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REVIEW

SPECIAL ISSUE: RECONSTITUTING CELL BIOLOGY

Minimal *in vitro* systems shed light on cell polarity

Kim J. A. Vendel*, Sophie Tschirpke*, Fayezeh Shamsi, Marileen Dogterom and Liedewij Laan‡

ABSTRACT

Cell polarity – the morphological and functional differentiation of cellular compartments in a directional manner – is required for processes such as orientation of cell division, directed cellular growth and motility. How the interplay of components within the complexity of a cell leads to cell polarity is still heavily debated. In this Review, we focus on one specific aspect of cell polarity: the non-uniform accumulation of proteins on the cell membrane. In cells, this is achieved through reaction–diffusion and/or cytoskeleton-based mechanisms. In reaction–diffusion systems, components are transformed into each other by chemical reactions and are moving through space by diffusion. In cytoskeleton-based processes, cellular components (i.e. proteins) are actively transported by microtubules (MTs) and actin filaments to specific locations in the cell. We examine how minimal systems – *in vitro* reconstitutions of a particular cellular function with a minimal number of components – are designed, how they contribute to our understanding of cell polarity (i.e. protein accumulation), and how they complement *in vivo* investigations. We start by discussing the Min protein system from *Escherichia coli*, which represents a reaction–diffusion system with a well-established minimal system. This is followed by a discussion of MT-based directed transport for cell polarity markers as an example of a cytoskeleton-based mechanism. To conclude, we discuss, as an example, the interplay of reaction–diffusion and cytoskeleton-based mechanisms during polarity establishment in budding yeast.

KEY WORDS: Cdc42, Min proteins, Cell polarity, Microtubules, Minimal systems, Reconstitution

Introduction

According to the second law of thermodynamics, any system of particles naturally tends to maximize its entropy, increasing the disorder of the system. How is it then possible that cells are intracellularly structured and organized? Spatial organization in cells – the non-uniform distribution of cellular components – is the result of dynamic interactions between molecules under dissipation of energy (Karsenti, 2008). Cell polarity is a special form of spatial organization that refers to the morphological and functional differentiation of cellular compartments in a directional manner (Thery et al., 2006), which is important for processes where spatial separation is necessary (e.g. growth, division, differentiation and motility). In this Review, we will focus on one specific aspect of cell polarity: the non-uniform accumulation of proteins at the cell membrane. An example is the accumulation of the cell division control protein Cdc42 at the location of the bud-site in budding yeast cells, acting as a division precursor (Mazel, 2017). Cells employ reaction–diffusion and cytoskeleton-based mechanisms to distribute and accumulate proteins (i.e. establish polarity). In reaction–diffusion systems, components are transformed into each other by chemical reactions and are moving in space by diffusion. Under specific conditions, these systems can establish polarity (Box 1). In cytoskeleton-based processes, cellular components (e.g. proteins) are actively transported by microtubules (MTs) and actin filaments to specific locations in the cell.

Cells are complex systems, and combinations of *in vivo*, *in vitro* and *in silico* approaches are required to elucidate the principles of polarity establishment (Box 2). Here, we will discuss the role and importance of *in vitro* approaches in general, and minimal system approaches in particular. Minimal systems are a subgroup of the *in vitro* methodology in which a particular cellular function is reconstituted with a minimal number of required components.

We will start by examining a reaction–diffusion system: the Min protein system in *Escherichia coli*. Here, three proteins (MinC, MinD and MinE) oscillate between the cell poles (i.e. accumulate alternately at the membrane of one of the cell poles). These oscillations result in a time-averaged protein gradient that differentiates the cell center from the cell poles. The protein oscillations were reconstituted in a minimal system in 2008 (Loose et al., 2008). We will show what led to this reconstitution and discuss what the follow-up investigations taught us.

Thereafter, we will investigate how cytoskeleton-based processes, focusing on MTs, establish polarity. Although MT organization has been reconstituted in minimal systems, a minimal system for MT-based polarity has not yet been established. However, ongoing scientific strategies to build such a system are following a similar path to those of the bacterial Min system. We will examine what we have learned from *in vivo* and *in vitro* approaches, and discuss which steps towards a minimal system still need to be taken. Finally, we will discuss Cdc42-based polarization in budding yeast where both reaction–diffusion and cytoskeleton-based mechanisms come together to establish polarity.

Reaction–diffusion-based mechanism – the Min system

The Min protein system

E. coli cells divide by binary fission, a process in which the cell divides in its center into two equally sized daughter cells. Two mechanisms that are independent from each other – nucleoid occlusion to prevent chromosome bisection and the Min system – ensure together that this occurs at the right time and place (Wu and Errington, 2012; Rico et al., 2013; Laloux and Jacobs-Wagner, 2014).

The Min system consists of three proteins, MinC, MinD and MinE (de Boer et al., 1989), which oscillate due to reaction–diffusion processes between the cell poles (Hu and Lutkenhaus, 1999; Raskin and de Boer, 1999a,b). These oscillations create a time-averaged protein gradient of all three Min proteins with the maxima at the cell poles (Fig. 1A). In presence of a membrane, only MinD and MinE are

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Box 1. Polarity establishment through reaction–diffusion processes

In reaction–diffusion systems, components are transformed into each other by chemical reactions and are moving through space owing to diffusion. Systems subject to diffusion are generally spatially uniform (i.e. unordered). However, the unordered state can become unstable if a small perturbation (i.e. a small local deviation from the well-mixed uniform state) gets amplified and thus drives the system towards a non-uniform (i.e. ordered) state. This concept is called a dynamic instability, and hereby cell polarity can be established.

One biologically relevant example is the so-called Turing instability (Turing, 1952). In this case, the reaction–diffusion system consists of components whose diffusion constants are of different orders of magnitudes. Order emerges from the combination of molecular diffusion and feedback loops in the reaction system, as diffusive coupling can lead to an instability that gets amplified through the feedback loops.

required for oscillations (Raskin and de Boer, 1999a) whereas MinC inhibits polymerization of the protein filamenting temperature-sensitive mutant Z (FtsZ) (Bi and Lutkenhaus, 1991; de Boer et al., 1992). Thus, the polymerization of FtsZ into the Z-ring only occurs at middle of the cell, where it establishes the cell division protein complex, the divisome (Vicente and Rico, 2006).

