POSITIONING AND ASSEMBLY OF DIVISION MACHINERY IN FISSION YEAST

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SUMMARY

Cytokinesis is a terminal event in the cell cycle during which a parent cell physically separates into two daughter cells. The cytoskeleton plays an important role in cytokinesis. Microtubules are involved in chromosome segregation, whereas filamentous-actin is required for cell cleavage. Cytokinesis is mediated by an actomyosin-based contractile ring in various organisms, such as animal cells, yeasts and fungi. Uncovering how the actomyosin rings are positioned and assembled is crucial to understanding the global regulation of cytokinesis.

The fission yeast *Schizosaccharomyces pombe*, like many eukaryotes, divides utilizing an actomyosin based contractile ring and is an attractive model for the study of cytokinesis. Successful cytokinesis depends on the proper positioning and assembly of the cell division machinery. Work in fission yeast has previously identified Mid1p, a protein with properties similar to anillin from animal cells, as a key molecule in division plane positioning. Mid1p ensures medial fission of *S. pombe* cells by recruiting the division machinery to the medial region of the cell. After specification of the division plane, an actomyosin based contractile ring is assembled and maintained in the middle of the cell. Recent studies have led to a 'search, catch, pull and release' model in which actomyosin ring assembly is initiated from multiple membrane anchored nodes. These nodes contain Mid1p, the formin-related protein Cdc12p, type II myosin and IQGAP-related protein Rng2p. The

formation of these membrane-associated nodes has been shown to be dependent on Mid1p.

In this study, I address some questions related to the mechanisms of division plane positioning and actomyosin ring assembly in fission yeast.

In chapter III, I demonstrate that although *mid1* mutants misplace the division septa, the misplaced septa are occluded from cell ends, indicating that an additional negative mechanism inhibits the incorrect positioning of division plane at the cell ends. This process, which I refer to as 'tip-occlusion', requires cell-end localized polarity determinants Tea1p, Tea4p / Wsh3p, and the Dyrk- related kinase Pom1p. This mechanism is essential in the cells lacking Mid1p and is important for the fidelity of division plane positioning in small cells. The FER/CIP homology protein Cdc15p, which is required for actomyosin ring maintenance and division septum assembly, appears to mediate the formation of tip-septa. Partial compromise of Cdc15p function restores tip-occlusion, and thereby prevents the formation of tip-septa.

In chapter IV, I test the current 'search, catch, pull and release' model for actomyosin ring assembly in certain mutants that are devoid of membrane-associated nodes. I find that cells lacking cortical nodes are able to organize orthogonal actomyosin rings of normal appearance, suggesting that cortical nodes are not essential for the orthogonal ring formation. Instead, activated septation initiation network appear be

sufficient to promote orthogonal ring formation, even in the absence of Mid1p or cortical nodes.

Finally, in chapter V, I establish a genetic method to reliably and efficiently generate fission yeast cells lacking nuclei and spindle pole bodies. Utilizing this approach, I investigate the mechanism of microtubules assembly and actomyosin ring formation in cells lacking nucleus and SPBs. I have found that the assembly of microtubules does not require nuclear associated microtubule organizing centers and SPBs. I also show that the nucleus and SPBs are not essential for the formation of actomyosin rings.

Collectively, my work provides some mechanisms involved in actomyosin ring assembly and positioning. These mechanisms may be relevant to other organisms as well.

Key words: *S. pombe*, cytokinesis, actomyosin ring, division plane

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LIST OF ABBREVIATIONS

APC/C	Anaphase promoting complex. cyclosome
CDK	Cyclin-dependent kinase
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DYRK	Dual-specificity tyrosine-phosphorylation-regulated kinase
eMTOC	Equatorial microtubule organizing center
FRAP	Fluorescence recovery after photobleaching
GAP	GTPase activation protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
HU	Hydroxyurea
LatA	Latrunculin A
MBC	Methyl-1-(butylcarbamoyl)-2-benzimidazolecarbamate
MEN	Mitotic exit network
NES	Nuclear export sequences
NETO	New end take off
NLS	Nuclear localization sequence
PAA	Post-anaphase array
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIN	Septation initiation network
SPB	Spindle pole body
Ura	Uracil

LIST OF PUBLICATIONS

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1. Introduction

1. 1 A general introduction to cytokinesis

The eukaryotic cell cycle is a sequential and coordinated set of events that leads to the division and propagation of cells. The cell cycle can be primarily divided into interphase and mitosis. During interphase, cells grow, acquire nutrients from their environment and duplicate their genetic material. During mitosis, the duplicated chromosomes are segregated into the daughter nuclei. Following this, the process of cytokinesis divides the cytoplasm, leading to the formation of two daughter cells.

Cytokinesis, the final step of cell cycle, partitions the cellular constituents of a mother cell into two daughter cells. Although occupying a comparatively brief period in the cell cycle, cytokinesis is critical for genome stability. Failure of cytokinesis can lead to polyploidy, which in metazoans can contribute to cancer progression. In many eukaryotes the cytokinetic machinery consists of an actin and myosin based contractile ring. The contractile ring is positioned beneath the plasma membrane in a plane perpendicular to axis of the mitotic spindle. Cell cleavage is achieved through ring constriction, the forces for which are generated by the sliding of myosin-II motor over actin filaments.

Cytokinesis is temporally and spatially regulated to ensure correct segregation of genetic material into two daughter cells. However, the underlying mechanisms of assembly and positioning of the cell division machinery are not fully understood.

1. 2 Division plane positioning in prokaryotes and animal cells

To ensure genome stability, cell cleavage takes place between the segregated daughter nuclei. Such division ensures that daughter cells receive a complete set of chromosomes, together with a full set of other organelles and cytoplasm constitute. To achieve this, cytokinesis is tightly coordinated spatially and temporally with chromosome segregation. The mechanisms of positioning the division plane have been intensively studied in prokaryotic and eukaryotic cells (Balasubramanian et al., 2004; Glotzer, 2004; Lutkenhaus, 2007). Different organisms appear to have evolved distinct mechanisms to select the division site. Nevertheless, there is growing evidence suggesting that multiple, overlapping mechanisms operate in a single cell (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003; Glotzer, 2004; Motegi et al., 2006).

1. 2. 1 Positioning of division plane in prokaryotes

In most prokaryotes, cytokinesis is achieved through the use of the so called Z-ring which is composed of polymers of FtsZ, the ancestral homologue of tubulin (Bramhill and Thompson, 1994; Erickson, 1997). The position of the FtsZ ring is determined by a

series of inhibitory mechanisms that prevent division at the cell ends and over segregating DNA (Margolin and Bernander, 2004; Wu and Errington, 2004). Rod-shaped prokaryotes appear to use the chromosome as a spatial cue for selection of a division site (Mulder and Woldringh, 1989). Chromosomes function as a negative regulator for positioning the division plane in prokaryotes. This mechanism, referred as 'nucleoid occlusion', blocks the assembly of the FtsZ ring in the vicinity of an unsegregated nucleoid (Mulder and Woldringh, 1989). In support of this model, when the nucleoid is retained in the middle of the cell, either by blocking DNA replication or chromosome segregation, non-medial FtsZ rings form beside the nucleoid (Harry et al., 1999; Sun and Margolin, 2001; Sun et al., 1998). The molecular mechanisms of nucleoid occlusion have begun to be elucidated. Recently two DNA-binding proteins, SlmA in *E. coli* and Noc in *B. subtilis,* have been shown to be at least in part responsible for nucleoid occlusion (Bernhardt and de Boer, 2005; Wu and Errington, 2004). Blocking initiation of DNA replication in *SlmA/Noc* mutant cells leads to assembly of Z rings over the unsegregated nucleoid. How SlmA and Noc achieve the nucleoid occlusion function is still unclear. Since SlmA physically interacts with FtsZ, it is suggested that SlmA might compete for FtsZ binding with FtsA and ZipA, which attach FtsZ to the membrane (Bernhardt and de Boer, 2005).

