with the behavior of wild type MinC, we found that the self-organizing Min protein patterns were unperturbed at low concentrations of MinC-G10D, and that the Min patterns were weakened by high concentrations of MinC-G10D (***Figure 3A***). The weakening of Min patterns for high concentrations of MinC is likely due to an overlap of the binding sites for MinC and MinE on MinD (***Ma et al., 2004***; ***Wu et al., 2011***), which might result in a competition of MinC with MinE for binding sites on MinD at large MinC concentrations. The weakening of Min Protein patterns therefore suggests that MinC-G10D binds to MinD. However, in contrast to MinC the MinC-G10D mutant was inefficient in depolymerizing FtsZ-mts on flat membranes, as well as at the poles, when co-reconstituted in microcompartments (***Figure 3B,C***, ***Figure 3—figure supplement 1***). These observations confirm that the inhibitory activity in the synthetic system originates from the N-terminal domain of MinC and that the inhibitory activity of the N-terminal domain is sufficient to inhibit FtsZ-polymerization at compartment poles in the context of a minimal system.

Establishment of protein gradients is dependent on compartment geometry

Gradient formation and FtsZ localization dependent on compartment length

Experiments with aberrantly shaped cells, such as round and filamentous *E. coli*, have shown that dynamic pattern formation depends on compartment geometry. However, the mode of pole-to-pole oscillations should be stable over a substantial variation in length. As cells grow and double their length before division, stable pole-to-pole oscillations of the Min proteins need to be established over a spatial range of at least two times the length of the daughter cells. We therefore hypothesized that stable pole-to-pole oscillations should be found in microstructures of different length, where the range should vary at least by a factor of two, if this length-dependent robustness is intrinsic to the Min system. To test this hypothesis, we reconstituted the oscillations of MinD and MinE in microcompartments of systematically varying length from 12 μm to 245 μm and investigated the stability of Min patterns at different compartment lengths. In compartments with a constant width of 10 μm, the Min system oscillated along the long axis of the compartments (***Zieske and Schwille, 2013***) (***Video 1***). We observed oscillations with multiple minima and maxima in long compartments, and found that every oscillation mode occurred at a defined range of the compartment length (***Figure 4A***). In most of the short compartments with an aspect ratio (length to width) below 4.5, we observed pole-to-pole oscillations, in rare cases they prevailed up to aspect ratios of 5.5.

In particular, these experiments demonstrate that pole-to-pole oscillations are stable (i.e., no other oscillation mode occurs in those compartments) over a significant length range of 15 μm–35 μm. In other words, although the length of these cavities varies by about a factor of 2.3, the oscillatory behaviour is conserved. This result shows that no auxiliary proteins besides the MinDE protein system are required to adopt the Min wavelength to the varying length of a growing bacterium, demonstrating the power of this system as a robust spatial cue.

In the shortest of these compartments, with an aspect ratio of 1.2, we occasionally observed an aberration of the oscillation axis from the long axis (***Video 2***), which is indicative of a transition from stable pole to-pole oscillations to the patterns observed in round compartments and cells (***Zieske and Schwille, 2013***). ***Figure 4A*** provides an overview of the oscillation modes with respect to the length-to-width ratio of the compartment.

While the length dependent increase of oscillation modes was well conserved in our experiments, the oscillation period was prone to small perturbation. In particular, we observed a slight increase of the oscillation period over time, which might be a result of buffer evaporation (***Figure 4—figure supplement 1***). Thus, oscillation periods between less than a minute and 4 min were measured. This time scale is well in agreement with measurements of 0.5–2 min for the oscillation period in living cells (***Raskin and de Boer, 1999b***).

The strong fluctuation of oscillation periods in living cells, as well as between different samples of the synthetic system, render a systematic characterization of cell length dependent protein oscillations challenging. To overcome these problems and to better characterize the dependence on compartment length, we acquired time-lapse movies, capturing numerous compartments simultaneously. Then, we

