Nine unanswered questions about cytokinesis

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Experiments on model systems have revealed that cytokinesis in cells with contractile rings (amoebas, fungi, and animals) depends on shared molecular mechanisms in spite of some differences that emerged during a billion years of divergent evolution. Understanding these fundamental mechanisms depends on identifying the participating proteins and characterizing the mechanisms that position the furrow, assemble the contractile ring, anchor the ring to the plasma membrane, trigger ring constriction, produce force to form a furrow, disassemble the ring, expand the plasma membrane in the furrow, and separate the daughter cell membranes. This review reveals that fascinating questions remain about each step.

Introduction

After reading recent reviews on cytokinesis (D'Avino et al., 2015; Willet et al., 2015b; Cheffings et al., 2016; Glotzer, 2016; Meitinger and Palani, 2016; Bhavsar-Jog and Bi, 2017), a new research assistant in a friend's laboratory asked, "Because so much is known about cytokinesis, why is research on cytokinesis still important?" Although much has been learned in the past 40 yr since cell biologists reported in the *Journal of Cell Biology* the first molecules contributing to cytokinesis, actin filaments (Schroeder, 1972), and myosin-II (Fujiwara and Pollard, 1976; Mabuchi and Okuno, 1977), the process is so complicated that many fundamental questions remain. I will use this review on the molecular mechanism of cytokinesis to highlight what we do not know. I focus on the contractile ring of actin filaments and myosin-II, which drives the formation of the cytokinetic furrow in animals, fungi, and amoebas. Nine questions, most posed 40 to 50 yr ago, remain unanswered or incompletely understood (Fig. 1). I will use evolution to guide the discussion toward the core mechanisms shared by organisms on our branch of the phylogenetic tree.

Evolution of cytokinetic machinery

Eukaryotic cells appeared between 1 and 2 billion years ago and donated their genes to the last eukaryotic common ancestor (LECA) that gave rise to all contemporary eukaryotes ∼1 billion years ago (Adl et al., 2012). LECA inherited genes for actin, small GTPases, and endosomal sorting complexes required for transport (ESCRTs) from its archaeal progenitor (Spang et al., 2015). The genes for aurora kinase, BAR domain proteins, capping protein, cyclin-dependent kinases, formins, kinesins, profilin, polo-like kinases, myosin-I and myosin-V, and SNARES evolved in early eukaryotes (D'Avino et al., 2015; Willet et al., 2015b; Cheffings et al., 2016; Glotzer, 2016; Meitinger and Palani, 2016). Evolution of the myosin-II gene on the branch leading amoebas, fungi, and animals (Odronitz and Kollmar, 2007) may have been the key event that allowed these cells to form contractile rings, which make cytokinesis more efficient in these organisms. Eukaryotes on other branches, including algae, plants, and countless unicellular eukaryotes, lack myosin-II and use other mechanisms for cytokinesis. For example, plants use membrane traffic guided by Rab GTPases, tethers, and SNARES to build a new plasma membrane and cell wall to separate daughter cells. As explained here under question 9, cells with contractile rings continue to use this ancient membrane fusion machinery for abscission, the final topological resolution of the daughter cells.

Model systems

I assume that contemporary amoebas, fungi, and animals use elements of ancient mechanisms that evolved in the LECA to position, assemble, constrict, and disassemble contractile rings. Although contemporary organisms inherited their cytokinesis genes from ancient cells and many ancient molecular mechanisms have been conserved, the functions of some proteins diverged over the past billion years. For example, Rho-family GTPases establish the position of the contractile ring in animals (D'Avino et al., 2015), but they regulate septum assembly in fission yeast cells (Perez and Rincón, 2010). Thus, species redeployed available molecules for new purposes as they adapted under different pressures on evolutionary timescales. Often, debates about these genuine differences have dissipated energy from finding general principles, which is the focus here.

Diverse methods contribute to understanding mechanisms of cytokinesis (Table S1). Physical manipulations of cells provided some of the earliest insights. Characterizing cells with deletion, hypomorphic, or conditional mutations or after depletion of mRNAs and proteins has linked proteins to specific steps in cytokinesis. Although genetics has been more useful than biochemistry for discovering cytokinesis proteins, biochemical and biophysical experiments are required to provide the molecular structures, kinetic constants, and thermodynamic parameters necessary to propose molecular mechanisms. Quantitative fluorescence microscopy and superresolution fluorescence micros-

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Abbreviations used: ESCRT, endosomal sorting complex required for transport; LECA, last eukaryotic common ancestor; SIN, septation initiation network.