In addition to nucleoid occlusion, cylindrical bacteria utilize another inhibitory mechanism, the MinCDE system, to prevent Z ring assembly at the cell poles. This mechanism was identified on the basis of mini-cell mutations in which cell division is aberrantly positioned at the cell poles as well as in the cell middle. Such asymmetric

division leads to the formation of mini-cells lacking DNA (Adler et al., 1967). At least three proteins have been shown to be responsible for this mechanism: MinC, MinD and MinE (Lutkenhaus, 2007). Depending on the species, Min proteins localize to the cells pole either statically or dynamically (Errington et al., 2003). In *E. coli*, the inhibitory system is achieved through the oscillation of MinC and MinD proteins (Raskin and de Boer, 1999). By contrast, MinC and MinD in *B. subtilis* constitutively localize at the cell poles (Marston et al., 1998). The complex of MinCD then inhibits polymerization of FtsZ at cell ends. Simultaneous overproduction of MinC and MinD prevents assembly of the Z ring and cytokinesis (Levin et al., 2001; Pichoff and Lutkenhaus, 2001). MinE, the topological regulator of MinCD, releases this inhibition at the cell center (Lutkenhaus, 2007). Consistently, MinE is observed as a ring at the cell center (Raskin and de Boer, 1997).

Using these two inhibitory mechanisms, nucleoid occlusion and the MinCDE system, the division site in bacteria is precisely positioned in the middle of cells. Loss of the Min system and nucleoid occlusion leads to the failure of cell division, and FtsZ is distributed sporadically in arcs or rings along the filament (Bernhardt and de Boer, 2005; Wu and Errington, 2004).

1. 2. 2 Positioning of division plane in animal cells

In animal cells, the division site is determined in late anaphase when chromosomes are well separated. Early studies using inhibitors or cold treatment to de-polymerize

microtubules established that microtubules play an important role in positioning the division plane in animal cells (Beams, W. H. 1940). However, despite decades of studies, the identity of microtubules responsible for specification of the position of the cleavage furrow in animal cells is still being actively debated. Currently, there are three models that account for specification of the cleavage furrow in animal cells: the astral stimulation model, the polar/astral relaxation model and the central spindle stimulation model (Glotzer, 2004).

The astral stimulation model proposes that overlapping astral microtubules determine the cleavage furrow position. This model is based on the classic 'torus experiment' that was performed in sand dollar eggs by Rappaport in 1961 (Rappaport, 1961). It was observed that by micromanipulation, furrows were induced between asters at the sites lacking chromosomes and midzone microtubules. This indicated that only astral microtubules are required to position the cleavage furrow. This hypothesis was further supported by experiments performed in amphibian zygotes and sea urchin embryos (Sawai, 1998; Schroeder, 1987). However, the molecular mechanisms of astral stimulation remain unclear. One interpretation is that astral microtubules transport certain factors to the cell cortex to stimulate cleavage furrow formation. Since overlapping astral microtubules from two poles influences the equatorial region, the concentration of such factors is the highest at the equatorial cortex. As a result, the cleavage furrow is induced at the equatorial cortex where the strength of this stimulus is highest. This interpretation is further supported by computer modeling studies (Devore et al., 1989; Harris and Gewalt, 1989).

In contrast to the astral stimulation model, the astral relaxation model states that astral microtubules relay signals that inhibit contractility to the polar cortex. According to this model, the density of astral microtubules is higher at the cell poles than at the equatorial site. The difference of contractility between the cell poles and equatorial site allows the cleavage furrow to be positioned at the equatorial region, where the cell cortex contractility is the highest. This model is supported by the experimental finding that furrows are induced all over the cortex in a *Caenorhabditis elegans* mutants with shortened astral microtubules (Severson and Bowerman, 2003). Consistent with this model, mathematical modeling and direct measurement of microtubule distribution suggests that the density of microtubules at the cell poles is higher than that at the future division site (Dechant and Glotzer, 2003; Yoshigaki, 2003).

The central spindle stimulation models asserts that the central spindle, which comprises overlapping microtubules in the center of the spindle, is required for the specification of the cleavage furrow. This idea is supported by the observation that cells lacking astral microtubules are able to induce furrow formation at the proper position (Alsop and Zhang, 2003). Removing centrosomes and chromosomes from grasshopper spermatocytes does not cause a failure in positioning the cleavage furrow. On the contrary, the remaining microtubules rearrange into a central spindle-like structure and appear to induce the furrow formation (Alsop and Zhang, 2003). Similarly, genetically removing centrioles from fly cells causes the absence of astral microtubules, but the cleavage furrow is still able to assemble and is positioned normally (Basto et al., 2006). These experiments

indicate that the astral microtubules are dispensable, whereas the central spindle is required to position the cleavage furrow in these cell types.

Different types of animal cells appear to utilize different mechanisms to position the division plane. In large cells, such as those in early marine invertebrate embryos, the positioning of the cleavage furrow seems to be determined by interactions between the cortex and the astral microtubules (Rappaport, 1985). In smaller cells, such as most somatic cells in vertebrate and *Drosophila*, the site of cleavage furrow formation is specified by the central spindle (Bonaccorsi et al., 1998; Cao and Wang, 1996; Giansanti et al., 2001). However, recent studies have suggested that overlapping mechanisms operate in cleavage plane specification in *C. elegans* (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003; Glotzer, 2004; Motegi et al., 2006).

1. 3 Actomyosin ring assembly in animal cells

After specifying the position of the division plane, the division apparatus is assembled. In many organisms, the division apparatus is an actomyosin contractile ring which is assembled underlying the plasma membrane. To date, over 50 proteins have been determined to be present in actomyosin ring (Balasubramanian et al., 2004). Interestingly, there is significant evolutionarily conservation of essential actomyosin ring components (Balasubramanian et al., 2004).

The critical molecule to induce cleavage furrow formation in animal cells is the small GTPase Rho. When RhoA is depleted genetically or inactivated using the bacterial enzyme C3, furrow formation is abolished (Jantsch-Plunger et al., 2000; Kishi et al., 1993). Rho family GTPases act as molecular switches that cycle between inactive GDP-bound forms and active GTP-bound forms. Their ability to exchange and hydrolyze GTP is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). RhoA is activated by Rho GEF, which is encoded by ECT2 in humans, pebble in flies and LET-21 in worms (Piekny et al., 2005). Activated RhoA accumulates in the equatorial cortex before furrow formation (Bement et al., 2005; Yuce et al., 2005). It has been suggested that activated RhoA promotes nucleation, elongation and sliding of actin filaments through the coordinated activation of formin and myosin II motors, and this in turn induces the assembly of the cleavage furrow (Kawano et al., 1999; Li and Higgs, 2003; Piekny and Mains, 2002; Sagot et al., 2002).