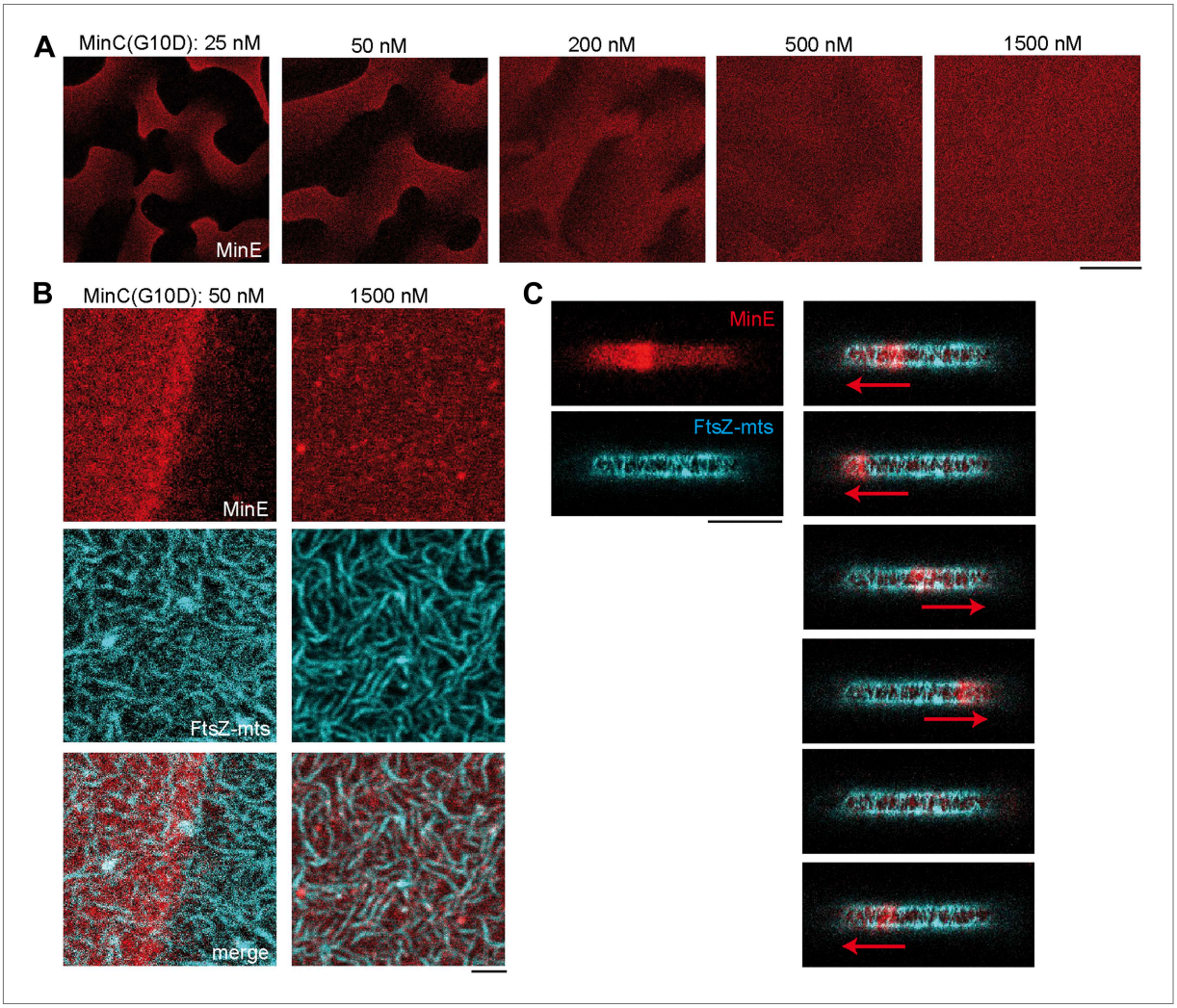

Figure 3. MinC-G10D does not inhibit polymerization of FtsZ-mts. (**A**–**C**) FtsZ-mts (blue), MinD, MinE (5% labeled with Atto655: red) were reconstituted with the MinC-G10D mutant. Concentrations of MinD, MinE and FtsZ-mts are constant for all images. (**A**) Confocal images of Min protein pattern (MinE.Atto655: red) on flat supported membranes at different concentrations of MinC-G10D. High concentrations of MinC-G10D disturb Min protein patterns. The contrast between images is not comparable and is increased for higher concentrations of MinC-G10D, because the MinE intensity at the membrane decreased with higher concentrations of MinC-G10D. Scale bar: 50 μm. (**B**) Confocal images of the Min system (MinE.Atto655:red) and FtsZ-mts (blue) on flat membranes at different concentrations of MinC-G10D demonstrate that MinC-G10D is inefficient in disturbing FtsZ-mts networks. Scale bar: 2 μm. (**C**, ***Figure 3—figure supplement 1***) In cell-shaped micro compartments with MinD, MinE, FtsZ-mts and 50 nM MinC-G10D the Min system (MinE.Atto655: red) oscillates from pole-to-pole. However MinC-G10D does not inhibit polymerization of FtsZ-mts (blue) at the compartment poles. Time between frames: 90 s, scale bar: 10 μm.

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The following figure supplement is available for figure 3:

Figure supplement 1. MinC-G10D does not inhibit polymerization of FtsZ-mts at compartment poles.

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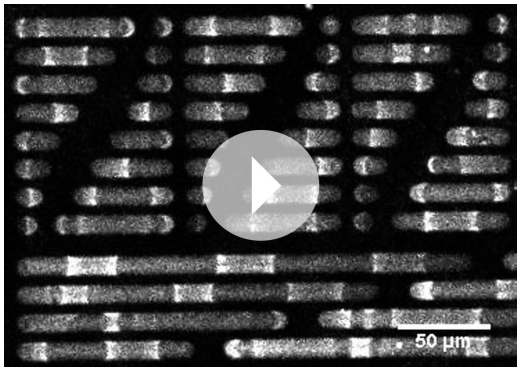
compared Min oscillations in micro compartments with different aspect ratios up to 5.5, which were acquired at the same time on the same chip. Imaging multiple compartments on the same chip allowed us to analyse Min oscillations in about 200 compartments under the same experimental conditions at the same time. We found that the oscillation period between aspect ratios of 1.5 and 5.5 increased from 79 s to about 165 s. For higher aspect ratios we only observed higher oscillation modes. For very low aspect ratios of 1.2 the mean value for the oscillation period was slightly higher than for 1.5 (***Figure 4B***). Note that in very small cells a stochastic switching of Min protein pattern between the poles have been observed (***Fischer-Friedrich et al., 2010***) and that a longer oscillation period for very



Video 1. Reconstituted Min protein oscillations in microcompartments of different length. This video shows a confocal time lapse movie of reconstituted MinD/MinE pattern in cell-shaped micro-compartments. 10% MinE are labeled with Alexa 488. The movie follows the dynamics of Min protein pattern for 17 min.
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small compartments might represent a transition from normal pole-to-pole oscillation to different dynamics in very small compartments.

To address how downstream processes of the Min gradient are affected by the length dependent oscillation modes in compartments of different length, we investigated FtsZ-mts localization in these compartments. We found that higher oscillation modes resulted in multiple minima of the MinC profile and consequently in multiple distinct regions of FtsZ-mts assembly (***Figure 5***).