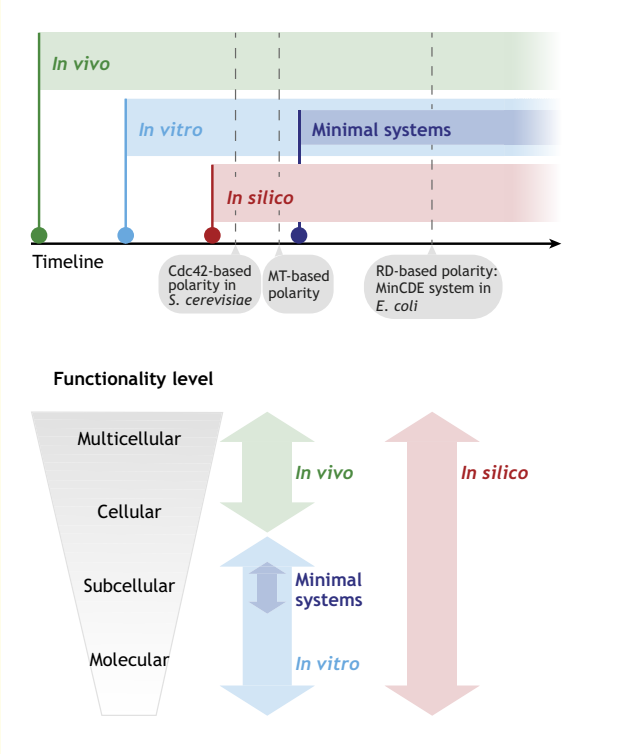
Towards a minimal Min protein system

The reconstituted Min system was not the result of a single methodology, but was established by the synergy of findings from *in vivo*, *in vitro* and *in silico* approaches (Box 2). *In vivo* approaches showed which proteins are responsible for the oscillations, what their oscillation patterns look like and which protein domains are required for the oscillations to occur (de Boer et al., 1989, 1991; Hu and Lutkenhaus, 1999, 2001; Raskin and de Boer, 1999a,b; Rowland et al., 2000; Fu et al., 2001; Hale et al., 2001). Furthermore, they allowed the elucidation of processes involved in the assembly of the global structure of the division machinery, like the polymerization of FtsZ into a Z-ring (Bi and Lutkenhaus, 1991) or those of MinE into the E-ring (Raskin and de Boer, 1997). *In vitro* investigations refined this picture by adding mechanistic details; the oscillation mechanism was found through insights on which protein–protein interactions take place (Huang et al., 1996) and further elaborated through the addition of knowledge on domain specificity (Hu and Lutkenhaus, 2000; Szeto et al., 2001; Dajkovic et al., 2008). Importantly, *in vitro* experiments revealed the biochemical basis of the oscillations; they showed that MinD exhibits ATPase activity (de Boer et al., 1991) and binds in an ATP-dependent (Hu et al., 2002; Suefuji et al., 2002) and cooperative (Lackner et al., 2003; Mileykovskaya et al., 2003) fashion to the membrane. It forms dimers (Hu et al., 2002, 2003; Suefuji et al., 2002; Hu and Lutkenhaus, 2003; Mileykovskaya et al., 2003), recruits MinC and MinE (Hu et al., 2003; Lackner et al., 2003) and is displaced from the membrane upon MinE-stimulated ATP hydrolysis (Hu and Lutkenhaus, 2001; Hu et al., 2002, 2003; Suefuji et al., 2002; Lackner et al., 2003) (Fig. 1A).

These observations of the mechanistic details of molecular events represent the core element for the development of mathematical models, defining which specific reactions take place. Furthermore, *in vitro* experiments contributed to the accuracy of model predictions (that depend on the used parameters) through quantification of the involved reactions, for example, the analysis of reaction kinetics (de Boer et al., 1991). *In silico* work suggested that the Min oscillations can be reconstituted *in vitro* (Kruse, 2002) and in an open geometry (Fischer-Friedrich et al., 2007) – proposing an experimentally easily accessible setup.

Box 2. Interplay of *in vivo*, *in vitro* and *in silico* approaches

In vivo experiments deal with complex living systems, reveal the components behind cellular functionalities and characterize their interplay within an organism. Traditionally, this is how biological experiments are conducted. In addition, *in vitro* experiments play an increasingly important role. *In vitro* experiments use purified components to dissect exact molecular mechanisms and obtain more quantitative information. Both *in vivo* and *in vitro* results contribute to the design of *in silico* models. Based on the knowledge from *in vivo* and *in vitro* experiments and guided by model predictions, minimal systems can be established. Minimal systems are specific types of *in vitro* systems that contain enough complexity to reconstitute a specific cellular function (e.g. the formation of a protein gradient), while still using a minimal number of components. Ideally, this allows the conclusive comparison of theoretical predictions and experimental results. The figure below is a schematic representation of the development of *in vivo*, *in vitro* and *in silico* approaches and the functionality levels the different methodologies deal with, including an indication of the current state of the three systems discussed in this Review.



What did we learn from the minimal Min protein system?

The first reconstitution of the Min dynamics showed that, in presence of ATP, MinD and MinE spontaneously self-organize on a flat, supported lipid bilayer into traveling waves and spirals (Loose et al., 2008) (Fig. 1B). This observation revealed the minimal requirements for Min patterns: MinD, MinE, a membrane and ATP. Furthermore, the reconstitution established a highly controlled and adjustable environment for the dissection of the molecular mechanism and the systematic manipulation of the system. Mechanistic insights that were gained are: (1) that the proteins self-organize from a homogeneous state into protein patterns (i.e. require no spatial markers), (2) that ATP is required for the

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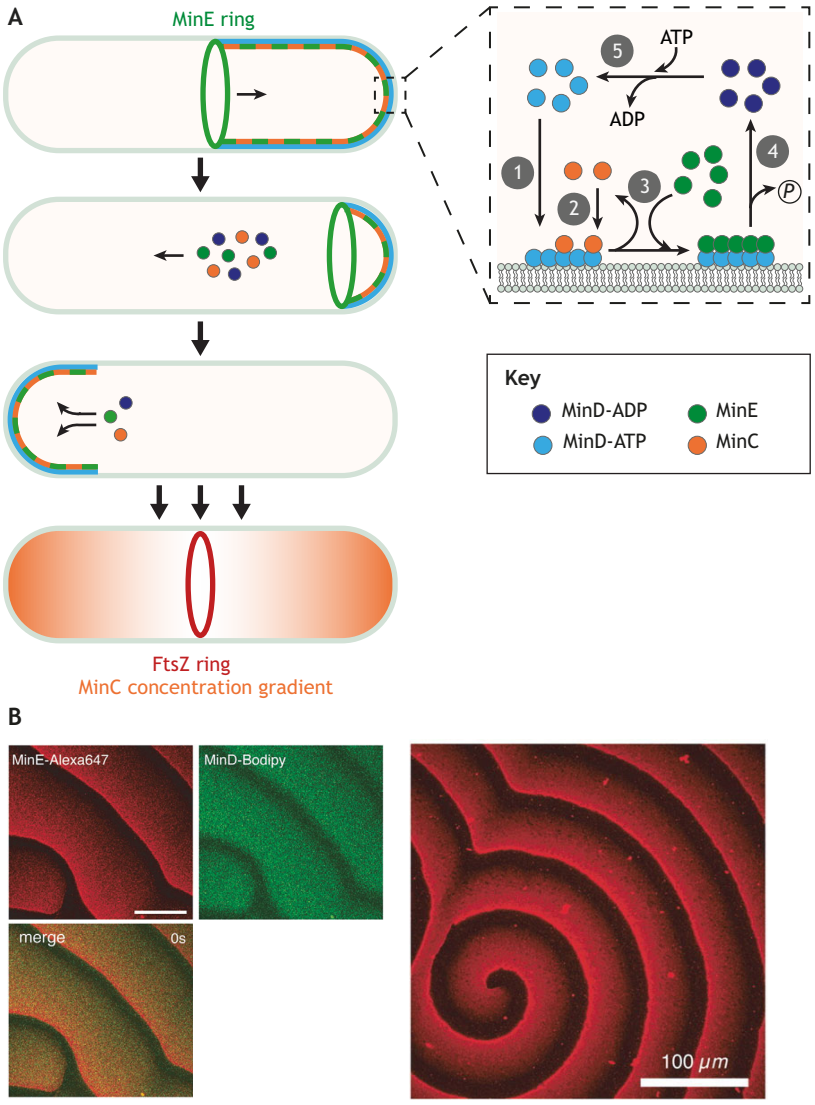