The central spindle plays an important role in cleavage furrow induction. In *Drosophila*, defects in the formation of the central spindle prevents the formation of the cleavage furrow (Adams et al., 1998). Recently, it has been suggested that the central spindle executes its regulation of cleavage furrow formation by delivering the Rho regulators to the cortex (Piekny et al., 2005). These Rho regulators include GDP-GTP exchange factor (GEF) ECT2/Pebble/LET-21 and GTPase-activating factor (GAP) MgcRacGAP/CYK-4/RacGAP50C (Piekny et al., 2005; Somers and Saint, 2003). Rho GEF, Rho GAP and motor proteins form a complex known as centralspindlin, which is required for central spindle assembly (Jantsch-Plunger et al., 2000; Mishima et al., 2002). In *Drosophila*,

RacGAP50C physically interacts with Pebble, and then promotes Rho activation (D'Avino et al., 2004; Somers and Saint, 2003). Localization of the Rho GEF and Rho GAP complex to the central spindle is dependent on the motor protein Mklp1/Zen-4/Pav (Yuce et al., 2005).

The chromosome passenger complex, which localizes initially to the chromosomes and centromeres and relocates to the central spindle, appears to be involved in the control of early furrowing events (Earnshaw and Cooke, 1991). The major chromosome passenger proteins are serine/threonine kinase Aurora B, the inner centromere protein INCENP and the IAP repeat protein Survivin (Vader et al., 2006; Vagnarelli and Earnshaw, 2004). A dominant negative form of INCENP causes failure in cytokinesis, suggesting that INCENP is essential for cytokinesis (Mackay et al., 1998). RNA interference experiments and knockout experiments have confirmed the importance of INCENP in cytokinesis (Adams et al., 2001; Kaitna et al., 2000). In the absence of Aurora B, both central spindle formation and cleavage furrow induction fails, indicating that Aurora B is involved in cytokinesis (Adams et al., 2001; Kaitna et al., 2000). Recently two proteins, Mklp1 and MgcRacGAP, have been identified as substrates of Aurora B (Guse et al., 2005; Minoshima et al., 2003; Neef et al., 2006). Both of these substrates are components of the centralspindlin complex and are critical regulators of Rho A. This suggests that Aurora B, through modulation of Mklp1 and Rho GAP, regulates Rho A to influence cleavage furrow formation.

Another kinase implicated in cleavage furrow formation is Plk1 (Polo-like kinase 1). Cdc5, the Polo-like kinase in budding yeast, is required for the recruitment of two Rho GEFs, Tus1 and Rom2, to the division site (Yoshida et al., 2006). Therefore Cdc5 is suggested to be necessary for recruitment and activation of Rho1 and in turn to be important to induce actomyosin ring formation (Yoshida et al., 2006). A similar mechanism also functions in animal cells (Brennan et al., 2007; Burkard et al., 2007; Petronczki et al., 2007; Santamaria et al., 2007). At metaphase–anaphase transition, Plk1 undergoes a dramatic relocalization from the centrosome and kinetochores to the spindle midzone, suggesting a role in regulation of cytokinesis (Barr et al., 2004). However, defining the precise role of Plk1 in cytokinesis is difficult because of the early prometaphase arrest upon Plk1 depletion (Glotzer, 2005; Sumara et al., 2004). This difficulty was overcome by a recently developed specific small molecule inhibitor for Plk1 and a genetically engineered Plk1 allele (Brennan et al., 2007; Burkard et al., 2007; Petronczki et al., 2007; Santamaria et al., 2007). These new technologies allowed the precise temporal control of Plk1 inactivation and facilitated the study of Plk1 function in late mitosis without interfering with its earlier mitotic function (Brennan et al., 2007; Burkard et al., 2007; Petronczki et al., 2007; Santamaria et al., 2007). By the use of these technologies, Plk1 was found to be required for the recruitment of Rho GEF ECT2 to the central spindle in human cells, which in turn is thought to trigger the initiation of cytokinesis. The failure of this mechanism results in the mis-localization of RhoA to the cell cortex, which causes a defect in cleavage furrow formation.

1. 4 Fission yeast as a model system to study cytokinesis

Schizosaccharomyces pombe, a simple and unicellular fungus, has become an attractive model to study cytokinesis. *S. pombe* cells are cylindrical in shape and grow in a polarized fashion (Mitchison and Nurse, 1985). It can be readily grown and manipulated in the laboratory using a variety of molecular and genetic methods (Nurse, 1975; Nurse et al., 1976). The genome of *S. pombe* is fully sequenced (Wood et al., 2002) and a large bank of conditional mutants defective in various aspects of cytokinesis is available (Balasubramanian et al., 1998; Chang et al., 1996; Nurse, 1975). More importantly, *S. pombe* cells divide through the constriction of an actomyosin-based ring, in a manner similar to that observed in animal cells. The major components of the actomyosin ring in *S. pombe*, such as actin, myosin-II, formin, profilin, cofilin and PCH proteins, have also been found in animals (Balasubramanian et al., 2004; Balasubramanian et al., 1994; Chang et al., 1997; Fankhauser et al., 1995; Marks et al., 1986; Nakano and Mabuchi, 2006). Moreover, Septation Initiation Network (SIN) which is required for actomyosin ring maintenance and septum formation in *S. pombe*, is highly analogous to the mitotic exit network (MEN) in budding yeast (Balasubramanian et al., 2004). Thus, studies on the mechanism of cytokinesis in *S. pombe* have provided important insights into the mechanism of cytokinesis in higher organisms.

1. 5 Regulation of polarity in fission yeast

Cell polarity is a fundamental property of cells from unicellular to multicellular organisms. Establishment of proper cell polarity is important for cell division, differentiation, migration, and signaling (Martin and Chang, 2003). The use of genetics and easily screenable phenotypes make *S. pombe* an appealing model for the study of cell polarity.

1. 5. 1 Cell growth is temporally regulated during cell cycle

In *S. pombe*, cell growth is tightly regulated during the cell cycle (Mitchison and Nurse, 1985). A newly divided cell has an old and a new end. In G1 phase, the cell growth is restricted to the 'old end', which is the cell tip that existed in the mother cell before cell division. At this stage, actin patches, which are required for vesicle secretion and necessary for cell growth, are concentrated at the old end (Marks and Hyams, 1985). During early G2 phase, actin patches relocate to the 'new end'. As a result, the new end initiates growth. The cells then grow from both the old and new ends until they enter mitosis. This transition from monopolar growth to bipolar growth is referred as 'new end take off' (NETO). To trigger NETO, cells are required to reach a certain size (Castagnetti et al., 2007; Frazier et al., 1998). During mitosis, actin is redistributed to the middle of cell.

1. 5. 2 Microtubules play an important role in regulating cell polarity

The interphase microtubules play an important role in the regulation of cell polarity. Interphase microtubules are organized as anti-parallel bundles extending the length of the fission yeast cells. The plus ends of microtubules face both cell tips, while the minus ends of microtubules are located near the nucleus. The plus end of microtubules keep elongating until they reach the cell ends, and then undergo catastrophe and shrink back (Brunner and Nurse, 2000; Drummond and Cross, 2000; Tran et al., 2001). Genetic or drug disruption of microtubules leads to bent or T-shaped cell morphology, indicating a critical role for microtubules in regulating cell polarity (Castagnetti et al., 2007; Hagan, 1998). For example, in *mal3Δ* mutant cells (a microtubule end binding protein), microtubules undergo catastrophe even before they reach cell cortex, leading to short interphase microtubules. Such a defect in microtubules results in an abnormal cell shape and aberrant cell growth pattern (Busch and Brunner, 2004).

1. 5. 3 Cell end localized polarity factors

Microtubules function in the regulation of cell polarity through deposition of Tea1p (a Kelch-repeat protein) and Tea4p/Wsh3p (a SH3 domain protein) at the cell tips. Tea1p physically interacts with Tea4p (Martin et al., 2005; Tatebe et al., 2005). Both proteins serve as a landmark for cell growth, as they are localized to both cell ends throughout the cell cycle and mark regions for growth. Targeting of Tea1p to the cell ends is essential for its function (Behrens and Nurse, 2002). Mutation in *tea1/tea4* leads to the bent or T-shape cell morphology, and cells grow in a monopolar manner (Mata and Nurse, 1997; Verde et al., 1995). Tea1p-Tea4p associates with the growing plus end of microtubules.