Gradient formation dependent on compartment width

How does the cell diameter affect cell organization, and in particular gradient formation? Tools to answer this question for the chosen system in vivo are limited, because in living *E.coli* bacteria it is challenging to systematically modify the width of a cell. Thus, it is unknown whether the constant diameter of cells plays a significant role as a geometric cue for septum positioning. Compared to living cells, our in vitro system is much more flexible in pre-setting a defined geometry and reaction volume, enabling us to address for the first time the role of cell width for Min gradient formation and thus, as a geometrical cue for cell organization. In compartments with a small width of 10 µm, only oscillations along the long axis were supported (***Video 1***, ***Figure 6A***). Interestingly however, larger widths also resulted in oscillations along the short axis of a compartment (***Figure 6B***, ***Video 3***). Even larger widths resulted in more complex oscillation patterns (***Figure 6—figure supplement 1***, ***Video 4***). These experiments revealed that a fixed small width of the cell is important for Min gradient formation along the long axis.

Geometry modulated partitioning of min proteins during septum closure

A dramatic local change in cell width occurs during every cell division in *E. coli* in the middle of the cell due to septum closure. Theoretical modeling suggested that this change in geometry might account for an equal partitioning of Min proteins to both daughter cells. (***Di Ventura and Sourjik, 2011***) In other words, septum constriction is hypothesized to provide a geometrical cue to switch from the pole-to-pole oscillations of Min proteins to a symmetric double oscillation, which enables the daughter cells to trap equal amounts of Min proteins. In live cells it is challenging to directly observe the partitioning of proteins, because the constricting septum of *E. coli* becomes smaller than the optical resolution limit during the proposed process. At the same time, the Min protein system is a highly dynamic system, rendering time-lapse imaging with current superresolution techniques difficult. To experimentally test the proposed partitioning of Min proteins during septum constriction, compartments that mimicked morphologically different stages of bacterial cell division were designed. Using cell-shaped compartments with a systematically varying width of the artificial septum, we analyzed whether different patterns occurred in more 'constricted' compartments, as compared to 'non-constricted' compartments.

We found that double oscillations occurred indeed in compartments with a small neck in the middle of the compartment. As a negative control, normal pole-to-pole oscillations were sustained in compartments with the same length, but constant cell width. Finally, geometries mimicking the full septum closure, that is, separation in two cells, induced independent pole-to-pole oscillations in each 'daughter' compartment (***Figure 6C***, ***Figure 6—figure supplement 2 and 3***, ***Video 5***). This finding presents the first direct experimental evidence that equal Min protein distribution during cell division is a result of geometrical cues. Furthermore, it strikingly confirms theoretical modeling predictions, where septum constriction supports partitioning of the Min proteins (***Di Ventura and Sourjik, 2011***).

To address the role of cell length for equal partitioning of Min proteins during septum closure, we performed similar experiments using cell shaped compartments with different length. We demonstrated