Fig. 1. Schematic of Min dynamics in *E. coli* and *in vitro* reconstitution of Min dynamics. (A) Schematic of Min dynamics in *E. coli*. Left, MinD-ATP (blue) binds to the membrane and recruits MinC (orange) and MinE (green). A high MinE concentration (MinE ring) diffuses from the middle of the cell towards the poles, causing protein displacement from the membrane. All three proteins diffuse through the cytoplasm and rebind to the plasma membrane. These oscillations result in a MinC concentration gradient that directs FtsZ ring formation to the middle of the cell. Right, the biochemical reactions near and at the membrane. The ATPase MinD (de Boer et al., 1991), in its ATP-bound form (Hu et al., 2002; Suefuji et al., 2002), binds cooperatively (Lackner et al., 2003; Mileykovskaya et al., 2003; Loose et al., 2011a; Renner and Weibel, 2012; Miyagi et al., 2018) to the cell membrane (1), dimerizes (Hu et al., 2002, 2003; Suefuji et al., 2002; Hu and Lutkenhaus, 2003; Mileykovskaya et al., 2003) and recruits MinC (2), forming a MinC–MinD complex (Hu et al., 2003; Lackner et al., 2003). Concomitantly, MinD recruits the ATPase-activating protein MinE, which displaces MinC (3) and subsequently triggers ATP hydrolysis that results in the detachment of ADP-bound MinD (dark blue) from the membrane (Hu and Lutkenhaus, 2001; Hu et al., 2002, 2003; Suefuji et al., 2002; Lackner et al., 2003) (4). MinD undergoes nucleotide exchange, diffuses through the cytoplasm and rebinds to the membrane of the opposite cell pole (Raskin and de Boer, 1999b) (5). (B) *In vitro* reconstitution of Min protein dynamics. MinD (green), supplemented with Bodipy-labeled MinD (green), and MinE, supplemented with Alexa Fluor 647-labeled MinE (red), form dynamic surface waves and rotating spirals on a supported lipid bilayer in presence of ATP. Scale bar: 50 µm, if not indicated otherwise. The images in A have been adapted from Loose et al. (2011b) with permission conveyed through Copyright Clearance Center, Inc; the images in B are adapted from Loose et al. (2008), reprinted with permission from AAAS.

oscillations to occur, and (3) that the emerging protein waves are based on reaction–diffusion processes, namely the attachment and detachment of proteins on a membrane. Next to this qualitative information, the reconstituted system also facilitated investigations on how features of the system quantitatively influence the protein dynamics, revealing that the MinE to MinD ratio influences the wave velocity and wavelength (see Table S2).

The Min oscillations have also been studied intensively *in silico* (Meinhardt and de Boer, 2001; Howard et al., 2001; Kruse, 2002; Huang et al., 2003; Howard and Rutenberg, 2003; Meacci and Kruse, 2005; Drew et al., 2005; Kerr et al., 2006; Pavin et al., 2006; Tostevin and Howard, 2006; Fange and Elf, 2006; Cytrynbaum and Marshall, 2007; Fischer-Friedrich et al., 2007; Arjunan and Tomita, 2010; Halatek and Frey, 2012; Bonny et al., 2013; Hoffmann and Schwarz, 2014). However, the proposed models differed in some fundamental properties, such as the origin of the dynamic instability (Fischer-Friedrich et al., 2007). The establishment of the minimal system provided a tool to experimentally test the model predictions and the validity of their assumptions. For example, the first Min reconstitution experiment pointed out one source of the dynamic instability: the reversible, cooperative and energy-dependent membrane-binding of proteins and their subsequent detachment (Loose et al., 2008).

Interestingly, although the reconstituted Min waves had a great resemblance to the observations made *in vivo* (Hale et al., 2001), they displayed an ∼10 times greater length scale than those in bacterial cells (Loose et al., 2008). As it turned out, this discrepancy became one of the strongest driving forces for future investigations.

What insights were gained from further reconstitution experiments?

The development and details of the Min reconstitution experiments are reviewed plentifully (Loose et al., 2011b; Rowlett and Margolin, 2015; Kretschmer and Schwille, 2016; Brauns et al., 2018; Kretschmer et al., 2018; Wettmann and Kruse, 2018). In this Review, we will use the example of the Min reconstitutions to show the diversity of minimal system investigations and the knowledge that can be gained from it.

Dissecting the influence of single factors on the properties of the system

Minimal systems are ideal environments to inspect the contribution of single factors to the properties of the system, as they facilitate highly controlled, adaptable and reproducible experimental conditions. As mentioned above, the main property that distinguished the reconstituted (Loose et al., 2008) and the *in vivo* situation was the specific length scale of the Min protein patterns. Many investigations

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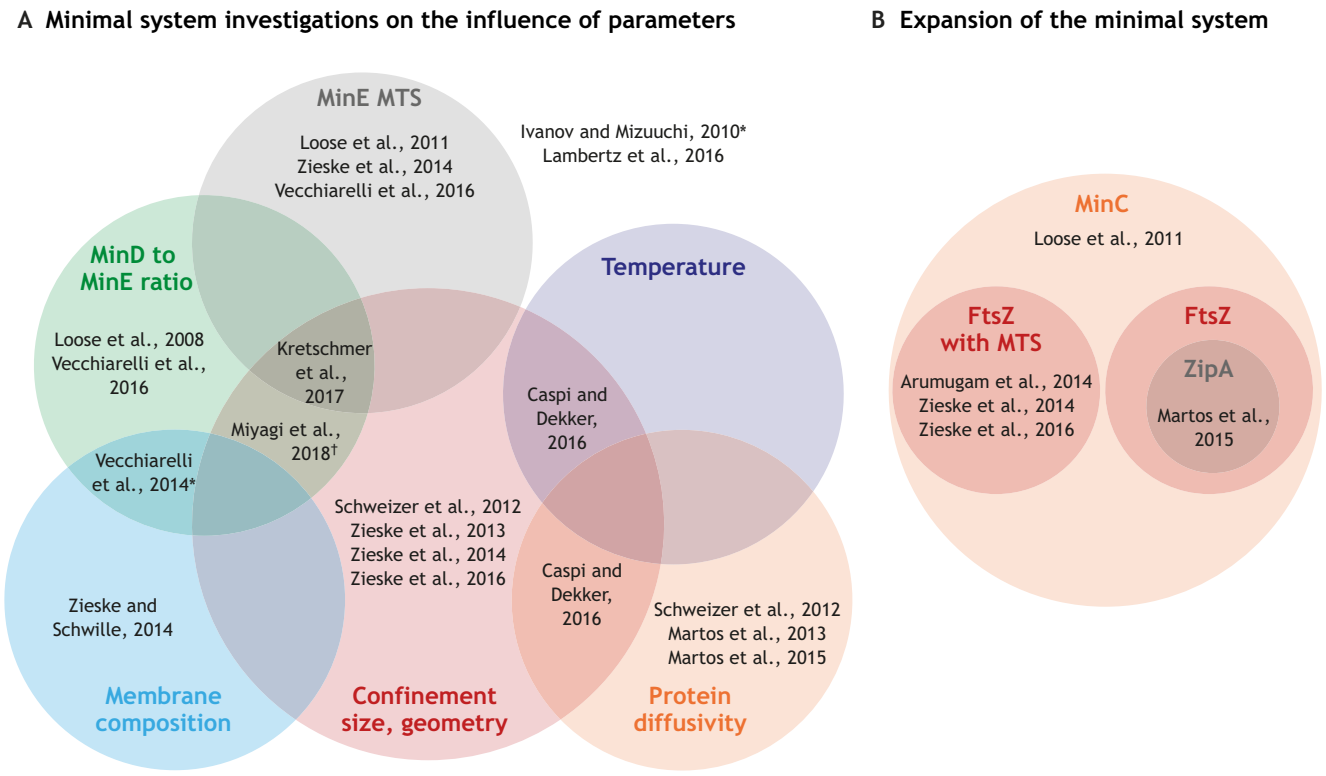