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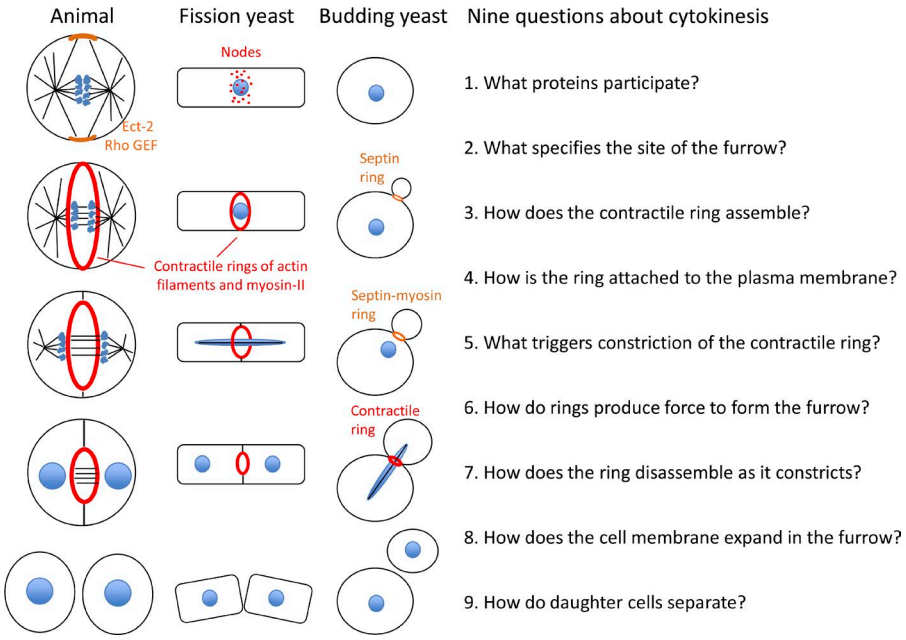

Figure 1. **Pathways of cytokinesis for animal, fission yeast, and budding yeast cells with the questions addressed in this review.**

copy of live cells have been invaluable for counting molecules and suggesting cellular mechanisms. The most rigorous way to test hypotheses regarding mechanisms in a dynamical system such as cytokinesis is cycles of experimentation and computer simulations of mathematical models. Characterization of isolated contractile rings (Huang et al., 2016), reconstitution of cytokinesis components (Nguyen et al., 2014; Miyazaki et al., 2015), and electron microscopy are still underused strategies.

Knowing the timing of the events during cytokinesis empowers research by providing a standard to detect defects in experimentally modified cells and allow for comparison of data between laboratories. Most laboratories working on fission yeast now use a timescale based on the separation of spindle pole bodies (Wu et al., 2003). Events in wild-type cells are precisely timed with standard deviations of just a few minutes around the mean times. The onset of anaphase is a convenient time marker in animal cells (Davies et al., 2014), but full timelines are rarely used.

Common problems that compromise the interpretation of experiments on cytokinesis

Protein deletion or depletion experiments established that model organisms use a common set of proteins for cytokinesis, but they often fail to reveal mechanisms. A study may conclude that protein X is required for cytokinesis, but we are often left with the questions of when and why it is required. The problem is often a chicken and egg question: Interpreting the results of experiments is difficult without information about the mechanism being studied (Meitinger and Palani, 2016). For example, "cortical contractility" is used to describe forces produced by myosin and actin without specifying how the proteins are organized to produce such forces. Consequently, it is challenging to infer molecular mechanisms from the experiments. Advancing mechanistic understanding is hard work, but it provides a rich context to design and interpret more informative experiments.

Analysis of yeast revealed that cytokinetic mechanisms are robust (D'Avino et al., 2015; Willet et al., 2015b; Cheffings et al., 2016; Glotzer, 2016; Meitinger and Palani, 2016; Bhavsar-Jog and Bi, 2017), with redundant pathways. Therefore, when cells are compromised experimentally, they use the remaining proteins to complete cytokinesis by alternative pathways that usually delay one or more steps. Sorting out these outcomes requires careful quantitative measurements.

Experiments using gene deletion or RNAi have additional problems. Secondary mutations may allow cells to survive deletion of an important gene but can confuse the interpretation of the experiment. RNAi experiments lack the time resolution required to determine exactly when a protein functions during cytokinesis. Following the time course of the depletion of a protein in an RNAi experiment provides valuable information about the concentration dependence of an emerging phenotype (Oegema and Hyman, 2006), but this is rarely done. The best-available approach to pinpoint when each protein is required to advance of cytokinesis along its normal time course is using fast temperature shifts in strains with rapidly acting, reversible, temperature-sensitive mutations (Davies et al., 2014), although the number of appropriate strains is limited. Backcrossing a conditional mutation can eliminate suppressor mutations.

Question 1: What genes are required for normal cytokinesis?