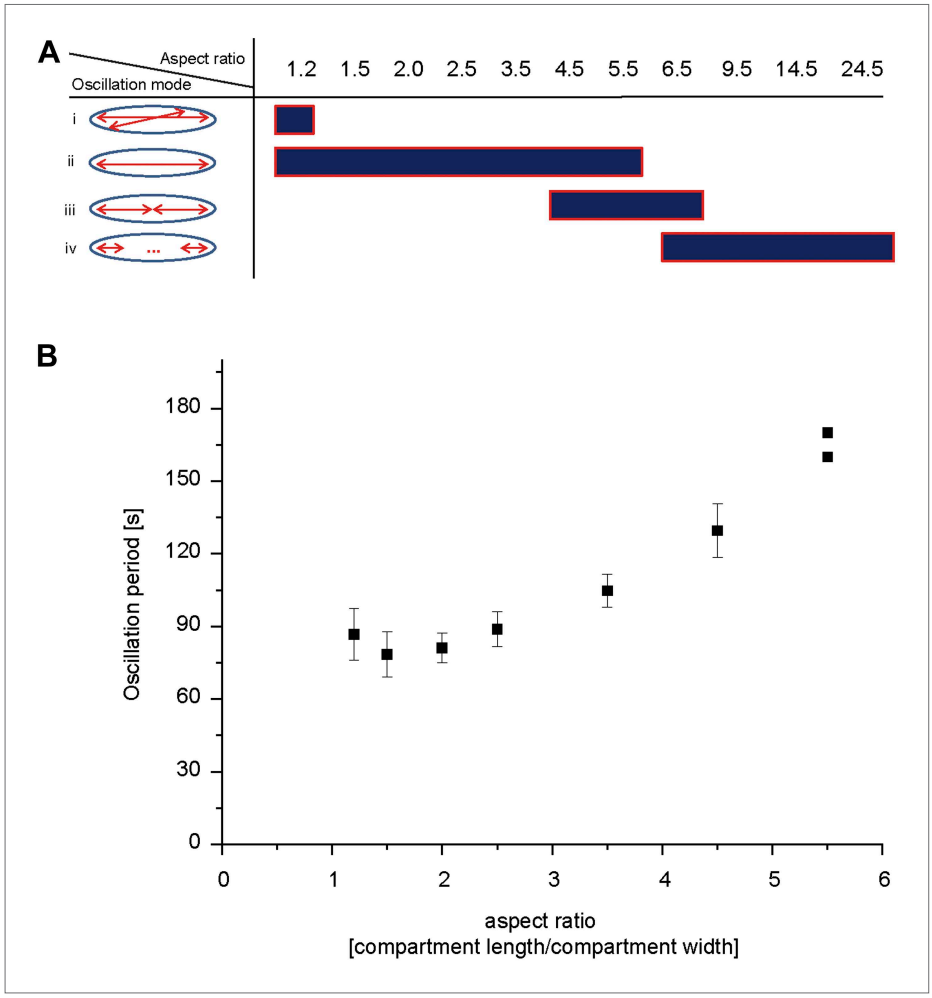

Figure 4. Compartment length dependent protein oscillations. (**A**) Experiments such as in ***Video 1*** were analyzed for the oscillation mode in the corresponding compartments and categorized as (i) oscillations with aberration from the long axis of the compartment, (ii) pole-to-pole oscillations, (iii) double oscillations, (iv) higher oscillation mode (at least triple oscillations). (**B**) Oscillation period of pole to pole oscillations in dependence of compartment length. Simultaneous time-lapse acquisition of Min protein patterns in multiple compartments allowed highly comparable experimental conditions. In total, 27 to 29 compartments of seven different aspect ratios (1.2, 1.5, 2.0, 2.5, 3.5, 4.5, 5.5) on the same time lapse movie were analyzed. Out of 27 compartments with an aspect ratio of 1.2, one compartment harboured oscillation that deviated from the length axis. Out of 28 compartments with an aspect ratio of 4.5 we observed 16 compartments, and out of 29 compartments with an aspect ratio of 5.5 we observed 27 compartments with double oscillations or oscillations which switched between pole-to-pole oscillations and double oscillations. All remaining compartments harboured pole-to-pole oscillations for which the mean oscillation period and standard deviation was calculated. For an aspect ratio of 5.5 the two data points are directly plotted, due to the small sample size. ***Figure 4—figure supplement 1*** demonstrates that the oscillation period increases over time.

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The following figure supplement is available for figure 4:

Figure supplement 1. Kymograph of MinE oscillations.

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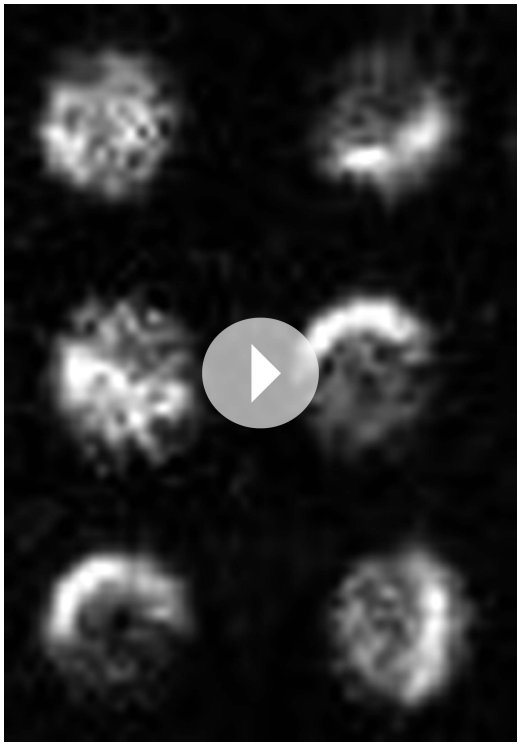
that a switch from asymmetric pole-to-pole oscillations to symmetric double oscillations occurred at a certain cell length. However, in longer compartments, double oscillations occurred already in unconstricted cells und thus, a constriction in the middle of the cell had no additional effect on pattern formation. In very short cells, on the other hand, a constriction in the middle of the compartment resulted in a new length axis of the two compartments perpendicular to the length axis of the unconstricted



Video 2. Min protein pattern in short compartments. This movie shows confocal time-lapse movies of a MinD and MinE patterns (doped with 10% MinE.Alexa488) in 12 μm long and 10 μm wide compartments. Six time-lapse movies with compartments in which the Min patterns aberrate from a stable pole-to-pole axis are stitched together. The protein patterns are followed over a time of about 17 min.
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'mother' compartment (***Figure 6D***). Thus, a reorientation of the pattern occurred during constriction, from oscillations along the long axis in the unconstricted compartment, to perpendicular new length axes. These findings demonstrate that an optimal range of cell length exists were Min gradients represent an efficient cue for septum formation. In addition, the Min partitioning to both daughter cells can be controlled by changing the local compartment width during septum closure in the middle of the cell.

Discussion

We demonstrated that coordinated spatial control by intracellular protein gradients can be reconstituted in cell-free systems, and that these gradients are modulated by geometric parameters. The reconstitution of self-organizing protein systems in membrane clad soft-polymer compartments represents a simple approach to study membrane interacting proteins in a defined sample volume and geometry. Applying this system, we particularly tackled three major questions. First, what are the minimal requirements for protein gradient formation? Second, can the organized localization to predefined sites be achieved by reading out the positional information of a minimal protein gradient system? And finally, what are the geometric boundary conditions to generate and modulate protein gradients?

To address the minimal requirements for gradient formation, we considered the Min protein system as a prototype for a gradient forming system and accomplished the reconstitution of a time-averaged nonhomogeneous concentration profile with the lowest concentration in the middle of the compartment. We demonstrated that the minimal requirements for Min gradient formation comprised only a negatively charged membrane, a cell-shaped compartment, ATP as an energy source, and the two antagonistic proteins MinD and MinE.