Fig. 2. Overview of established minimal Min protein systems. Studies illustrating the investigated parameters in the minimal Min protein systems (A) and components added to expand the system (B). *The experiment in this paper was conducted under constant flow of proteins. †Label-free methods were used in this paper. MTS, membrane-targeting sequence.

explored factors that could cause this difference, thereby contributing to the characterization of modulators of the Min dynamics (Fig. 2A). The role of geometrical confinement was investigated most, since theoretical models (Varma et al., 2008; Fischer-Friedrich et al., 2010; Halatek and Frey, 2012) and *in vivo* investigations (Raskin and de Boer, 1999b; Varma et al., 2008; Corbin et al., 2002; Shih et al., 2005) had already shown that the Min oscillations are influenced by compartment geometry. The aim to reconstitute the Min oscillations in cell-shaped *in vitro* environments stimulated the development of systems with defined shapes, both *in vitro* (see Table S1) and *in vivo* (Mannik et al., 2009; Männik et al., 2012; Wu et al., 2015, 2016). Experiments in these setups elaborated on how confinement influences the Min dynamics: confinement length and width affects the orientation and period of the oscillations (Schweizer et al., 2012; Zieske and Schwille, 2013, 2014; Caspi and Dekker, 2016). In addition, the specific length scale, which is 10 times bigger on flat membranes (Loose et al., 2008) than *in vivo*, is brought closer to the *in vivo* situation through confinement in 3D (Caspi and Dekker, 2016). Furthermore, these studies elucidated another feature of the Min dynamics – their adaptability and variety: depending on the chosen confinement, different dynamics and Min patterns occurred (Ivanov and Mizuuchi, 2010; Zieske and Schwille, 2013, 2014; Caspi and Dekker, 2016; Vecchiarelli et al., 2016; Zieske et al., 2016). Reaction–diffusion systems are sensitive to parameter changes. Therefore, factors such as temperature (Touhami et al., 2006; Caspi and Dekker, 2016), membrane composition (Mileykovskaya and Dowhan, 2000; Koppelman et al., 2001; Mileykovskaya et al., 2003; Szeto et al., 2003; Hsieh et al., 2010; Renner and Weibel, 2011, 2012; Shih et al., 2011; Vecchiarelli et al., 2014; Zieske and Schwille, 2014), diffusion in the cytosol (Meacci et al., 2006; Schweizer et al., 2012; Martos et al., 2015; Caspi and Dekker, 2016) and on the membrane (Meacci et al., 2006; Martos et al., 2013), the concentration ratio of MinD to MinE (Raskin and de Boer, 1999b; Shih et al., 2002; Loose et al., 2008; Vecchiarelli et al., 2016; Kretschmer et al., 2017; Miyagi et al., 2018) and interaction of MinE with the membrane (Hsieh et al., 2010; Loose et al., 2011a; Park et al., 2011; Shih et al., 2011; Zieske and Schwille, 2014; Vecchiarelli et al., 2016; Kretschmer et al., 2017) can also modulate the Min behavior and cause a difference in the specific length scale in *in vivo* and reconstituted systems (see Table S2). Reconstitution experiments helped, for example, to characterize the role of the membrane-targeting sequence of MinE; Kretschmer et al. showed that membrane binding of MinE is not a requirement for Min oscillations, but that it modulates the length scale of the pattern (Kretschmer et al., 2017). Experiments with higher diffusion constants, representing the absence of molecular crowding in the cytosol and on the membrane, showed that these factors account for the increased length scale *in vitro* (Martos et al., 2013, 2015; Caspi and Dekker, 2016). By contrast, cardiolipin, which has been speculated to act as a structural cue for MinD membrane binding (Drew et al., 2005; Mileykovskaya and Dowhan, 2005; Cytrynbaum and Marshall, 2007; Shih et al., 2011), is not required for oscillations (Vecchiarelli et al., 2014; Zieske and Schwille, 2014). In summary, the reconstituted environment has been a powerful tool for dissecting which factors are responsible for altering the dynamic behavior of the Min proteins.

Quantitative characterization and mechanistic details

Reconstitution experiments have helped to disentangle the molecular mechanisms underlying MinC, MinD and MinE propagation. Loose et al. showed that MinD binds cooperatively to the membrane and that MinE can persist on it even after MinD is

released. At the rear of the protein wave, MinE does not inhibit binding of MinC to MinD, but collectively displaces it from membrane-bound MinD (Loose et al., 2011a). Miyagi et al. elaborated on the MinD association and dissociation processes; MinD binds as a monolayer to the membrane but detaches in supramolecular structures from large membrane subareas (Miyagi et al., 2018). The details and the kinetic characterization are given in Miyagi et al. (2018). How previously discussed parameters, such as confinement or temperature, influence the Min patterns quantitatively is illustrated in Table S2.

Exchange with theoretical investigations

Owing to defined and adjustable conditions, minimal systems provide an experimental setup in which model assumptions from *in silico* approaches can be tested. Several models assumed that MinD binds cooperatively to the membrane (Hale et al., 2001; Huang et al., 2003) and that the underlying instability leading to protein patterns *in vivo* as well as *in vitro* is of the Turing type (Box 1) (Meinhardt and de Boer, 2001; Huang et al., 2003; Meacci and Kruse, 2005; Fange and Elf, 2006; Loose et al., 2008). However, reconstitution experiments could verify the cooperativity of MinD membrane binding (Loose et al., 2011a), but brought to notion that *in vitro* Min protein patterns might be based on a different kind of instability (Caspi and Dekker, 2016) – thereby influencing *in silico* approaches (Halatek and Frey, 2018). At the same time, theoretical knowledge of the dynamics of a system did improve experiments. The mathematical description of reaction–diffusion systems implies that these systems are parameter-sensitive. Earlier Min reconstitutions only investigated the influence of one parameter and were carried out under different conditions each time, meaning the results could not be compared. Only in the past few years has the sensitivity of the system to parameter changes been considered as a factor itself, and systematic variations of geometry in interplay with other parameters were investigated (Caspi and Dekker, 2016; Kretschmer et al., 2017; Miyagi et al., 2018) (Fig. 2A). These studies experimentally illustrated the significance of parameter interplay, created comprehensive datasets for comparisons with simulations, and clarified, for example, the highly discussed role of membrane binding for MinE (Kretschmer et al., 2017).