Such association is dependent on the kinesin Tea2p (Browning et al., 2000) and the CLIP170-like protein Tip1p (Brunner and Nurse, 2000). Tea2p transports Tea1p to the plus ends of microtubules (Browning et al., 2003; Browning et al., 2000). Growing microtubules deliver Tea1p-Tea4p to the cell tip. At the cell tip Tea1p-Tea4p transfer from microtubule plus ends to the cell cortex. It is proposed that Tea1p retention at the cell end occurs via multistep and multimodal mechanisms (Snaith et al., 2005). At the nongrowing end, Tea1p C-terminus interacts with another Kelch-repeat protein, Tea3p (Arellano et al., 2002). Tea3p is anchored at the cortex through binding with Mod5p, a plasma membrane protein (Snaith et al., 2005; Snaith and Sawin, 2003). At the growing end, Tea1p is thought to anchor to the cell cortex through interaction with Mod5p and unknown factors, which function similar to Tea3p at the nongrowing end. Cell end localized Tea1p then functions to regulate cell polarity by recruiting the polarisome, a large protein complex of cell polarity factors. Bud6p, the actin-binding protein, is first recruited by Tea1p (Glynn et al., 2001), followed by the recruitment of formin For3p (Feierbach et al., 2004). Mutation in *tea1* leads to the mis-localization of Bud6p and For3p. Bud6p is required for the proper localization of For3p (Feierbach and Chang, 2001) and might also stimulate For3p activity by regulating the autoinhibition of For3p (Martin et al., 2007). For3p contributes to polarized growth activation by promoting actin cable formation (Feierbach and Chang, 2001). The activity and localization of For3p is highly dynamic on a time scale of seconds (Martin and Chang, 2006). For3p and newly synthesized short actin filaments may be released from the cell tip and carried onto assembling actin cables. For3p is regulated by autoinhibition through an intramolecular interaction and such autoinhibition is thought to be relieved by Cdc42p

(Martin et al., 2007). Tea4p functions as the molecular bridge between Tea1p and For3p by direct physical interaction (Martin et al., 2005). By these interactions, microtubules are linked with actin cytoskeleton, contributing to the establishment of cell polarity in *S. pombe*.

Pom1p, the dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK) (Becker and Joost, 1999), may act as a downstream effector of cell-end polarity factors. As with other polarity factors, Pom1p localizes to the cell ends (Bahler and Pringle, 1998). Mutations in *pom1* results in a defect in cell shape and NETO, leading to a phenotype similar to those displayed by cell-end polarity factor mutants (Bahler and Pringle, 1998). Pom1p kinase activity is stimulated during bipolar growth and is inhibited during monopolar growth (Bahler and Nurse, 2001; Bahler and Pringle, 1998). Both localization and kinase activity are important for the cellular function of Pom1p (Bahler and Nurse, 2001; Bahler and Pringle, 1998). Localization of Pom1p to the cell ends is dependent on Tea1p and Tea4p, but Pom1p is dispensable for the recruitment of Tea1p and Tea4p to the cell ends (Bahler and Nurse, 2001; Bahler and Pringle, 1998; Martin et al., 2005). This suggests that Pom1p may act downstream of Tea1p-Tea4p. Recent work has established that Pom1p achieves its function in polarity regulation through modulation of the activity of Cdc42p (Tatebe et al., 2008), the activated form of which is required to develop F-actin structures and in turn is important for cell growth (Miller and Johnson, 1994). Pom1p physically interacts with Rga4p, the GAP of Cdc42p (Tatebe et al., 2008). Exclusion of Rga4p from cell ends is crucial for the activation of Cdc42p. However, *pom1* mutant cells fail to exclude Rga4p from the cell ends, leading to the inactivation of

Cdc42p at the cell ends (Tatebe et al., 2008). Therefore, it has been proposed that Pom1p regulates localization of Rga4p to ensure the bipolar activation of Cdc42 in *S. pombe* (Tatebe et al., 2008).

1. 6 Positioning and assembly of division plane in fission yeast

1. 6. 1 Positioning of division plane in fission yeast

In *S. pombe*, the actomyosin ring is assembled at medial region of the cell upon entry into mitosis. The division site is determined in G2 by the nucleus, which is maintained at the cell center. There is a tight correlation between the position of the interphase nucleus and that of the division site. The interphase nucleus is maintained in the middle of cell by pushing forces generated by interphase microtubules (Tran et al., 2001). In *S. pombe*, interphase microtubules are organized in an antiparallel array, with plus end facing the cell ends and minus ends of microtubules associated with nuclear membrane. When microtubules undergo polymerization, the pushing force exerted on the nuclear membrane positions the nucleus in the middle of the cells. Mutants defective in tubulin misposition the nucleus and therefore misplace the division plane (Chang et al., 1996). Physical manipulation of the position of the nucleus by high speed centrifugation leads to the assembly of misplaced actomyosin rings (Daga and Chang, 2005). In addition, when the position of the nucleus is displaced from cell center by optical tweezers, the cells divide asymmetrically (Tolic-Norrelykke et al., 2005). In cells manipulated by optical tweezers, the division plane is no longer placed in the cell center; rather, it is tightly correlated with the position of the displaced nucleus. These experiments further indicate

the critical role of the nuclear position in division site selection. The specification of the division plane by the nucleus occurs prior to mitosis. Displacement of the nucleus during prometaphase, by either centrifugation or optical tweezers, does not affect the position of division site. In contrast, displacement of the nucleus during prophase leads to asymmetrical cell division (Daga and Chang, 2005; Tolic-Norrelykke et al., 2005). All these results support the hypothesis that the nucleus specifies the division site before metaphase.

Genetic screens have identified three genes, *mid1*, *plo1* and *pom1*, which are involved in the positioning the division site in *S. pombe* (Bahler et al., 1998; Chang et al., 1996; Sohrmann et al., 1996). Mutants defective in any of these genes display a frequent mislocalization and misorientation of actomyosin ring and division septa, although the position of the centrally located interphase nucleus is normal.

Mid1p shares some structural similarities in the PH domain with *Drosophila* contractile ring protein anillin (Field and Alberts, 1995). Both proteins shuttle between the cortex and nucleus in interphase, and localize to the contractile ring in cytokinesis. Mid1p contains two leucine-rich nuclear export sequences (NES), a putative nuclear localization sequence (NLS), a proline-rich domain and a C-terminal pleckstrin homology domain (PH domain) (Sohrmann et al., 1996). PH domains are known to target some proteins to the cell surface through direct interaction with membrane phospholipids (Lemmon et al., 1995). However, the PH domain of Mid1p is dispensable for Mid1p localization to the membrane, and for the cellular function of Mid1p (Paoletti and Chang, 2000). The

molecular function of the PH domain in Mid1p is presently unclear. Mutations in *mid1* lead to the assembly of misplaced and mis-oriented actomyosin rings and division septa. However, the position of nucleus is unaffected in *mid1* mutant cell. This phenotype indicates that Mid1p is one of the candidate proteins that couples the division plane with the interphase nucleus.