To investigate whether the reconstituted gradient provides an efficient cue to organize cellular space by positioning down-stream targets, we co-reconstituted the MinD/E system with the cell division protein FtsZ-mts and the FtsZ inhibitor MinC. This cell-free bottom-up system recapitulated remarkably cell-like properties, such as coordinated spatial coordinated localization of FtsZ-mts to the middle of a cell-like compartment. Interestingly, although it is proposed that MinC has two inhibitory domains which act on FtsZ–FtsZ interactions and on the ternary system FtsZ-FtsA/ZipA, respectively (***Shen and Lutkenhaus, 2009***, ***2010***), our data suggest that MinC can already inhibit FtsZ-localization at compartment poles by only disrupting FtsZ–FtsZ interactions. While we do not exclude that the interaction of MinC with the conserved C-terminal domain, which interacts with FtsA and ZipA, is a parallel mechanism to inhibit FtsZ polymerization at cell poles and possibly make FtsZ structures more sensitive to the action of the Min system, our results show that the minimal biochemical requirements to displace FtsZ from compartment poles by the Min system do not involve the disruption of FtsZ-membrane binding sites.

Moreover, we demonstrated that very long compartments harbored a MinC protein concentration profile with multiple minima. While a normal growing wild type cells typically comprises a Min concentration gradient with only one minimum for division protein assembly, multiple minima resulting in more than one sides for cell division, are potentially advantageous in filamentous cells. If transient unfavorable environmental conditions inhibit cell division but not cell elongation, divisions at multiple

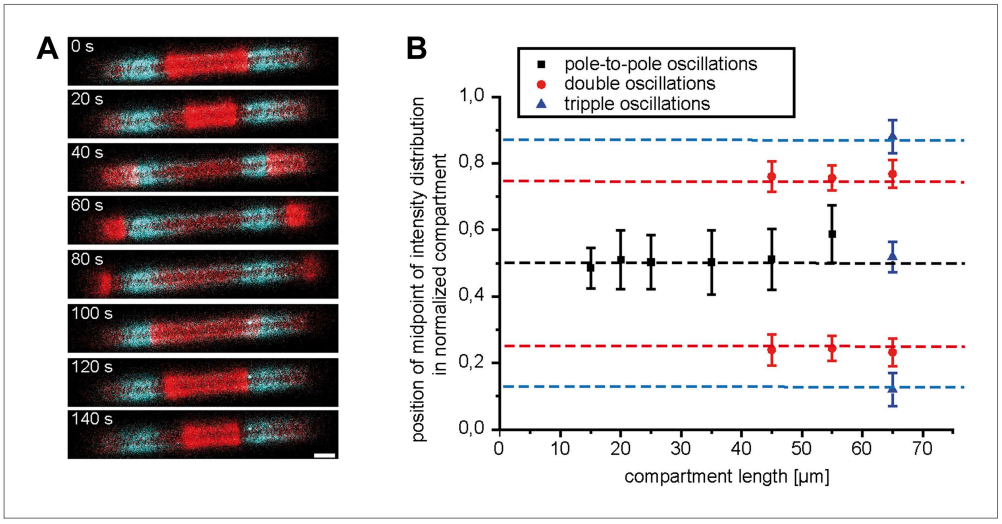

Figure 5. Compartment length affects FtsZ localization. (**A**) In compartments with double oscillations of the Min system (red: MinE.Atto655), FtsZ-mts (blue) localizes to two regions along the cell length. Scale bar: 5 µm. (**B**) Localization of FtsZ-mts along the length axis of an artificial cell for pole-to-pole oscillations (black), double oscillations (red) and triple oscillations (blue) in comparison to expected values (dotted lines), considering the symmetries of oscillation modes of Min protein along the long axis of a compartment. For pole-to-pole oscillations in 55 µm long compartments: n = 3 (For 55 µm the majority of structures revealed double oscillations and only in a minor fraction of these long structured occured pole-to-pole oscillations). For all other values, n >9.

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sides could ensure the re-establishment of a normal cell length when conditions, which support division, reoccur.

To systematically address how compartment geometry modulates Min protein gradients, we reconstituted Min protein patterns in compartments with systematically varying shape. Varying the length of the compartments, we found that robust pole-to-pole oscillations occur in compartments, even if the length of these compartments was varied by a factor of 2.3. In a live cell, this robustness of the Min oscillations with respect to cell length should be highly significant, because the length of living cells is not constant over time, and during the life cycle of *E. coli*, the cell doubles in length. The observation that the Min protein patterns in vitro deviate from a stable axis in very short compartments, and that higher order oscillations systematically occur the longer the compartment is, confirm that the MinD/E patterns in synthetic compartments are strikingly similar to the Min pattern of round, wild type and filamentous cells in vivo (***Fu et al., 2001***; ***Hale et al., 2001***; ***Corbin et al., 2002***; ***Shih et al., 2005***; ***Raskin and de Boer, 1999b***).

Interestingly, when we increased the width of the compartments, the Min proteins oscillated along the short axis of the compartment. The cause of oscillations switching along the length axis to oscillations along the short axis is not known. However, the width at which this switching occurs might be correlated with the wavelength of the Min protein patterns and therefore related to the mechanism for determining the wavelength of the Min system. It is also unknown whether a similar gradient along the short axis of the cell is used in living cells, and which factors might contribute on the bacterial scale to regulate gradients along the short axis.

However, note that FtsZ involving cell-division in certain bacteria, such as *Laxus oneistus*, occurs along the long axis. (***Polz et al., 1992***; ***Leisch et al., 2012***) Although the spatial cues to determine the position of their division plane are unknown, it is intriguing to speculate that gradients along the short axis might also provide an effective cue to spatially organize space along the short axis Thus, protein gradients might also orient the cell division plane in cells which divide along the length axis of the cell.