Expanding the system

Reconstituted systems facilitate the functional characterization of a component in a system through their 'plug and play' property, by which the components of the system can be added or removed at will. Min oscillations ensure the positioning of the Z-ring at the middle of the cell (Wu and Errington, 2012; Rico et al., 2013; Laloux and Jacobs-Wagner, 2014). Although the assembly of FtsZ into the Z-ring was already reconstituted *in vitro* (Osawa et al., 2008), the influence of Min oscillations on this process, as well as the mechanism of FtsZ inhibition by MinC, were poorly understood. Reconstitutions containing all Min proteins, FtsZ and the protein ZipA helped to clarify these processes (Arumugam et al., 2014; Zieske and Schwille, 2014; Martos et al., 2015; Zieske et al., 2016). It showed, for example, that the Min oscillations alone are sufficient to position FtsZ (Zieske et al., 2016). An overview of which components have been added is given in Fig. 2B.

Cytoskeleton-based cell polarity

Polarized cells are characterized by an asymmetric internal organization of components, and the cytoskeleton is often also asymmetrically oriented (Li and Gundersen, 2008). A main role of the cytoskeleton in polarity establishment and maintenance is the active, directed transport of cargo (e.g. proteins, vesicles or even organelles) through the cell; for example, by motor proteins walking along cytoskeletal filaments or cytoplasmic flows that are generated by cytoskeletal network contractions (Theurkauf, 1994; Serbus et al., 2005; Yi et al., 2013; Suzuki et al., 2017). Both MTs and actin filaments are involved in cell polarity, but this Review focuses on MTs – highly dynamic intrinsically polarized filaments whose properties can be influenced by a wealth of MT-associated proteins (MAPs). Besides MAPs themselves, post-translational modifications of tubulin influence MT dynamics by regulating MAP binding and affecting the behavior of molecular motors (Westermann and Weber, 2003; Janke and Bulinski, 2011; de Forges et al., 2012).

What do we know from *in vivo* work about the role of MTs in cell polarity?

In vivo experiments led to the discovery of proteins that are involved in cell polarity, and revealed the roles of MTs in the polarization of different cell types (Drubin and Nelson, 1996; Siegrist and Doe, 2007; Li and Gundersen, 2008; Chang and Martin, 2009; Huisman and Brunner, 2011; St Johnston, 2018). In fission yeast, for example, tip elongation aberrant protein 1 (Tea1) is a protein that is involved in MT-dependent polarized cell growth (Behrens and Nurse, 2002; Sawin and Snaith, 2004). Tea1 is transported to the cell poles through MTs by association with, among others, a molecular motor (Tea2) and a MT-tip-binding protein (Mal3) (Mata and Nurse, 1997; Brunner and Nurse, 2000) (Fig. 3A). At the cell membrane, Tea1 interacts with anchoring proteins like Mod5 to bind to the membrane and establish an accumulation of Tea1 at the cell poles (Snaith and Sawin, 2003). Mod5 itself operates in a positive-feedback loop with Tea1, which is in a complex with Tea4, as Mod5 gets localized to the cell poles only in the presence of Tea1, a process that promotes further anchoring of MT-delivered Tea1 (Snaith and Sawin, 2003). Remarkably, most of the involved proteins are MAPs, which are known to influence MT length and dynamics by controlling nucleation, orientation, forces and dynamics. This suggests additional feedback loops; for example, stable MTs can deliver proteins during longer time periods resulting in an increased accumulation of Tea1 at the cell poles (Siegrist and Doe, 2007). Two features of the mechanism for MT-based polarity establishment emerge from this example (Fig. 3A): (1) MTs transport and deliver cargo (e.g. polarity proteins) to the cell membrane, therefore MT architecture strongly influences polarity by determining where polarity proteins are delivered; and (2) polarity proteins can influence MT stability and architecture directly or indirectly through MAPs, often in a (positive) feedback loop between MTs and those proteins.

Studies in other cell systems, such as fibroblast migration, neuronal growth cone formation and apical–basal polarity in epithelial cells (Siegrist and Doe, 2007) (Fig. 3B,C) show that these same features play a role beyond fission yeast, only with different molecular components (Fukata et al., 2002; Watanabe et al., 2004; Siegrist and Doe, 2007). The essential difference is that fission yeast cells have elongated shapes. For geometrical reasons, MTs primarily grow towards the cell poles to deliver proteins (Fig. 3A); this automatically results in an asymmetric protein accumulation. In less-elongated cells with more symmetric MT conformations, additional feedback between polarity proteins and MTs (feature 2) is required to achieve asymmetric protein accumulation. As observed for fission yeast, a possible feedback loop is the stabilization of MTs by delivered proteins (Li and Gundersen,

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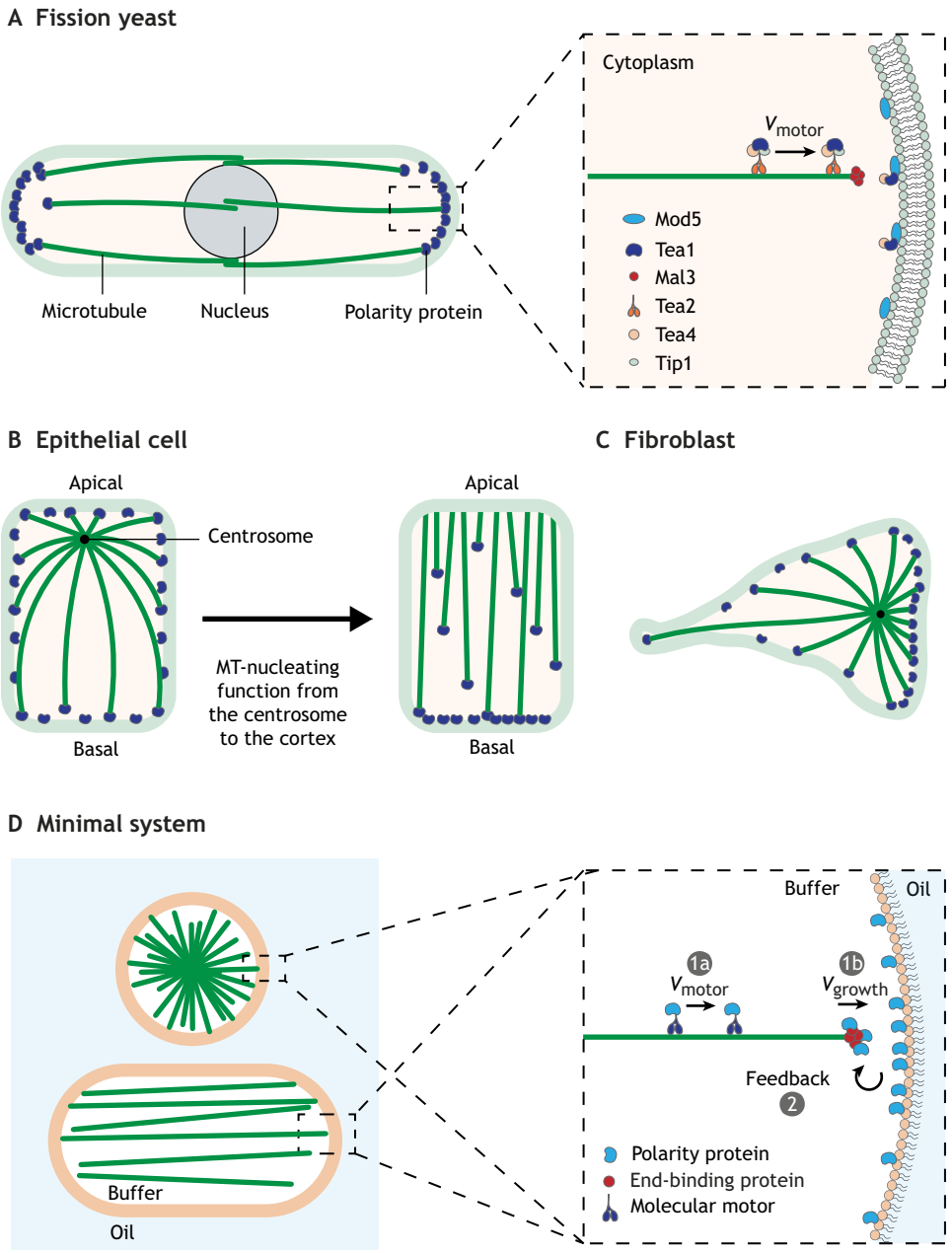