Identifying the participating molecules is essential for understanding cytokinesis, but the gene inventory is unlikely to be complete for any organism (Table S2). Forward genetic screens for conditional mutants identified >100 cytokinesis genes in budding yeast (Meitinger and Palani, 2016) and >150 in fission yeast (Pollard and Wu, 2010). Proteomic and RNAi screens of metazoan model organisms have found dozens of cytokinesis proteins, most also used by yeast. One large screen used mass spectrometry to identify candidate proteins in isolated midbodies from CHO cells followed by depletion of homologous proteins from *Caenorhabditis elegans* zygotes by RNAi (Skop et al., 2004). Depletion of 48 proteins resulted in early or late cytokinesis defects, whereas depletion of other known cytokinesis proteins (actin, Cdk1, citron kinase, and cofilin) were embryonic lethal. Seven of the 48 worm cytokinesis proteins overlapped with cytokinesis proteins identified in one or more of three RNAi screens of *Drosophila melanogaster* S2 cells

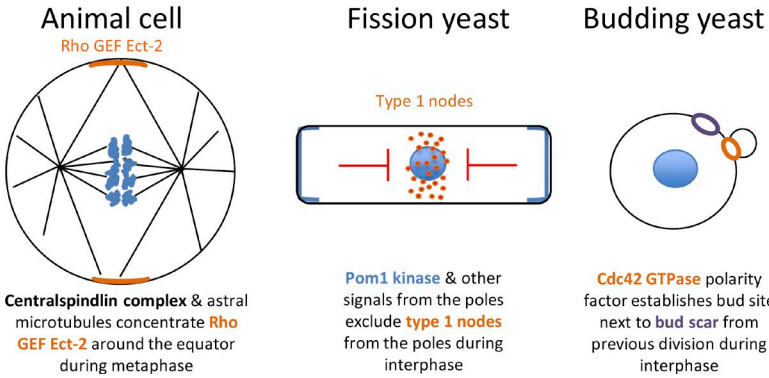

Figure 2. **Simplified, schematic mechanisms that specify the plane of cytokinesis in animal cells, fission yeast, and budding yeast.** The text explains that each mechanism involves additional elements.

(Pollard, 2003), whereas the fly screens identified seven known cytokinesis proteins (including anillin, Rho1, Rho-GAP, and Rho-GEF) missed in the *C. elegans* screen. Completing the inventory of cytokinesis genes in animals is an unmet need. Conditional mutations would be valuable, because many cytokinesis genes are essential for viability.

Question 2: How do cells position the contractile ring?

Perhaps the greatest source of the curiosity in the field has been how cells position the division plane for cytokinesis. Pioneering cellular manipulation experiments on echinoderm embryos by Rappaport and Hiramoto identified the mitotic spindle as one source of positioning signals (Rappaport, 1996). Identifying and characterizing these signaling pathways has been challenging, although it is now clear that the two yeasts and animal cells each uses different, redundant mechanisms to specify the cleavage site (Fig. 2).

Budding yeast divide where a bud grows from the mother cell (Meitinger and Palani, 2016; Bhavsar-Jog and Bi, 2017). Very early in the cell cycle, polarity signals involving the GTPase Cdc42 establish the bud site (Balasubramanian and Tao, 2013). Secondarily, the contractile ring assembles from septins, myosin-II, formins, and actin filaments in the neck between the mother and bud. Rather than specifying the cleavage site, the mitotic spindle positions one daughter nucleus in the bud, whereas the other stays behind in the mother cell.

Fission yeast cells establish the cleavage position early in interphase but can adjust later if necessary (Cheffings et al., 2016). Contractile ring precursors called type 1 nodes assemble from proteins on the inside of the plasma membrane in a band around the centrally located nucleus. The protein kinase Cdr2p is a scaffold for protein kinases Cdr1p and Wee1p in these nodes (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). Type 1 nodes assemble when the activity of the septation initiation network (SIN) declines at the end of mitosis (Pu et al., 2015), because the SIN kinase Sid2p blocks their assembly by phosphorylating Cdr2p (Rincon et al., 2017). Signals from the poles of the cell, including the Pom1p kinase, exclude type 1 nodes from the poles, so they concentrate in the middle of the cell (Fig. 2). An intermolecular autophosphorylation mechanism establishes the gradient of Pom1p at both poles (Hersch et al., 2015). A second type of node composed of adapter protein Blt1p, guanine nucleotide exchange factor Gef2p, and kinesin-3 (Klp8p) emerges from the previous contractile ring as it disassembles (Moseley et al., 2009; Akamatsu et al., 2014). These type 2 nodes diffuse on the inside of the plasma membrane until they are captured by stationary type 1 nodes around the equator. During late interphase, polo kinase stimulates movement of anillin Mid1p from the nucleus to the nodes, and the position of the nucleus can influence the position of the nodes (Almonacid et al., 2011). When the cell enters mitosis, nodes accumulate contractile ring proteins (see Question 3). This elegant positioning mechanism is still incompletely understood. We do not understand how Pom1 excludes nodes from the poles. Furthermore, one or more components of the positioning mechanism are missing, because type 1 nodes are excluded from only one pole in the absence of Pom1 function (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009).