During interphase, Mid1p resides primarily in the nucleus (Paoletti and Chang, 2000). Upon entry into mitosis, Mid1p is phosphorylated in a Plo1p-dependent manner and exits the nucleus (Bahler et al., 1998; Paoletti and Chang, 2000). Overexpression of Plo1p causes a reduced mobility of Mid1p on gels, which indicates a hyperphosphorylated form of Mid1p (Bahler et al., 1998). But direct phosphorylation of Mid1p by Plo1p has not been demonstrated. The nuclear-localized Mid1p is believed to be non-functional because deletion of nuclear export signal (NES) sequences prevents nuclear exit of Mid1p, resulting in misplacement of division site, a phenotype similar to that observed in *mid1* mutant (Paoletti and Chang, 2000).

Subsequently, Mid1p is anchored to the cortex to form a broad band overlying the nucleus. Fluorescence recovery after photobleaching assays (FRAP) have revealed that upon reaching cell cortex Mid1p remains stably bound to the cell cortex, unlike other highly dynamic actomyosin proteins such as myosin and actin (Clifford et al., 2008). The positioning of the Mid1p band is tightly linked to the position of the nucleus. Displacing the nucleus by high speed centrifugation leads to the displacement of the Mid1p broad band (Daga and Chang, 2005). In cells with multiple nuclei, Mid1p is present on the cell

surface near each nucleus. It has been shown that Mid1p associates with the cell cortex via a dual binding mechanism (Motegi et al., 2004). Firstly, the C-terminus of Mid1p associates with the cell cortex through a putative amphipathic helix (Motegi et al., 2004). Secondly, the N-terminus of Mid1p is able to form faint patches at the medial cortex and is sufficient to form a tight ring (Motegi et al., 2004). Mechanisms by which the Mid1p band is restricted in the medial region are currently unclear. Recent studies suggest that Pom1p, the dual-specificity tyrosine-phosphorylation-regulated kinase (DYRK), excludes Mid1p from the non-growing tips (Celton-Morizur et al., 2006; Padte et al., 2006). Mutation in *pom1* leads to the uncoupling of the Mid1p broad band with the nucleus. Interestingly, Mid1p distribution expands toward the non-growing cell ends in *pom1* mutant cells, indicating that Pom1p localized to the cell ends negatively regulates the association of Mid1p with cell cortex at the non-growing ends. However, the inhibitory factors that prevent Mid1p localization to the growing tips remain to be identified.

The cell cortex-anchored Mid1p acts as a scaffold protein and recruits type II myosin heavy chain Myo2p by the physical interaction. This promotes the recruitment of other actomyosin ring components to the medial region (Motegi et al., 2004). The interaction between Mid1p and the C-terminal tail of Myo2p is temporally regulated by dephosphorylation of Myo2p (Motegi et al., 2004). A recent study reveals that Mid1p also physically associates with Clp1p (Clifford et al., 2008), a phosphatase involved in cytokinesis checkpoint (Mishra et al., 2005; Mishra et al., 2004). The recruitment of Clp1p by Mid1p to the actomyosin ring contributes to the robustness of cytokinesis. All actomyosin ring components that are recruited by Mid1p are then compacted into a tight

ring precisely in the middle of the cell. Whether Mid1p physically interacts with other components of the ring remains unknown.

At late anaphase, prior to actomyosin ring constriction, Mid1p relocalizes to the nucleus. The NLS sequence is predominantly responsible for nuclear import activity of Mid1p (Paoletti and Chang, 2000). In a *mid1* mutant with a defective NLS sequence, the nuclear localization of Mid1p is greatly reduced, but not totally abolished (Paoletti and Chang, 2000). This suggests that other unknown mechanisms also account for the nuclear import of Mid1p. NLS mutant cells undergo symmetrical division, indicating that the NLS is not required for Mid1p function (Paoletti and Chang, 2000).

In addition to Mid1p, two other molecules, Plo1p and Pom1p, have been implicated in the spatial regulation of division plane (Bahler and Pringle, 1998; Bahler et al., 1998). Plo1p belongs to a conserved family of Ser/Thr protein kinases, the polo-like kinase family (Ohkura et al., 1995). In addition to its conserved function in bipolar spindle formation (Ohkura et al., 1995), Plo1p has been found to play a crucial role in defining the position of the division sites (Bahler et al. , 1998). *plo1* temperature-sensitive mutants display defects in the selection of division site, in a manner similar to *mid1* mutants (Bahler et al., 1998). Such a phenotype may be due to the incapability of Mid1p to exit the nucleus, since *plo1* mutants display an increasing nuclear localization of Mid1p, even in mitosis. Overexpression of Plo1p leads to premature export of the hyperphosphorylated form of Mid1p from nucleus. Both loss-of-function and gain-of-function analysis suggest that Plo1p is required for phosphorylation of Mid1p and export

of Mid1p out of nucleus. However, a subsequent study revealed that Mid1p is able to form a broad band, but is unable to incorporate into the contractile ring in *plo1*-1 mutant cells (Paoletti and Chang, 2000). This indicates that Plo1p may also play a role in regulating the cytoplasmic activity of Mid1p, probably through facilitating the interaction between Mid1p and other ring components. Plo1p is considered to function in a common pathway with Mid1p, because the *mid1*-18 *plo1*-1 double mutant is viable and the phenotype of the double mutant is indistinguishable from either single mutant in terms of division site selection. Mid1p phosphorylation and release from the nucleus is dependent on Plo1p (Bahler et al., 1998). However, a *mid1* mutant that with defects in the nuclear localization signal is unable to rescue the ring-positioning defect in *plo1*-1 mutant cell (Paoletti and Chang, 2000). This suggests that a defect in the nuclear export of Mid1p is not the sole cause of the defect in division site selection in a *plo1*-1 mutant. Apart from regulation through Mid1p, Plo1p might have additional functions in the regulation of actomyosin ring positioning.

Pom1p, a protein kinase of the DYRK family, localizes to the cell ends during interphase and to mid-cell during cell division (Bahler and Pringle, 1998). *pom1* mutant displays defects in cell polarity, as well as in division site selection, indicating Pom1p plays dual functions in positioning the division plane and the growth zone (Bahler and Pringle, 1998). Recent studies suggest that Pom1p inhibits Mid1p association with the cortex at the non-growing tips (Celton-Morizur et al., 2006; Padte et al., 2006). In *pom1* mutant cells, the Mid1p broad band expands toward the non-growing ends, which in turn causes the mis-positioning of the actomyosin ring toward the non-growing cell ends. Therefore

Pom1p is suggested to act in the same pathway as Mid1p, by regulating Mid1p localization (Celton-Morizur et al., 2006; Padte et al., 2006). However, Pom1p displays genetic interactions with Mid1p (Bahler and Pringle, 1998). The double mutant, defective in *pom1* and *mid1* is lethal (Bahler and Pringle, 1998). This suggests that in addition to regulating Mid1p localization, Pom1p appears to play a distinct role from Mid1p to specify the division plane.

Once the actomyosin ring is assembled in the medial region of *S. pombe* cells, the ring is maintained at the cell center until late anaphase when the actomyosin ring starts to constrict. Microtubules are believed to account for the medial maintenance of actomyosin rings at this stage (Pardo and Nurse, 2003). At late mitosis, microtubules are nucleated from the cell division site by an equatorial microtubule organizing center (eMTOC) to form a post-anaphase array (PAA), a ring of microtubules underneath the actomyosin ring (Hagan, 1998). The PAA is thought to be required for maintenance of the actomyosin ring in the medial region of cells after the assembly of actomyosin ring (Pardo and Nurse, 2003). Studies carried out on a *cps1* mutant, in which septum formation is compromised (Ishiguro et al., 1997; Liu et al., 2002; Liu et al., 1999), allows for the analysis of the behavior of the actomyosin ring without the perturbation of the septum. Disruption of PAA in such a mutant by a microtubules inhibitory drug leads to the migration of actomyosin ring away from cell center in a membrane traffic-dependent manner, which suggests that PAA is essential to anchor the actomyosin ring in its medial location.