Fig. 3. Schematic representation of MT architectures in three different cell types and the minimal system. (A–C) Examples from three different cells types that illustrate how MT architecture can influence cell polarity by defining where proteins are delivered by MTs, either through molecular motors or through association with growing MT plus ends. (A) In fission yeast cells, MTs nucleate from the nucleus and orient longitudinally, thereby mainly delivering proteins to the cell poles. The magnified view shows the proteins involved, where polarity protein Tea1 is transported to the cell membrane by, among others, the molecular motor Tea2 and end-binding protein Mal3. At the cell membrane, Mod5 helps to anchor Tea1, working in a positive-feedback loop with Tea1 and Tea4. (B) Epithelial cell where the MT architecture and, therefore, delivery location of polarity proteins depends on the origin of MT nucleation, since it can shift from being centrosome-based to being located at the cell cortex. (C) In a migrating fibroblast, MTs nucleate from the centrosome, which is located on one side of the cell, resulting in an asymmetric MT architecture and protein delivery. (D) Schematic example of a minimal system in emulsion droplets of aqueous buffer in oil (left), where both features of MT-based polarity establishment [protein transport by MTs (1a and 1b) and feedback of polarity proteins on MT architecture (2)] are included (right). Transport can be achieved either through molecular motors (1a) or through transient interaction with tip-tracking proteins (1b). MT architecture strongly depends on the size and shape of droplet confinement, as illustrated by the spherical and elongated (yeast-sized) droplet; therefore, exact conditions and requirements for the establishment of a polarized cortical protein distribution will depend on this as well.

2008; Yoo et al., 2012; Jiang et al., 2015), resulting in increased delivery of proteins by molecular motors. Other examples of feedback loops are enhanced binding of polarity proteins to the cortex like the Tea1–Mod5 positive-feedback loop in fission yeast (Snaith and Sawin, 2003; Bicho et al., 2010) and local actin assembly as a result of MT-based delivery of polarity proteins (Glynn et al., 2001; Martin et al., 2005; Minc et al., 2009). Thus, in the latter, both cytoskeletal filaments work together to break symmetry. Since MT architecture dictates where polarity proteins are delivered, many *in vivo* studies have investigated how proteins that are involved in cell polarity change MT architecture. MTs often nucleate from a centrosome, and proteins that are functionally associated with cell polarity, such as the partitioning defective (Par) proteins or moesin are found to influence centrosome position in a cell (Feldman and Priess, 2012; Inaba et al., 2015; Jiang et al., 2015; Abeysundara et al., 2018). This could change the balance from a symmetric to an asymmetric MT network and is crucial for proper polarization of cells (Burute et al., 2017). In contrast, in epithelial cells, endothelial cells and mouse airway cilia, regulatory proteins ensure that MTs grow mainly from the apical membrane, rather than from the centrosome (Feldman and Priess, 2012; Vladar et al., 2012; Guillabert-Gourgues et al., 2016; Toya et al., 2016). The result is that MTs grow in parallel bundles instead of a radial conformation, which leads to different transport directions (Fig. 3B,C). Although it has been well established that MTs play a role in the establishment of polarity, additional proteins are still being discovered and many questions remain. For example, we do not know what the minimum requirements for MT-based symmetry breaking are, what exactly the roles of the different regulatory proteins are, and how MTs and proteins interact to break symmetry. The complexity of the cells makes it difficult to answer these questions with *in vivo* studies. Reducing complexity by performing *in vitro* reconstitutions has provided additional understanding on a molecular level.

How has *in vitro* work contributed to our knowledge?

Polarity is the result of an intricate interplay between MTs, proteins and membranes. Thus, the first step to understand the impact of each contribution is to investigate the separate components of the two aforementioned features for MT-based polarity establishment. MAPs regulate MT nucleation, dynamics, orientation and transport of proteins along MTs (Kinoshita et al., 2001; Faivre-Moskalenko and Dogterom, 2002; Jiang and Akhmanova, 2011; Akhmanova and Steinmetz, 2015). Therefore, specific combinations of MAPs can guide the self-organization of MTs into functional architectures (Alfaro-Aco and Petry, 2015). Examples of MAPs are the MT polymerase XMAP215, the MT depolymerase mitotic centromere-associated kinesin (MCAK), the dynein and kinesin molecular motors, and the end-binding proteins (EB proteins, also known as MAPRE proteins), which recruits other MAPs to the growing MT tip (Bieling et al., 2007; Honnappa et al., 2009; Zanic et al., 2013; Duellberg et al., 2014; Alfaro-Aco and Petry, 2015). Their influence on MT dynamics has typically been studied *in vitro* by reconstitution of dynamic MTs (Mitchison and Kirschner, 1984a,b; Alfaro-Aco and Petry, 2015) together with purified proteins, for example, on a glass slide or in an optical trap (see Table S3). For several motor proteins, the walking mechanism and quantitative information, such as step size, absolute force and binding–unbinding kinetics have been elucidated (Vale et al., 1985; Svoboda et al., 1993; Hirokawa, 1998; Vale and Milligan, 2000; Reck-Peterson et al., 2006; Block, 2007; Gennerich et al., 2007; Gennerich and Vale, 2009), which is relevant both for MT architecture and for directed transport of cargo along MTs. In summary, *in vitro* experiments on single MTs provide qualitative and quantitative information (Table S3) on basic molecular mechanisms that contribute to polarity establishment. However, one molecular mechanism on its own, such as protein transport by a molecular motor, does not establish cell polarity *in vitro*. To accomplish that, *in vitro* experiments are needed that combine multiple of the features known to contribute to polarity, to study how MTs, proteins and membranes work together in confinement to break symmetry of the protein distribution, namely a minimal system (Box 2).

What did we learn from the first minimal systems for MT organization?