In line with our in vitro results, Min oscillations along the short axis of a bacterial cell might be accomplished by an increased width as compared to an *E.coli* cell, or by smaller scales of Min protein patterns through modified reaction and/or diffusion rates of the proteins. However, also other systems which generate gradients along the length axis of a bacterial cell, such as activity gradients of

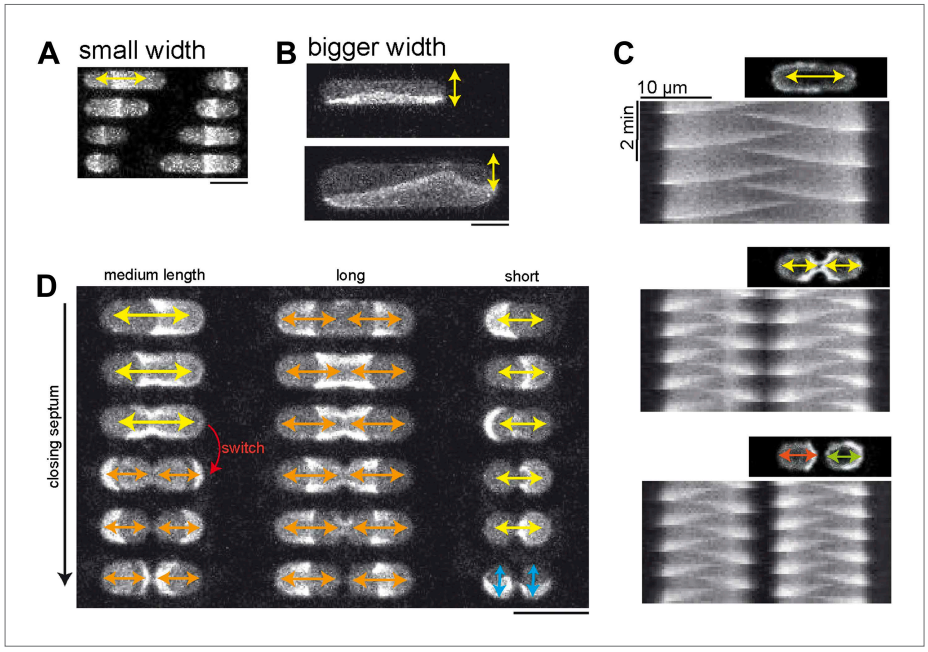

Figure 6. Compartment width affects gradient formation. (**A**) Pole to pole oscillations occur is narrow compartments. Scale bar: 20 µm. (**B**) In wider compartments the proteins oscillate parallel to the length axis of the compartment. Scale bar: 20 µm. In compartments with an even larger width more complex patterns occur as depicted in ***Figure 6—figure supplement 1***. (**C**) Confocal images and kymographs of MinE pattern in compartments with different degrees of 'constriction' depict pole-to-pole oscillation in compartments with a constant width (upper panel). In compartments with a narrow width in the middle of the compartment double ascillations occur. In separated compartments independent pole-to-pole oscillations are observed. (**D**) Oscillation modes in compartments with different lengths and stepwise narrowing width at the middle of the compartment. Left: Compartments without constriction or only slight decrease of the width in the middle harbor pole-to-pole oscillations (asymmetric oscillations, yellow arrows). Compartments with a narrow width in the middle harbor double oscillations (symmetric oscillations, orange arrows). A series of time-lapse images as well as kymographs of the third and fourth compartment, in which the transition from pole-to-pole to double oscillation occurs are shown in ***Figure 6—figure supplement 2***. ***Video 5*** shows the dynamic pattern of the third and fourth compartment in this image. Middle: In longer compartments double-oscillations occur due to the increased length, even if the width along the length of the compartment is constant. Right: In very short cells the constriction of the compartments results in two connected compartments, with length axis perpendicular to the length axis in non-constricting compartments. Thus the Min proteins oscillate along the new length axis, which is perpendicular to the oscillation axis in non-constricting short compartments (blue arrows). 1 µM MinE (doped with 10% MinE.Atto655), 1 µM MinD, Scale bar: 20 µm ***Figure 6—figure supplement 3*** demonstrates that oscillations happen between each 'constricting septum' if the compartment harbors two septa.

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The following figure supplements are available for figure 6:

Figure supplement 1. Min proteins self-assemble into complex pattern in large compartments.

DOI: 10.7554/eLife.03949.019

Figure supplement 2. Min proteins patterns switch from asymmetric pole-to-pole oscillations to symmetric double oscillation when the neck in the middle of a compartment decreases.

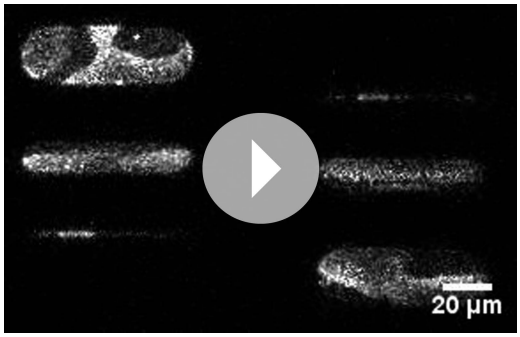
DOI: 10.7554/eLife.03949.020

Figure supplement 3. Min oscillations in compartments with multiple septa.

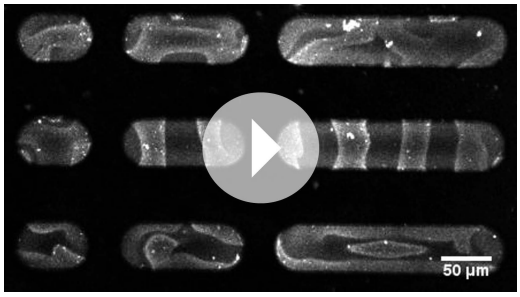
DOI: 10.7554/eLife.03949.021

phosphorylated proteins (***Chen et al., 2011***), might provide mechanisms to generate gradients along the short axis.