1. 6. 2 Actomyosin ring assembly in fission yeast

In *S. pombe*, the actomyosin ring assembles during early M phase, but cytokinesis does not occur until late anaphase. The major components of the actomyosin ring are actin and myosin. In interphase, F-actin forms patches structure at the growing ends. Upon entry into mitosis, F-actin patches are lost from cell ends and an F-actin ring is assembled in the cell center (Pelham and Chang, 2001). Formin Cdc12p, profilin Cdc3p, tropomyosin Cdc8p and IQGAP-related protein Rng2p are involved in actin ring formation (Balasubramanian et al., 1992; Balasubramanian et al., 1994; Balasubramanian et al., 1998; Chang et al., 1997; Eng et al., 1998; Lord and Pollard, 2004; Nurse et al., 1976). Cdc15p is a FCH (FER-CIP4 homology) family protein and is thought to play a role in rearrangement of the F-actin cytoskeleton during cell division (Fankhauser et al., 1995). Mutants defective in cdc15 are unable to maintain actomyosin ring in late mitosis (Wachtler et al., 2006). A Cdc15p-related protein, Imp2p, might also be involved in regulating actomyosin ring dynamics (Demeter and Sazer, 1998). α-actinin Ain1p and fimbrin Fim1p, which crosslink the actin filaments, appear to have overlapping and essential functions during cytokinesis (Wu et al., 2001). The formation of actomyosin ring requires not only the assembly of actin filaments, but also the depolymerization of F-actin. Adf1, an actin-depolymerizing protein is suggested to play a role in actomyosin ring assembly and maintenance (Nakano and Mabuchi, 2006). Recent studies have uncovered a novel actomyosin ring component, Pxl1p, a paxillin-related protein. Pxl1p is thought to modulate Rho1 activity (Pinar et al. , 2008) and to stabilize actomyosin ring during cytokinesis in *S. pombe* (Ge and Balasubramanian, 2008; Pinar et al., 2008).

There are two type II myosins in *S. pombe*: myosin heavy chains Myo2p and Myp2p, and the essential light chain Cdc4p and the regulatory light chain Rlc1p (Bezanilla et al., 1997; Bezanilla et al., 2000; Kitayama et al., 1997; Le Goff et al., 2000; McCollum et al., 1995). Only Myo2p is essential for the cell viability, whereas Myp2p is required for cytokinesis only under conditions of stress (Bezanilla et al., 1997; Kitayama et al., 1997). Rng3p and Chs2p play a role in myosin regulation. Rng3p, the UCS (UNC-45, Cro1p and She4p) protein is suggested to act as a myosin chaperone (Wong et al., 2000). In vitro motility assays demonstrate that Rng3p promotes the efficient interaction of Myo2 with actin filaments, and in turn activates actin filament gliding (Lord and Pollard, 2004). Chs2p, the chitin synthases related protein, has been shown to physically interact with Myp2p and is required for the integrity of actomyosin rings (Martin-Garcia and Valdivieso, 2006).

Assembly of the actomyosin ring in *S. pombe* occurs in several steps starting in late G2 phase. Mid1p exits from the nucleus, forming a broad band overlying the interphase nucleus. This leads to the marking of the future division site at the cell center. Other components of actomyosin ring are recruited sequentially to the medial region in a Mid1p-dependent manner (Wu et al., 2003). Mid1p physically interacts with myosin heavy chain Myo2p and then recruits Myo2p associated light chain proteins (Rlc1p and Cdc4p) and IQGAP protein Rng2p to the cell medial region. It is followed by the accumulation of Cdc12p and Cdc15p to the midzone of the cell. Formin cdc12p physically associates with Cdc15p, contributing to actin filament formation (Carnahan

and Gould, 2003; Chang et al., 1997). Next, actin begins to polymerize and bind to tropomyosin Cdc8p. Actin filaments are crosslinked by α-actinin Ain1p and fimbrin Fim1p, which may contribute to the parallel array of actin cable in the ring (Kamasaki et al., 2007; Wu et al., 2003). Meanwhile, the broad band containing Mid1p and myosin-II coalesces into a compact and well-focused ring structure. During anaphase B, the actomyosin ring matures through addition of unconventional myosin II Myp2p (Wu et al., 2003).

The actomyosin ring is believed to be continuously remodeled and reassembled. This assumption is based on fluorescence recovery after photobleaching assays (FRAP) (Wong et al., 2002). Fluorescence recovery occurs less than one minute after bleaching fluorescence in either the entire actomyosin ring or in a part of it. This suggests that the components of the actomyosin ring turn over dramatically. Consistent with this, permeabilized cell assays reveal that profilin Cdc3p, formin Cdc12p and Arp3p are required for the assembly of F-actin at the division site, and labeled G-actin monomers are quickly incorporated into the actomyosin ring (Pelham and Chang, 2002). This suggests that actin is continuously assembled and disassembled from the ring. In addition to this evidence, treatment of cells with Latrunculin A, which depolymerizes F-actin filaments by binding to monomeric actin, leads to the disassembly of the actomyosin ring within a short time (Bahler and Pringle, 1998; Naqvi et al., 1999; Pelham and Chang, 2002), reinforcing the dynamic nature of the actomyosin ring.

How are these different components assembled into a ring structure? Currently, there are two models, the 'leading cable model' and the 'search and capture model', which account for the mechanism of actomyosin ring assembly (Mishra and Oliferenko, 2008).

The leading cable model postulates that actomyosin rings are generated from actomyosin cables spreading out from a spot-like structure (Wong et al., 2002). The actomyosin cables then encircle the cell circumference, forming a ring structure. This model came from the observation that the actomyosin ring arises from a myosin-II containing spot (Arai and Mabuchi, 2002; Chang, 1999; Wong et al., 2002). Removal of the spot structure by genetic inactivation of Rng3p prevents actomyosin ring assembly in the subsequent mitosis. This suggests the importance of myosin-II containing spot in actomyosin ring assembly. Interestingly, Cdc12p and Cdc15p are also detected as a spot in interphase cells (Carnahan and Gould, 2003), although it is claimed that the spot containing Cdc12p and Cdc15p is distinct from myosin-II containing spot (Chang, 1999; Wong et al., 2002). Investigation of F-actin ring formation by optical sectioning and three-dimensional microscopy reveals that a single F-actin cable extends from the aster-like structure and encircles the cell to form the primary F-actin ring (Arai and Mabuchi, 2002). Ultra-structural examination of direction of actin filaments, by decorating with myosin S1, establishes that during early cytokinesis, the ring consists of two semicircular populations of parallel filaments with opposite directionality, which orient toward to a common point (Kamasaki et al., 2007). Both studies suggest that the ring initiates from a single site at the division plane, which is consistent with the leading cable model.

Recent studies using a combination of fluorescence imaging of live cells and computational approaches, have led to another model known as 'search and capture model' (Wu et al., 2006). According to this model, actomyosin rings do not initiate from the spot structure, instead they arise from a broad medial band of nodes, each of which has been shown to contain seven proteins: Mid1p, Myo2p, Rlc1p, Cdc4p, Rng2p, Cdc12p and Cdc15p. Mid1p anchors the protein complex to the cell membrane. It is suggested that Cdc12p promotes nucleation of actin filaments, which are then captured by myosin in the adjacent nodes. The force generated by myosin sliding on actin filaments then pulls the nodes together and therefore promotes the formation of a ring. The formation of cortical nodes is dependent on Mid1p. Mutation in *mid1* leads to the absence of membrane-associated nodes and therefore causes the defective positioning of the division plane. This model is further supported by a computational simulation, which establishes that the transient connection between myosin and actin filament is important for a tight ring formation (Vavylonis et al., 2008).