Animal cells depend on at least three signaling mechanisms to position and assemble a contractile ring (D'Avino et al., 2015; Glotzer, 2016; Figs. 2 and 3). First, active Rho GTPase around the equator regulates formins that polymerize actin filaments and kinases that activate myosin-II. The nucleotide exchange factor (GEF) Ect-2 activates Rho by catalyzing the exchange of GDP for GTP. Local activation of a Rho-GEF is sufficient to localize Rho-GTP and initiate the formation of a furrow even in interphase cells (Wagner and Glotzer, 2016). The centralspindlin complex of proteins (a dimeric Rac-GAP called MgcRacGAP/Cyk-4 and a dimeric kinesin-6 called MKLP1/ZEN-4) not only targets Ect-2 to the plasma membrane (Kotýnková et al., 2016) but also regulates its GEF activity (Zhang and Glotzer, 2015). Polo kinase participates by phosphorylating MgcRacGAP to form a binding site for Ect-2 (Kim et al., 2014). The mechanisms targeting centralspindlin, Ect-2, and Rho to the cleavage site are still being investigated, but they include transport along astral microtubules to the equator (Su et al., 2014) and tethering to microtubule plus ends by end-binding proteins (Breznau et al., 2017). Some aspects of this mechanism have been reconstituted in vitro (Nguyen et al., 2014). Second, during cytokinesis cells inhibit the GTPase Rac, which may reduce cortical stiffness from branched actin filaments. The centralspindlin RacGAP promotes the hydrolysis of GTP bound to Rac (Zhuravlev et al., 2017), and the Rac GEF Trio is inhibited (Cannet et al., 2014). Third, Aurora B kinase in the chromosomal passenger complex antagonizes global inhibition of centralspindlin oligomerization by PAR-5/14-3-3 (Basant et al., 2015) but is only required before the ring starts to assemble (Davies et al., 2014). The separating chromosomes also influence cytokinesis, because the kinetochores carry a PP1 protein phosphatase to the poles of the spindle, where it dephosphorylates moesin and reduces polymerized actin locally (Rodrigues et al., 2015). This may explain the "polar relaxation" that has been speculated for decades to contribute indirectly to cleavage furrow formation at the equator. These broad outlines of the positioning process in animal cells are a good start, but

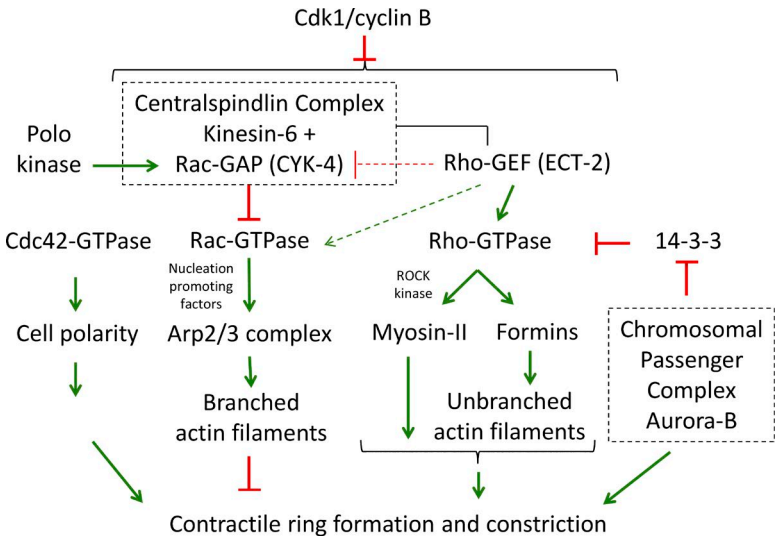

Figure 3. **Signaling pathways controlling the assembly of contractile rings in animals.** This schematic is based on figures from Glotzer (2016) and Zhuravlev et al. (2017). It is a synthesis of information from experiments on nematode zygotes and vertebrate cells. This entire group of elements has not been established to operate in any cell type. Green arrows represent positive signals, and red lines with bars represent negative signals.

much interesting work remains to characterize how the system concentrates active Ect-2 and Rho around the equator.

Question 3: How do cells assemble cytokinetic contractile rings?

The eukaryotic model organisms considered here all use formins to produce actin filaments that are acted upon by myosin-II to produce forces to assemble contractile rings. Local concentrations of active RhoA stimulate contractile ring assembly in budding yeast (Meitinger and Palani, 2016; Bhavsar-Jog and Bi, 2017) and animal cells (D'Avino et al., 2015), but related GTPases participate in other ways in fission yeast (Perez and Rincón, 2010). The process also depends on adapter proteins, including anillin, IQGAPs, and F-BAR proteins, but much less is known about how they participate mechanistically.

Budding yeast cells build a contractile ring from the core cytokinesis proteins, but some aspects of the timing differ from animals and fission yeast (Meitinger and Palani, 2016; Bhavsar-Jog and Bi, 2017). A ring of septin filaments assembles in the bud neck and forms a scaffold for adapter protein Bni5 and myosin-II (Ong et al., 2014). An IQGAP anchors the myosin-II after Bni5 dissociates (Fang et al., 2010). Thereafter, the GTPase Rho1 activates the formin Bni1 to assemble actin filaments de novo (Tolliday et al., 2002; Balasubramanian and Tao, 2013). In spite of the fact that these contractile rings form in a preexisting furrow, the molecular interactions are closely related to those in cells where the contractile ring initiates the cytokinetic furrow. Deeper exploration of these interactions is likely to benefit our understanding of cytokinesis in other systems.