In addition to varying the overall width of a compartment, we mimicked septum closure in the middle of the compartments and thereby simulated morphologically different stages of bacterial cell division. While it has been controversial how the pool of Min proteins is distributed to the two daughter cells after cell division, we provide experimental evidence for the theoretical model that Min



Video 3. Compartment width dependent orientation of Min oscillations. This movie demonstrates how the oscillation axis switches depending on the width of the sample compartment. The confocal time-lapse movies of MinD and MinE patterns (doped with 10% MinE. Atto655) follows the dynamic oscillations over a time of 6 min.
DOI: 10.7554/eLife.03949.022



Video 4. Complex Min protein pattern in large compartments. This movie shows a confocal time-lapse movie of a MinD and MinE patterns (doped with 10% MinE.Alexa488) in 50 µm wide compartments. The protein patterns are followed over a time of 6 min.
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protein partitioning is a result of geometric variation during septum constriction (***Di Ventura and Sourjik, 2011***).

In summary, we reconstituted a cell-free bottom-up system which recapitulates cell-like properties, such as coordinated spatial control for division site placement by intracellular protein gradients. In particular, our results unravel the minimal requirements for localization of the cell division protein FtsZ. Moreover, our findings demonstrate that the interplay of geometrical cell boundaries and self-organizing Min proteins determine the orientation of Min gradients, the number of potential FtsZ assembly sites, as well as Min protein partitioning during cell division. Thus, our synthetic system opens a way to study complex organization principles in a simplified environment, and provides novel insights into the basic biophysical and biochemical requirements for gradient formation.

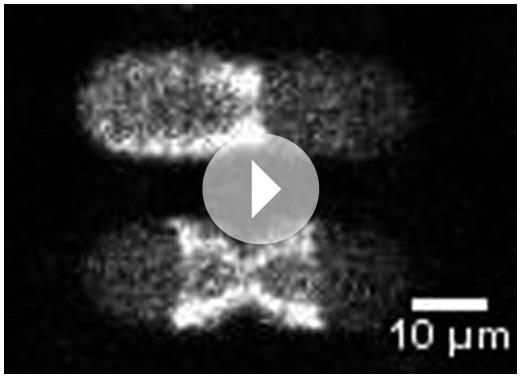
Material and methods

PDMS micro-compartments

Using photolithographic techniques, resist micropatterns were produced on Si wafers mainly as described in reference (***Zieske and Schwille, 2013***). Photoresist patterns (ma-P 1275, micro resist technology GmbH, Germany) of about 10 µm height on top of Si wafers (Si-Mat, Kaufering, Germany) were produced by photolithography. The chrome mask was purchased from Compugraphics Jena GmbH. To better mimic the curvature of the cellular membrane, we left a small gap between photoresist and mask during exposure of the mask to UV light, resulting in more tilted walls as compared to the contact mode. For reconstitution of Min protein oscillations along the length axis of a compartment, the mask patterns had a width of 10 µm. The Si wafers were coated with chlorotrimethylsilane (Sigma–Aldrich, St. Louis, MO) to prevent sticking of PDMS to the wafer. PDMS (Sylgard184, Dow Corning, Midland, MI) was mixed at a ratio (monomer to cross-linker) of 10:1 and degased under vacuum. The PDMS mixture was then poured on top of the wafer. Standard glass coverslides where then manually pressed into the liquid PDMS on top of the wafer, leaving a PDMS layer of about 30 µm between the wafer and the glass coverslide. After curing the PDMS at 80°C overnight, the bulk PDMS layer was peeled off. With the help of a razor blade, the glass coverslide with the about 30 µm thin, micro structured PDMS layer was carefully separated from the wafer and used as sample support (***Figure 1—figure supplement 2***). The microstructured PDMS/glass devices were stored at room temperature until further use. Before the microstructured PDMS was used as a membrane support, it was sonicated for 5 min in ethanol, washed with water, air dried, and treated with air plasma.

Design of microcompartments

The two-dimensional geometry at the upper level of the compartment is determined by the patterns on a chrome mask. The profiles of the micro compartments can be designed with a flat bottom or a tapered bottom (***Figure 1—figure supplement 1C***). The Min protein oscillations and Min gradient formation were supported by both profiles. However, the image quality was better when the bottom of the compartments was flat. The advantage of a tapered bottom was that these structures allowed



Video 5. Min protein pattern in compartments with a small neck in the middle of the compartment. This movie shows a confocal time-lapse movie of a MinD and MinE patterns (doped with 10% MinE.ATTO655) in compartments with a small neck in the middle. The width of the neck in the lower compartment is smaller as compared to the upper compartments and induces a switch from pole-to-pole oscillation to double oscillations. Time of the movie: 2 min.
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FtsZ-mts to align perpendicularly to the length axis of the compartments (***Figure 1—figure supplement 1D***). Thus, all compartments containing FtsZ-mts (***Figures 2,3,5***) had a tapered bottom. To keep the boundary conditions comparable, the MinCDE oscillations and gradients in ***Figure 1*** were reconstituted in tapered compartments as well.

To better visualize the oscillation mode and measure the oscillation period of MinD/E pattern, compartments with a flat bottom were employed (***Figure 4***, ***Video 1 and 2***). Note that the oscillation mode in flat compartments (***Figure 4A***) is in agreement with FtsZ localization in tapered compartments (***Figure 5B***). Compartments with constrictions and compartments for determining width dependent Min patterns had a flat bottom, as well (***Figure 6***, ***Video 3–5***).