1. 7 Septation initiation network (SIN) in fission yeast

During late anaphase and concomitant with ring constriction, the division septum is deposited in a centripetal manner. The initiation of actomyosin ring contraction and synthesis of the division septum are regulated by an elaborate signal transduction cascade known as septation initiation network (SIN), whose counterpart in *Saccharomyces cerevisiae* is referred to as the mitotic exit network (MEN) (Simanis, 2003). Failure in SIN signaling leads to defects in constriction of the actomyosin ring and formation of

division septum at the end of the nuclear cycle. However, the nuclear cycle in SIN defective mutant cells continues, despite the failure of cell division. As a result, SIN mutant cells undergo multiple rounds of S and M phases, giving rise to highly elongated, multi-nucleate cells (Simanis, 2003). Ectopic activation of SIN signaling causes the multiple rounds of assembly and constriction of the actomyosin ring, and formation of division septa without cell cleavage, giving rise to binucleate cells with multiple septa (Simanis, 2003). Activation of SIN signaling can even promote septum formation in interphase cells (Schmidt et al., 1997).

1. 7. 1 Components of SIN

SIN consists of a small GTPase (Spg1p), three protein kinases (Cdc7p, Sid1p and Sid2p) and their associated subunits. At the top of this signaling cascade is the small GTPase Spg1p (Schmidt et al., 1997). GTP-bound Spg1p activates the signaling pathway and therefore promotes the formation of the division septum. The nucleotide state of Spg1p is regulated by a GTPase activating protein (GAP), which comprises two proteins: Cdc16p (Fankhauser et al., 1993) and Byr4p (Song et al., 1996). Cdc16p is found to contain a 'GAP' domain (Furge et al., 1999; Furge et al., 1998), while Byr4p acts as a scaffold protein that interacts with Spg1p and Cdc16p (Furge et al., 1998). The signaling triggered by GTP binding of Spg1p transduces down the cascade through three protein kinases: Cdc7p, Sid1p and Sid2p. Cdc7p is the first kinase in this cascade and is found to interact with GTP-bound Spg1p (Sohrmann et al., 1998). Efficient transduction of the signal requires Cdc14p and Mob1p, which are binding partners of Sid1p and Sid2p,

respectively (Fankhauser et al., 1993; Guertin et al., 2000; Hou et al., 2004; Sparks et al., 1999). Most of these SIN proteins associate with the spindle pole body (SPB). The localization of SIN proteins to the SPB depends on a scaffold protein complex containing Sid4p and Cdc11p (Chang and Gould, 2000; Krapp et al., 2001; Tomlin et al., 2002).

SIN is activated during anaphase (Simanis, 2003). In interphase, Cdc16p-Byr4p localizes to SPB and hence keeps the SPB-localized Spg1p in the inactivated GDP-bound form (Cerutti and Simanis, 1999; Li et al., 2000a; Sohrmann et al., 1998). During metaphase, when the mitotic spindle forms, Cdc16p-Byr4p leaves both SPBs, leaving Spg1p in a GTP-bound state (Cerutti and Simanis, 1999; Li et al., 2000a). Activated Spg1p then recruits Cdc7p to both SPBs through direct interaction (Sohrmann et al., 1998). As activated signal transduces from Cdc7p to Sid1p-Cdc14p (Guertin et al., 2000), Cdc16p-Byr4p returns to one of the SPBs (Cerutti and Simanis, 1999; Li et al., 2000a). This results in the hydrolysis of GTP in one SPB and loss of Cdc7p from one SPB, giving rise to asymmetric localization of Cdc7p and Cdc16p-Byr4p. Activated signals then are thought to transduce from Sid1p-Cdc14p to Sid2p-Mob1p. Sid2p then translocates from SPB to the medial cortex, where it is believed to trigger actomyosin ring constriction (Guertin et al., 2000; Sparks et al., 1999).

1. 7. 2 Targets of SIN

How SIN contributes to regulating cytokinesis remains an open question. One of the possibilities is that SIN activation might lead to the targeting of cell wall synthetic

enzymes to the division site. An attractive candidate target of SIN is Cps1p, the 1, 3-β glucan synthase subunit. Cps1p is a stable integral membrane protein which is required for the assembly of the primary septum (Ishiguro et al., 1997; Liu et al., 2002; Liu et al., 1999). Cps1p localizes to the site of polarized growth in interphase and to the actomyosin ring in mitosis (Cortes et al., 2002; Liu et al., 2002). However, in mutants defective in SIN, Cps1p is unable to be recruited to the future division site, indicating the requirement of SIN for proper localization of Cps1p (Cortes et al., 2002; Liu et al., 2002). Moreover, there is a genetic interaction between *cps1* and *sid2*, the most downstream kinase in SIN cascade (Liu et al., 1999). Therefore, Cps1p might be an effector of Sid2p.

Clp1p, a phosphatase that is involved in cytokinesis checkpoint (Mishra et al., 2004), is another target of SIN. In interphase, Clp1p localizes in nucleolus and SPB. During mitosis, Clp1p accumulates in cytoplasm, actomyosin ring and spindle midzone (Cueille et al., 2001; Trautmann et al., 2001). Upon the minor perturbation of the cytokinetic machinery, Clp1p is maintained in the cytoplasm for longer periods and this is critical for the cell viability (Cueille et al., 2001; Mishra et al., 2005; Mishra et al., 2004; Trautmann et al., 2001). The maintenance of Clp1p in cytoplasm is achieved through the direct phosphorylation of Clp1p by Sid2p, the most downstream SIN component (Chen et al., 2006). Mutation in Sid2p phosphorylation sites on Clp1p causes loss of Clp1p from the cytoplasm and premature return to the nucleolus during cytokinesis. This improper localization of Clp1p renders cells sensitive to perturbation of the cell division machinery (Chen et al., 2006).

Genetic analysis of the interactions between *cdc15* and SIN components suggests that SIN may regulate the function of Cdc15p (Markset al., 1992). Upon cytokinesis delay, localization of two of the SIN components, Clp1p and Cdc7p, are independent of Cdc15p, indicating that Cdc15p does not act upstream of SIN and Clp1p (Wachtler et al., 2006). The assembly and maintenance of the ring upon activation of SIN require Cdc15p function (Wachtler et al., 2006). Moreover, Cdc15p is dephosphorylated in response to SIN activation and Clp1 phosphorylation (Hachet and Simanis, 2008; Wachtler et al., 2006). These suggest that both SIN and Clp1p contribute to the maintenance of the actomyosin ring in late mitosis through Cdc15p.

Thus presently, Cps1p, Clp1p and Cdc15p are thought to be downstream of SIN. Other downstream factors of SIN are still unknown.

1. 8 Aims and objectives of this thesis

My work aims to get a better understanding of spatial regulation of cytokinesis, and further understand the mechanism of actomyosin ring assembly in *S. pombe*. In my study, I further the investigation of spatial regulation of cytokinesis. My studies revealed an inhibitory mechanism that cooperates with the Mid1p-dependent mechanism. I also test the existing models for actomyosin ring assembly. A revised model for actomyosin ring assembly is proposed based on my work. Finally, I examine the requirements of the nucleus and SPBs for actomyosin ring assembly and find that the actomyosin ring can assemble in the absence of the nucleus and SPBs.