Fission yeast cells assemble contractile rings by a search-capture and pull mechanism (Vavylonis et al., 2008). Late interphase nodes containing anillin Mid1p are anchored to the plasma membrane in a band around the equator (Paoletti and Chang, 2000). As the cell enters mitosis, these nodes collect in succession stoichiometric ratios of IQGAP Rng2p, myosin-II Myo2, and F-BAR protein Cdc15p (Wu et al., 2003). The IQGAP probably anchors the C-terminal region of Myo2 to anillin in the node, and the Myo2 heads dangle into the cytoplasm (Laplante et al., 2016). Small numbers of formin Cdc12p join the nodes last through strong interactions with Cdc15p (Willet et al., 2015a). Each Cdc12p assembles an actin filament and presumably anchors its barbed end in the node. Filaments elongate at ∼70 subunits/s. If their pointed end approaches another node, Myo2 binds the filament and pulls the nodes together (Vavylonis et al., 2008). Myosin-V Myo51 plays a supporting role (Wang et al., 2014; Laplante et al., 2015), by virtue of being linked between filaments by proteins Rng8 and Rng9 (Tang et al., 2016). Computer simulations of this search, capture, pull, and release mechanism produce a contractile ring in ∼10 min (the time required by live cells), providing that the actin filament connections between nodes break a few times each minute. Cofilin is required to sever the filaments (Chen and Pollard, 2011). Repetition of this cycle of filament growth, capture, pulling and severing results in the continuous assembly of new contractile units and avoids the unproductive aggregation of nodes (Vavylonis et al., 2008). Still missing are the mechanisms that anchor nodes to the plasma membrane, recruit contractile ring proteins to nodes, and coordinate the forces produced by the two types of myosin.

Animal cells also depend on actin filaments and myosin-II to assemble contractile rings, but questions remain about the sources of contractile ring actin filaments. Schroeder (1972) observed no preexisting filaments around the equator of sea urchin eggs, so he suggested that the "contractile ring . . . is assembled in situ . . . beneath the plasma membrane." However, individual actin filaments are notoriously difficult to preserve during the preparation of thin sections, and fluorescence microscopy established that animal cells have a thin cortex of actin filaments (Chalut and Paluch, 2016) nucleated in part by Arp2/3 complex. Formins are required to assemble contractile rings, but multiple formin genes (15 in mammals) complicate their analysis. For example, formin mDia2 is required for cytokinesis of mouse 3T3 cells (Watanabe et al., 2008), but mouse embryos lacking both copies of the mDia2 gene undergo thousands of rounds of cytokinesis until cytokinesis fails in erythroblasts on embryonic day 11 (Watanabe et al., 2013). In *C. elegans*, formin CYK-1 is downstream of Rho and is required to assemble a contractile ring, although its activity is not required during late ring constriction (Davies et al., 2014). Other formins may participate or continued filament formation may not be required late in the process.

Injection of inhibitory antibodies (Mabuchi and Okuno, 1977), gene disruption in *Dictyostelium discoideum* (De Lozanne and Spudich, 1987), RNAi depletion in flies and worms

(Pollard, 2003; Skop et al., 2004), and treatment with the myosin inhibitor blebbistatin (Straight et al., 2003) established that myosin-II is required to assemble contractile rings in amoeboid and animal cells. Myosin-II and anillin accumulate around the equator of HeLa cells treated with blebbistatin, but no furrow forms. RhoA activates Rho-kinase, which phosphorylates the regulatory light chain and activates myosin-II enzyme activity and formation of bipolar filaments (Matsumura, 2005). Super-resolution fluorescence micrographs show bipolar myosin-II filaments in the contractile ring (Beach et al., 2014; Henson et al., 2017) and electron microscopy of replicas sea urchin cortices shows myosin-II filaments interconnected with actin filaments (Henson et al., 2017). Anillin and IQGAP contribute to contractile ring formation but little is known about their mechanisms or their structural organization.

Cortical flow dependent on myosin-II delivers actin filaments to the cleavage site and may help to organize filaments formed locally by formins. A careful quantitative analysis (Reymann et al., 2016) documented that cortical flow toward the future furrow aligns actin filaments in nematode embryos. The authors' mesoscopic flow model accounts for their observations, so they rejected search–capture and pull as the mechanism. However, it seems likely that cortical flow results from myosin pulling on dynamic actin filaments growing between clusters of myosin-II, as explained by the search–capture–pull-and-release mechanism. Thus, a molecularly explicit model of contractile ring assembly in animals is still lacking.

Question 4: How is the contractile ring attached to the plasma membrane?