Experiments with MinD/E pattern in compartments with constricting septum (***Figure 6*** and ***Figure 6—figure supplement 1–3***): The walls of the compartments were slightly tilted and the bottom of the compartment was flat. The width at the unconstricted region was about 12 µm at the top and 10 µm at the bottom of the compartments. The septal region of compartments, in which the switch of the oscillation modes occurred, had a width of about 2 µm at the top. The resolution limit in z-direction and the tilted PDMS-surface through which the laser beam had to pass, render an exact description of the profile at the septum challenging, However a confocal x/z-scan of the septum is depicted in ***Figure 6—figure supplement 3C***. Also note that a meniscus due to the surface tension of the buffer might render the actual cross-sectional area of the buffer smaller than the profile of the structure suggests.

Supported lipid membranes

E. coli lipid membrane within the micro compartment was prepared as described previously (***Zieske and Schwille, 2013***). Two-dimensional motility of the lipids within the membranes was confirmed by labelling the membrane with 0.1% DiI (FAST DiI, Invitrogen, Carlsbad, CA) and performing FRAP experiments. DiI labeled membranes were also applied to acquire compartment profiles.

Protein reconstitution assay

Proteins, ATP and/or GTP (depending on experiment) of defined concentration were added to a buffer reservoir of 200 µl on top of membrane clad supports. Dynamic Min patterns and/or FtsZ-mts bundles formed by self-organization on top of the membrane. At this step the Min patterns did not oscillate but formed travelling waves. Afterwards the buffer above the microstructures was removed by pipetting. Restricted to a small buffer compartments within the micro structures the Min protein patterns start to oscillate in cell-shaped geometries. To limit evaporation a lid was placed on top of the plastic ring surrounding numerous microstructures. The top of individual chambers was open with a buffer/air interface (***Figure 1—figure supplement 2***).

Proteins

MinC, eGFP-MinC, MinD, eGFP-MinD and MinE were purified with N-terminal His-tags as previously described (***Loose et al., 2008***, ***2011***). Purification of FtsZ-mts was originally described by the Erickson-group (***Osawa et al., 2008***). MinE was labeled with Alexa Fluor 488 C5 Maleimide (Molecular Probes, Carlsbad, CA) or ATTO655 Maleimide (ATTO-TEC, Siegen, Germany) according to the manufactures manual.

The plasmid for overexpression of MinC codes for an open reading frame for MinC connected to an N-terminal hexahistidine tag by a linker (***Loose et al., 2011***). The MinC-G10D mutant was obtained by site-directed mutagenesis (QuikChange II, Agilent Technologies, Santa Clara, CA) of this plasmid to obtain pET28a-MinC(G10D).

MinE without membrane targeting sequence (MinE(Δ3–8)) was generated by deleting the coding sequence for aminoacids 3–8 of MinE of the overexpression plasmid for MinE (***Loose et al., 2008***) using a 'GeneArt, Seamless Cloning and Assembly Enzyme Mix' (Invitrogen, Life Technologies, Carlsbad, CA) and primers TTCCGCGATGCGAATTCGGATCCGCGACC and AATTCGCATCGCGGAAGAAAAACACAGCCAACA to obtain pET28a-MinE(Δ3–8). MinC-G10D and MinE(Δ3–8) were purified like MinE. Purified MinE(Δ3–8) was labeled with Alexa647 maleimide (Molecular Probes, Carlsbad, CA) according to the manufactures manual.

The sample buffer in all experiments contained 25 mM Tris–HCl pH 7.5, 150 mM KCl, 5–15 mM $MgCl_2$. (5 mM $MgCl_2$ if only the MinD and MinE were reconstituted, 15 mM $MgCl_2$ if FtsZ-mts was reconstituted) When Min proteins where included in the experiments, the buffer was supplemented with 2.5 mM ATP. When FtsZ-mts was used 3 mM GTP was added to the buffer. Protein concentrations used in this study were as follows. MinC or eGFP-MinC: 0.05 μM, MinE: 1 μM, MinD: 1 μM (depending on the experiment MinD was supplemented with 10% eGFP-MinD), FtsZ-mts: 1 μM. When MinE was imaged 10% of MinE were substituted with the labeled version of MinE.

Note that these concentrations refer to the average concentrations in the sample buffer before the buffer volume was reduced below the upper level of the micro compartments.

Microscopy

Microscopy: Image acquisition was performed on a ZEISS (Jena, Germany) LSM780 confocal laser scanning microscope (inverted microscope) equipped with a ZEISS Plan-APO 25×/NA 0.8 objective and ZeissC-Apochromat 40×/1.20 objective.

For acquiring images of Min pattern and FtsZ bundles the focal plane was typically adjusted to the bottom of the compartments (if not stated otherwise). Note that the bottom area of the microstructures is smaller than the top area. Therefore the sizes of structures on the pictures might appear smaller than indicated in the text (text always refers to upper rim of microstructures).

The relative intensity profiles along the length axis of compartments can be more accurately determined if the focus plane is in the middle of the compartment, because at this plane the relative intensity profiles at different time steps are less prone to errors due to slight focal shift. Thus, data in ***Figure 1D*** and ***Figure 1—figure supplement 3*** were extracted from time-lapse images with the focal plane in the middle of the compartments.

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Author contributions

KZ, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; PS, Conception and design, Drafting or revising the article

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Publications

Zieske K and Schwille P (2014), Reconstitution of self-organizing protein gradients as spatial cues in cell-free systems. Elife, doi: 10.7554/eLife.03949. [Epub ahead of print]

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