In contrast to the Min system, a minimal system for MT-based cell polarity has not yet been established. What has been accomplished are minimal systems for spatial organization of MTs in cell-sized confinements, both 2D glass chambers (Holy et al., 1997; Nédélec et al., 1997; Laan et al., 2012a; Roth et al., 2014) and 3D emulsion droplets (Laan et al., 2012b; Baumann and Surrey, 2014; Roth et al., 2014; Juniper et al., 2018). These results showed that the confinement size strongly influences MT aster positioning (Holy et al., 1997; Laan et al., 2012a,b; Roth et al., 2014) and MT organization in the presence of molecular motors (Nédélec et al., 1997; Pinot et al., 2009; Baumann and Surrey, 2014; Juniper et al., 2018). In addition, they prove the possibility of encapsulating a MT system in confinement. These minimal systems for MT organization can be expanded to minimal systems for MT-based cell polarity by adding components to achieve protein transport by MTs and feedback from those proteins on the MT architecture (Fig. 3D). An alternative step towards a minimal system was made by Recouvreux et al. (2016) through an *in vivo* experiment with fission yeast cells containing a chimera protein that has only two functionalities: binding to the growing MT tip and binding to the membrane, where it can diffuse. Interestingly, this is sufficient to establish a polarized distribution of chimera proteins at the cell membrane. Since the experiment was performed inside a fission yeast cell, the influence of other cellular components could not be strictly excluded. In contrast, a minimal system would show if only these two functionalities are indeed sufficient. To create such a minimal system, elongated yeast-shaped confinements are needed. Techniques for the production of elongated glass wells and elongated water-in-oil emulsion droplets have been developed (Taberner et al., 2015). Furthermore, theoretical work on MT-based polarity in cells that are not elongated resulted in predictions about the mechanisms that are minimally needed for protein accumulation, for example on the role of MT geometry combined with directed transport through MTs (Hawkins et al., 2009; Bressloff and Xu, 2015; Xu and Bressloff, 2015; Foteinopoulos and Mulder, 2017). Some models predict that for an aster-like arrangement of cytoskeletal filaments, symmetry breaking can only be induced by an external cue, whereas spontaneous symmetry breaking is only possible for a cortical filament arrangement (Hawkins et al., 2009; Bressloff and Xu, 2015). On the other hand, Foteinopoulos and Mulder (2017) formulated a model with the minimal number of components to get stable spontaneous polarity with an aster-like MT network. Such predictions on the influence of MT architecture (Hawkins et al., 2009; Bressloff and Xu, 2015; Foteinopoulos and Mulder, 2017), but also on molecular motor parameters (Bressloff and Xu, 2015), non-linearity of the feedback mechanisms (Foteinopoulos and Mulder, 2017) and MT-length distributions (Xu and Bressloff, 2015) can guide the construction of a minimal system. At the same time, minimal systems provide a platform to test the different predictions on minimally required mechanisms for pattern formation in a spherically symmetric confinement. Taken together, a possible way to establish a minimal system for MT-based cell polarity is to start from the system for MT organization and add components using knowledge obtained by *in vivo* and *in vitro* studies and from theoretical predictions.

Conclusions and perspectives

The long-term goal – combining reaction–diffusion- and cytoskeleton-based systems

In the previous sections, we have introduced two different mechanisms, their role in pattern formation and the current state of the accompanying minimal system. Here, we will discuss why, in the future, we will need to combine minimal systems to understand how reaction–diffusion and cytoskeleton-based mechanisms interact. We use polarity establishment in budding yeast as an example.

Polarity establishment in budding yeast

Polarity establishment in budding yeast is a classical system for pattern formation (Bi and Park, 2012; Martin, 2015) (Fig. 4A), where a Cdc42-based protein pattern on the cell membrane marks the site of bud emergence (Bi and Park, 2012). Cdc42 is a highly conserved membrane-bound small GTPase (Diepeveen et al., 2018) with two states: an active, GTP-bound, state, and an inactive, GDP-bound, state. Switching between the two states is highly regulated and only Cdc42-GTP signaling towards the downstream processes is sufficient for bud formation. The genes and proteins that contribute to Cdc42 regulation in budding yeast have been identified. Four different molecular functions – typically shared between several different proteins – are relevant for Cdc42 regulation *in vivo*. First, GTP exchange factor (GEF) activity, which leads to activation of Cdc42 by enhancing nucleotide exchange. GEFs for Cdc42 are Cdc24 and bud site selection protein 3 (Bud3) (Hartwell et al., 1973; Sloat et al., 1981; Chant and

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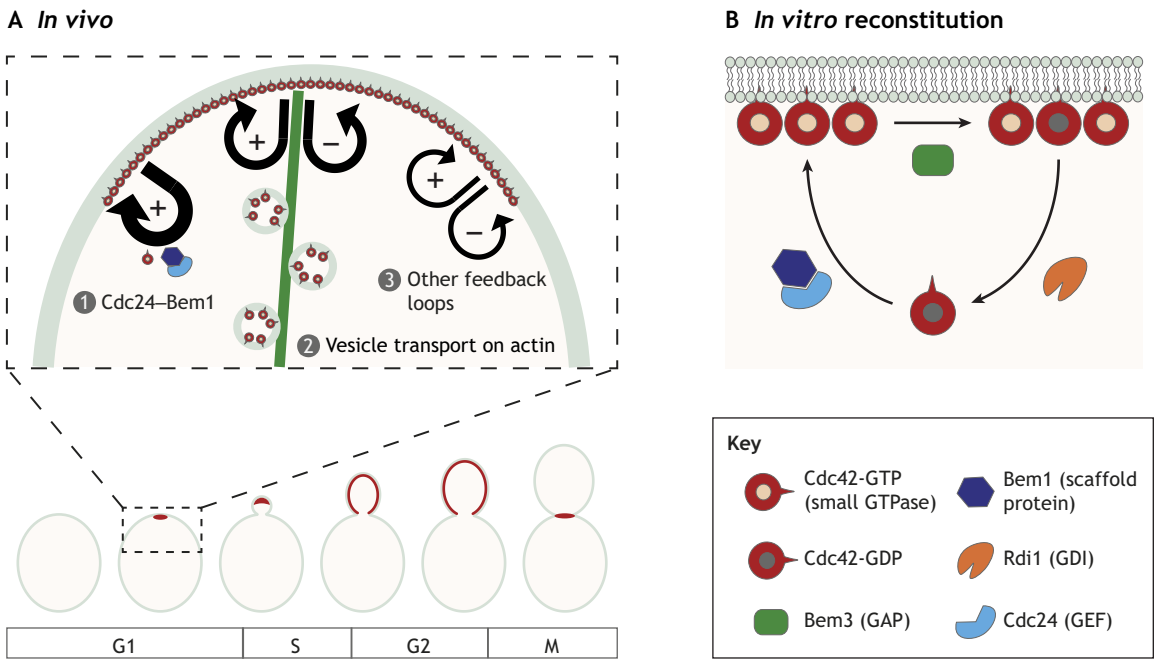

Fig. 4. Diagram of polarity establishment in *Saccharomyces cerevisiae in vivo* and *in vitro*. (A) The illustration on the bottom shows the role of polarity establishment in the yeast cell cycle. On the top, the different feedback loops that establish a Cdc42 protein pattern on the cell membrane are depicted. The cytoskeleton-based feedback loop is based on directed transport of vesicles along actin cables; the reaction–diffusion feedback depends on double-positive feedback between Cdc42 and the scaffold protein Bem1 and the GEF Cdc24, and a recently discovered (weak) feedback loop, which is at least partially independent from the other two depicted feedback loops. (B) Depiction of a schematic for a hypothetical minimal system for Cdc42 pattern formation by a reaction–diffusion mechanism. This is based on the recruitment and activation of Cdc42 to the membrane by the GEF Cdc24 and the scaffold protein Bem1 and possibly depending on the GDI Rdi1 for a high enough recycling rate, and on the GAP Bem3 for a high enough deactivation rate.