Physical connections between the contractile ring and the plasma membrane are required to form the cytokinetic furrow, but little is known in any system about number or nature of these anchors. Electron micrographs show actin filaments converging toward densities on the plasma membrane of dividing HeLa cells (Maupin and Pollard, 1986), but the components of these densities have not been identified.

Both lipid binding, peripheral membrane proteins, and transmembrane proteins are candidates to anchor the contractile ring. Among the peripheral membrane proteins, anillin has two lipid-binding domains, a CH2 domain and a PH domain (Sun et al., 2015), and contractile rings may detach from the plasma membrane when anillin is depleted. At least four fission yeast proteins with lipid-binding F-BAR domains participate in cytokinesis (McDonald et al., 2015), but less is known about BAR proteins during cytokinesis in animals. Interactions of peripheral membrane proteins with BAR domains with the inner surface of the plasma membrane bilayer may help to anchor the contractile ring.

I think that more substantial anchors by transmembrane proteins are likely to be required to anchor the contractile ring, but here, knowledge is still limited. Cadherins are candidates to anchor contractile rings in animal epithelial cells (Hoffman and Yap, 2015), but different proteins must fill this role in other cell types. Experiments on budding yeast (Meitinger and Palani, 2016) and fission yeast (Muñoz et al., 2013; Arasada and Pollard, 2014) identified transmembrane enzymes that synthesize the septum as possible anchors. Two groups of budding yeast proteins (including an IQGAP and an F-BAR protein) may link the contractile ring to the transmembrane cellulose synthase (Meitinger and Palani, 2016; Bhavsar-Jog and Bi, 2017). The protein Sbg1p is a good candidate for a linker in fission yeast, because it interacts with the transmembrane glucan synthase Bgs1p, F-BAR proteins (Rga7p, Imp2p, Cdc15p), and paxillin Pxl1p (Sethi et al., 2016). Contractile rings are unstable or slide along the membrane in fission yeast, if the linker or transmembrane proteins are deleted, mutated or depleted. Myosin-II remains in the furrow of sea urchin embryos without actin, so it may be anchored independently (Schroeder and Otto, 1988). Much more information is needed to understand how contractile rings are anchored.

Question 5: What triggers contractile ring constriction and cytokinetic furrow formation?

Cyclin-dependent kinases provide the top level of control for cytokinesis along with all other aspects of the cell cycle. In particular, the transition to anaphase and furrow formation in animal cells depends on degradation of cyclin B and the termination of Cdk1 kinase activity (Niiya et al., 2005; Potapova et al., 2006). The downstream pathways that trigger contractile ring constriction have not been established. A Ca^{2+} transient in the cytoplasm adjacent to cytokinetic furrow has been observed in fish eggs and may be a late step in these signaling pathways (Fluck et al., 1991). IP_3 receptors release this Ca^{2+} from the ER, but in large cells such as zebrafish embryos, the Ca^{2+} transient also depends on components of the "store-operated calcium entry" mechanism in the ER and plasma membrane (Chan et al., 2016). Ca^{2+} might bind calmodulin and activate myosin-light chain kinase to stimulate force production in the contractile ring (Matsumura, 2005). However, little is known about the mechanistic details or if Ca^{2+} transients participate in other cells.

Ring constriction in fungi depends on signaling pathways called the mitotic exit network in budding yeast and SIN in fission yeast (Simanis, 2015). These pathways consist of a GTPase and two or three protein kinases (related to the Hippo pathway in animals). In fission yeast, the SIN kinase Sid2p and its regulatory subunit, Mob1p, migrate from the spindle pole bodies to the contractile ring to activate constriction. However, the inventory of Sid2p substrates is incomplete, and it is not clear how constriction is initiated. Ring constriction also depends on the ring being anchored to the plasma membrane (Muñoz et al., 2013; Arasada and Pollard, 2014) and the initiation of septum synthesis (Proctor et al., 2012), both involving the large transmembrane β-glucan synthetases. Analysis of the trigger for ring constriction is still one of the least developed aspects of cytokinesis.

Question 6: How does the contractile ring produce force to form the cytokinetic furrow?

Inactivation or depletion of myosin-II in amoebas (DeLozanne and Spudich, 1987), animal cells (Mabuchi and Okuno, 1977; Straight et al., 2003), and fungi (Kitayama et al., 1997) stops the cytokinetic furrow, which led to the hypothesis that contractile rings constrict by a sliding filament mechanism like muscle (Schroeder, 1972). Observations of actin filaments with opposite polarities in contractile rings of animal (Schroeder, 1973; Sanger and Sanger, 1980) and fission yeast (Kamasaki et al., 2007) cells are consistent with a sliding filament mechanism. However, subsequent work raised questions about whether myosin-II acts alone. Budding yeast can divide without myosin-II but suffer from defects in cytokinesis and cell separation (Bi et al., 1998). They also divide remarkably well with myosin-II

lacking the head domain (Lord et al., 2005; Fang et al., 2010). Mammalian COS-7 cells can divide with enzymatically inactive myosin-II (Ma et al., 2012), and a temperature-sensitive mutation in the tail of myosin-II slows but does not stop ring constriction in nematode embryos (Davies et al., 2014). Thus, conventional myosin-II may cooperate with other myosins to produce tension in the ring. For example, a myosin-V and an unconventional myosin-II contribute in fission yeast (Laplante et al., 2015). Second, inward growth of the cell wall contributes to furrowing in fungi (Proctor et al., 2012). Third, a Brownian ratchet mechanism with actin filament disassembly coupled with cross-linking near filament barbed ends is proposed to produce force (Mendes Pinto et al., 2012), although the relevant proteins are not characterized.

Contractile rings of echinoderm eggs (Rappaport, 1967) and fission yeast protoplasts (Stachowiak et al., 2014) produced tensions similar to muscle (per cross-sectional area). Enough information is available in a few systems to formulate mathematical models of ring constriction that reproduce the forces produces by cells.

A model of the *C. elegans* contractile ring (Dorn et al., 2016) assumes an array of sarcomere-like units connected in series by elastic elements and associated laterally with the inner surface of the plasma membrane. The actin filaments are proposed to shorten during contraction. The model considers the energy associated with each of the elements, but not the actions of individual molecules. Cross-linking and interactions with the curved membrane tend to align the actin filaments around the circumference, which in turn favors contraction with positive feedback.

A molecularly explicit model of the fission yeast contractile ring (Stachowiak et al., 2014) is based on the numbers of the key proteins (formins to polymerize actin, actin filaments, clusters of myosin-II, and α-actinin to cross-link adjacent filaments), the rates of the reactions (rates of filament elongation and exchange of myosin and formins), the force produced by myosin, and estimates of drag from anchors and cross-links. Computer simulations of a two-dimensional model reliably assemble a contractile structure and produce tension similar to that measured in protoplasts. The simulations also explain why the experimentally observed exchange of formins, actin, and myosin with cytoplasmic pools is essential; without exchange, myosin accumulates in large clusters and the tension falls to zero. Exchanging the components avoids these problems by continuously rebuilding new contractile units.

The limited information about the arrangement of the proteins in contractile rings and the rates that these proteins exchange between the cytoplasm and contractile rings has constrained the formulation and testing of molecular models for constriction. It is assumed that the motors remain active during contractile ring constriction, but little is known about the mechanism.

Question 7: How does the contractile ring disassemble as it constricts?

Schroeder's analysis of electron micrographs showed that the volume of the contractile ring of echinoderm eggs declines in proportion to its circumference (Schroeder, 1972). Fluorescence microscopy showed the same is true for actin and associated proteins in fission yeast (Wu and Pollard, 2005; Courtemanche et al., 2016). The ratio of polymerized actin to formins declines in proportion to the circumference of constricting contractile rings of fission yeast (Courtemanche et al., 2016). This relationship suggests that the filaments shorten from ∼1.5 to 0.4 µm as the ring constricts. On the other hand, myosin-II concentrates in contractile rings of fission yeast (Wu and Pollard, 2005) and animal cells (Dorn et al., 2016) as they constrict.

The mechanisms consuming contractile ring actin filaments are not known. GFP-actin turns over with a half time of 26 s in the contractile rings of animal cells (Murthy and Wadsworth, 2005), but the mechanism has not been characterized. Compressive forces may break filaments, as observed in vitro (Murrell and Gardel, 2012). Severing of filaments by cofilin is not essential in fission yeast, because rings constrict at normal rates in cells dependent on mutant cofilin that severs filaments very slowly (Chen and Pollard, 2011). In addition, bundles of actin filaments peel off curved parts of the ring during constriction (Huang et al., 2016). Additional quantitative measurements of polymerized actin and formins in a variety of cells are needed to determine whether and how actin filaments are "consumed" during constriction of contractile rings. Genetic and biochemical experiments are needed to explore the mechanisms.

Question 8: How do cells expand the plasma membrane for the cytokinetic furrow?

Cytokinesis requires expansion of the plasma membrane in the cytokinetic furrow by ∼1.5-fold in a spherical cell and ∼1.1-fold in cylindrical fission yeast. Cellularization of the *Drosophila* blastoderm, a specialized type of cytokinesis, requires expansion of the plasma membrane area 25-fold (Figard et al., 2016). Animal cells store some of this membrane in filopodia and plasma membrane folds (Figard et al., 2016), but cells must expand the plasma membrane during cytokinesis by net addition of membrane.

Plants are the most extreme and best-characterized example, because they depend entirely on membrane expansion for cytokinesis. Plants create a new membrane compartment that expands to divide the daughter cells (Müller and Jürgens, 2016). Motors transport Golgi vesicles along microtubules to the division plane between the daughter nuclei, where they fuse into a new membrane compartment, the cell plate. Although plants lack contractile rings, a formin and myosin-VIII help to guide the expanding cell plate to the edge of the cell where fusion with the plasma membrane completes cytokinesis (Wu and Bezanilla, 2014). Membrane fusion to expand the cell plate depends on a GTPase related to Rab11, tethering complexes and SNAREs. The TRAPP-II tethering complex guides the lateral expansion of the cell plate, and the exocyst tethering complex participates during the late stages (Rybak et al., 2014). Transmembrane enzymes in the cell plate secrete callous and other extracellular matrix components (Müller and Jürgens, 2016), similar to septum formation in fungi.