Herskowitz, 1991; Zheng et al., 1994; Kang et al., 2014). Second, GTP-activating protein (GAP) activity, which leads to deactivation of Cdc42 by enhancing GTP hydrolysis. GAPs for Cdc42 are bud emergence protein 2 (Bem2), Bem3, Rho-type GTPase-activating protein 1 (Rga1) and Rga2 (Bender and Pringle, 1991; Zheng et al., 1993, 1994; Stevenson et al., 1995; Smith et al., 2002). Third, guanine nucleotide dissociation inhibitor (GDI) activity; this enhances dissociation of Cdc42 from the membrane, and promotes retention in the cytosol. The single GDI for Cdc42 is Rho GDP-dissociation inhibitor (Rdi1) (Dovas and Couchman, 2005; Dransart et al., 2005; Slaughter et al., 2009a; Boulter et al., 2010; Woods et al., 2016). Finally, a scaffolding function is needed; for example, binding through Bem1 strengthens the interaction between Cdc42 and Cdc24 (Bose et al., 2001; Irazoqui et al., 2003; Smith et al., 2013).

Pattern formation of active Cdc42 on the membrane arises from local activation and accumulation of Cdc42 through interconnected regulatory feedback loops (Fig. 4) (Howell et al., 2012; Freisinger et al., 2013; Wu and Lew, 2013). Through a combination of quantitative cell biological and theoretical approaches, at least three partially independent feedback loops have been identified (Bose et al., 2001; Wedlich-Soldner et al., 2003, 2004; Slaughter et al., 2009b; Howell et al., 2012; Rubinstein et al., 2012; Freisinger et al., 2013; Klünder et al., 2013; Wu and Lew, 2013; Martin, 2015): a feedback loop based on a reaction–diffusion system, the so-called GDI-based mechanism, another one based on the actin cytoskeleton (Wedlich-Soldner et al., 2003) and a third (weak) feedback loop, which is at least partly independent from both the GDI and actin (Bendezú et al., 2015). In brief, in the GDI-based reaction–diffusion mechanism, Cdc42 accumulation is suggested to be achieved by double-positive feedback through Cdc42-GTP-dependent recruitment of the GEF Cdc24 and the scaffold protein Bem1 to the membrane (Goryachev and Pokhilko, 2008; Kozubowski et al., 2008; Klünder et al., 2013; Wu and Lew, 2013; Witte et al., 2017). Localized concentrations of Cdc24 can lead to enhanced nucleotide exchange rates of Cdc42, thus increasing the local Cdc42-GTP concentration, which – together with Cdc42 recycling from the membrane to the cytosol through Rdi1 – can lead to pattern formation (DerMardirossian and Bokoch, 2005) (Fig. 4). However, the exact role of the different components is still to be determined. How the actin cytoskeleton-based pathway contributes to pattern formation is heavily debated (Martin, 2015). Several possible mechanisms have been proposed, but their relative importance and interaction is unclear. For example, Cdc42-GTP activates formins (Evangelista et al., 1997; Dong et al., 2003; Bi and Park, 2012; Chen et al., 2012), which nucleate actin cables, through which vesicles that contain Cdc42 are transported towards the membrane (Slaughter et al., 2013). The influx of membrane material and Cdc42 might result in a net dilution of the Cdc42 concentration at the membrane (Layton et al., 2011; Savage et al., 2012; Watson et al., 2014). Nevertheless, the formation of microdomains of Cdc42 on the membrane might counteract this dilution effect (Slaughter et al., 2013). Hence, both GDI-based reaction–diffusion mechanisms and actin cytoskeleton-dependent delivery and internalization of Cdc42 vesicles affect pattern formation, most likely combined with other, weaker, feedback loops. Whether they contribute to positive and/or negative feedback and what the exact molecular mechanisms are remains to be determined. Dissecting the molecular mechanisms and coupling between the different feedback loops is to date very controversial because of both parameter sensitivity and the high level of observed redundancy and interdependence within and between the feedback loops

(Wedlich-Soldner et al., 2004; Howell et al., 2012; Woods et al., 2016; Witte et al., 2017). This calls for the development of a minimal system for pattern formation in budding yeast.

Towards a minimal system for pattern formation in budding yeast

Currently, we are far from establishing a minimal system that combines reaction–diffusion-based and cytoskeleton-based feedback. The first step towards this goal is reconstituting pattern formation through a single minimal feedback loop (Fig. 4B). Theoretical work based on quantitative *in vitro* and *in vivo* experiments (Table S4) predicts that Cdc42, Bem1, and Cdc24 are sufficient to form Cdc42-based patterns on a spherical lipid membrane (e.g. a vesicle or water-in-oil emulsion droplet) through a reaction–diffusion mechanism (Goryachev and Pokhilko, 2008; Klünder et al., 2013). However, fine tuning of the reaction rates might require the addition of GAPs such as Bem3 and/or the GDI Rdi1 (Altschuler et al., 2008) (Fig. 4). *In vitro* work has revealed that recombinant Bem3 shows GAP activity and that Cdc24 shows GEF activity (Zheng et al., 1993, 1994). Rdi1 can extract Cdc42-GDP *in vitro* (and to a lesser extent Cdc42-GTP) from a lipid membrane (Johnson et al., 2009; Das et al., 2012), and Bem1 binds Cdc24 (Peterson et al., 1994) and enhances Cdc24 GEF activity (Rapali et al., 2017). Since the individual components have been characterized, the next step will be to combine Cdc24, Bem1 and a fluorescently labeled Cdc42 with a spherical lipid membrane to investigate whether pattern formation will occur as predicted by theory.

What more can we learn from a minimal system for pattern formation?

Polarity establishment in budding yeast is a highly regulated and precisely tuned process. Nevertheless, yeast can show evolutionary adaption of protein composition to compensate for the deletion of Bem1 through the stepwise deletion of Bem2, Bem3 and Nrp1 (Laan et al., 2015). How the functions of Bem1 are redistributed by removing the three other proteins remains to be discovered. How molecular functions are rearranged is also relevant beyond this specific experiment: comparative studies on 298 fungal strains and species showed that redistribution of functions over different proteins in the polarization network happens regularly over the fungal tree of life (Diepeveen et al., 2018), and theoretical work suggested that small changes in reaction rates or the topology of the polarization network can dramatically rearrange functions within the polarity network (Goryachev and Leda, 2017). A minimal system for pattern formation, where proteins can be selectively added and removed, might help the understanding of how molecular functions necessary for pattern formation can be redistributed during evolution.

In summary, we discussed the significance of *in vitro* and minimal system approaches in three model systems: the Min system in *E. coli*, polarity based on MT transport and Cdc42-based polarity establishment in budding yeast. Future experimental investigations in minimal systems, in combination with theoretical approaches, will further deepen our understanding on the subcellular level of living systems.

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Competing interests

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Supplementary information

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