These ancient membrane fusion mechanisms of plants are also used to expand the plasma membrane in the cytokinetic furrows of cells with contractile rings. Fission yeast expand the plasma membrane in the cytokinetic furrow by fusion of hundreds of vesicles and tubulovesicular carriers (Wang et al., 2016). Quantitative measurements and mutations showed that Rab8 GTPase and exocyst direct vesicles to the edge of the furrow where it invaginates from the surface plasma membrane, whereas Rab11 GTPase and TRAPP-II complex deliver vesicles to the bottom of the furrow adjacent to the contractile ring. TRAPP-II is present in puncta at delivery sites for only 8 s, so these events are easily missed. Budding yeast also use a Rab

GTPase (Sec4) and exocyst to deliver post-Golgi vesicles to the plasma membrane at the bud tip and division site. Phosphorylation of Sec4 early in mitosis inhibits vesicle fusion in the cytokinetic furrow, but phosphorylation declines late in cytokinesis (Lepore et al., 2016).

Cytokinesis by animal cells also depends on the Rab11 GTPase, the exocyst complex (Neto et al., 2013a), or TRAPP-II (Robinett et al., 2009) and SNARES (Neto et al., 2013b). Mutations in exocyst subunits stall furrow formation (Giansanti et al., 2015), and depletion of the Arf6 GTPase causes furrows to regress (Dyer et al., 2007). The prevailing hypothesis is that vesicles fuse with the plasma membrane deep in the furrow near the contractile ring, although quantitative information on sites of vesicle insertion is sparse (McCusker and Kellogg, 2012).

Many opportunities exist to improve our understanding of membrane expansion in the cytokinetic furrow. We need more information on whether the vesicles that expand the furrow membrane originate from constitutive secretory activity or have some special source. Better quantitative data on flux of membrane out by exocytosis and in by endocytosis will also be valuable.

Question 9: How do two daughter cells separate?

The actual division of daughter cells requires breaking their membranes apart, a process called abscission (Mierzwa and Gerlich, 2014). This separation depends on removing cytoplasmic structures that connect the cells, including the remnants of the contractile ring, mitotic spindle, and, in cells with closed mitosis, the nuclear envelope. The process is best understood in mammals, where the centralspindlin complex, adapter proteins, and SNARES recruit the ESCRT-III complex to the abscission site (Neto et al., 2013b). A conical spiral of ESCRT-III filaments on the cytoplasmic surface of the plasma membrane pulls the membranes together in the thin bridge between separating cells (Schöneberg et al., 2017). ESCRT-III also anchors the AAA ATPase spastin, which severs the microtubules left from the mitotic spindle (Connell et al., 2009). Rab35 recruits and activates the enzyme MICAL1, which oxidizes actin and promotes the depolymerization of the remnant of the contractile ring (Frémont et al., 2017). Abscission also depends on the AAA ATPase Vps4 that uses ATP hydrolysis to disassemble ESCRT-II filaments, although the mechanism is still being investigated (Schöneberg et al., 2017). The final separation of mammalian cells depends on fusion of the plasma membrane mediated by Rab GTPases, exocyst complex tethers, and SNAREs (Mierzwa and Gerlich, 2014), although the physical mechanism is still in question. As expected for such a pivotal event, signaling pathways monitor abscission, including negative feedback via Aurora B kinase to ESCRT-III, if chromosomes fail to segregate normally (Mierzwa and Gerlich, 2014).

Eukaryotes inherited ESCRT-III and Vps4 genes from archaea (Spang et al., 2015), but these proteins have not yet been firmly implicated in abscission in invertebrates and fungi. Fungi depend on cell wall formation and membrane fusion proteins such as Rabs and exocyst to separate daughter cells (Meitinger and Palani, 2016).

We now have a good inventory of the multitude of abscission proteins and biophysical characterization of some of them. However, we will not understand the complicated mechanics of membrane fusion without more information about the interactions of these molecules during each step in the process.

Conclusions

I hope that the research assistant mentioned in the introduction will now appreciate why research on cytokinesis is still important. A half-century of experimentation revealed that the genes for cytokinesis emerged in early eukaryotes and resulted in the invention of the contractile ring on the branch of life leading to amoebas, fungi, and animals. Now that we know some basic features of the process, we can look forward to a golden age of research on favorable model organisms that should answer the nine fundamental questions about cytokinesis and produce a satisfying understanding of the molecular mechanisms.

Online supplemental material

Table S1 shows methods used to study cytokinesis in various model systems and Table S2 shows mechanisms for steps in cytokinesis in various model systems.

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