2. Materials and Methods

2.1 *S. pombe* strains, reagents and genetic methods

2. 1. 1 *S. pombe* strains

Genotypes of *S. pombe* strains used in this study are listed in table I.

Table I : *Schizosaccharomyces pombe* strains used in this study

Strain number	Relevant genotype	Source
MBY102	*ade6*-210 *ura4*-D18 *leu1*-32 h+	Lab collection
MBY297	*mid1*-18 *ura4*-D18, *leu1*-32, *ade6*-21X, h+	Lab collection
MBY3562	*mid1*-366	Fred Chang Lab
MBY2017	*mid1::ura4+, leu1*-32, *ade6*-21?, h+	Lab collection
MBY 1287	*mid1*-18 rlc1::GFP ura4+ *ade6*-21x *leu1*-32 *ura4*-D18 h-	Lab collection
MBY2528	*tea1::ura4+, ura4*-D18, h+	Lab collection
MBY3197	*tea1(D946)::kanR leu1*-32 *ade6*-M216 *ura4*-D18 h-	Paul Nurse Lab
MBY2743	*tea2::his3+ His3*-D1 *leu*-32 *ura4*-D18 *ade6*-M2x h-	this study
MBY4238	*tea3::KanMX6 leu1*-32 *Ura4*-D18 h-	Paul Nurse Lab

MBY2832	*tea4::kanMX6 ade6*-M216 *leu1*-32 *ura4*-D18 h+	Fred Chang Lab
MBY2742	*mod5::kanMX6 ura4*-D18 *leu1*-32 *ade6*-M216 h+	this study
MBY2744	*bud6::KanMX6* h-	Fred Chang Lab
MBY1796	*pom1::ura4* h-	Lab collection
MBY2313	*wee1*-50, h+	Lab collection
MBY2699	*tea1::ura4*+ *mid1*-18 Rlc-GFP::ura4 h-	this study
MBY2878	*tea4::kanMX mid1*-18 Uch2-gfp::ura rlc1-gfp::leu	this study
MBY2877	*pom1::ura4 mid1*-18 Rlc1-GFP::leu	this study
MBY2745	*mod5::KanMX6 mid1*-18	this study
MBY2757	*bud6::KanMX6 mid1*-18 Uch-GFP::ura4 Rlc1-GFP::leu	this study
MBY2761	*tea2::his3*+ *mid1*-18 Uch2-GFP::ura4 Rlc1-GFP::leu	this study
MBY3218	*tea1(D946)::kanR mid1*-18	this study
MBY4281	*tea3::ura4 mid1*-18	this study
MBY2331	cdc15-GFP::KanR *ura4*-D18 *leu1*-32 *ade6*-M210 h-	Lab collection
MBY3208	cdc15-GFP *tea1::ura4*	this study
MBY3277	*cdc15*-gc1 *tea1::ura4 mid1*-18	this study
MBY383	mid1-GFP ura4+ *ade6*-210 *ura4*-D18 *leu1*-	Lab collection

	32 h-	
MBY3194	*wee1*-50 mid1GFP	this study
MBY4280	*wee1::ura4* mid1GFP::ura4	this study
MBY2792	*wee1*-50 *tea1::ura4* Uch2-gfp::ura4 Rlc1-gfp::leu	this study
MBY3210	*wee1*-50 *tea1::ura4* Rlc1-GFP	this study
MBY4213	*wee1*-50 *tea1::ura4 cdc15*-gc1	this study
MBY2972	*tea1::ura4* his+ ade+ leu+	this study
MBY2479	GFP-cps1 *ade6*-216 h+	Lab collection
MBY4189	GFP-Cps1 *tea1::ura4 mid1*-18	this study
MBY4211	GFP-cps1 *mid1*-18	this study
MBY4329	*tea1::ura4 mid1*-18 nmt1-GFP-cdc15::KanMX6	this study
MBy3890	*clp1::ura4 cdc15*-gc1	this study
MBY4258	*tea1::ura4 mid1*-18 Rng2GFP::ura4	this study
MBY4210	*tea1::ura4 mid1*-18 cdc12GFP	this study
MBY4223	*tea1::ura4 rlc1::ura4 mid1*-18	this study
MBY4257	*tea1::ura4 mid1*-18 *fim1::KanMx6*	this study
MBY4259	*tea1::ura4 mid1*-18 *ain1::KanMx6*	this study
MBY4246	*tea1::ura4 mid1*-18 *myp2::his7*	this study
MBY2995	*tea1::ura4 mid1*-18 *cdc7*-24	this study
MBY286	*cdc16*-116 , *leu1*-32, *ura4*-D18, *ade6*-M210, h+	Lab collection

MBY3007	*cdc16*-116 Pcp1GFP::ura4	this study
MBY2949	*cdc16*-116 Rad24GFP::ura4	this study
MBY4330	*cdc16*-116 Pcp1GFP::ura4 with pPDQ105 (GFP-Atb2p)	this study
MBY2603	*cdc16*-116 Uch2GFP::ura4 Rlc1GFP::leu1	this study
MBY5179	Rlc1-3GFP-KanMX6 *ade6*-M210 *leu1*-32 *ura4*-D18 h+	Wu Jian Qiu Lab
MBY5299	*mid1::ura4* Rlc1-3GFP h-	this study
MBY2019	*ade*-M216 *ura4*-D18 *leu1*-32 *dmf1::ura4* h- + pAP116 integrated (pmid1-dNES1+2 mid1 leu1+)	Fred Chang Lab
MBY 1463	*plo1*-1, *leu1*-32, *ura4*-D18, *ade 6*-21X, h-	Lab collection
MBY4123	cps1-191 mid1::ura4 Rlc1-GFP h+	this study
MBY2591	*cps1*-191 rlc1-GFP::ura4+ *ura4*-D18 *leu1*-32 *ade6*-M21x h+	Lab collection
MBY3957	mid1-18 cps1-191 rlc1-GFP::ura4+ ura4- leu1- ade6-21x h+	Lab collection
MBY5241	cps1-191 mid1-18 rlc1-3GFP	this study
MBY2697	cps1-191 dmf1::ura4 mid1NES1+2 leu+	this study
MBY5242	cps1-191 plo1-1 rlc1-3GFP	this study

2. 1. 2 Growth and maintenance of *S. pombe*

Cells were cultivated and maintained in yeast extract medium (YES), Edinburgh minimal medium (EMM) as described by Moreno *et al*. (1991). All solid media contains 2% Bactoagar (Difco). *cdc16*-116 *mid1*Δ cells were first cultured on YES agar plate containing 1. 2M sorbitol and then removed to YES liquid medium for further experiments. Temperature sensitive mutant cells were grown at 24^{o}C (permissive) and shifted to 36^{o}C (restrictive) for gene inactivation.

2. 1. 3 Mating and sporulation of *S. pombe*

YPD medium which contains 1% yeast extract (Gibco-BRL), 2% peptone (Gibco-BRL) and 2% glucose was used for mating and sporulation. After sporulation, tetrads were dissect on YES agar plate using a Singer MSM micromanipulator (Singer Instruments, Roadwater-Watchest, Somerset, UK) and then incubated at suitable temperature allowing spore germination.

2. 1. 4 Drugs used

To arrest cells in S phase, cells were first treated with 12 mM hydroxyurea (HU; Sigma) for four hours, and then treated with the same amount of HU for an additional two hours. Thiamine was used at a final concentration of 5μm to repress the transcription from the nmt1 promoter.

2. 1. 5 *S. pombe* transformation

A lithium acetate protocol was used in *S. pombe* transformation. 20ml cells were cultured to a density of 0. 5 -1 x 10^7 cells/ml (OD_{595} = 0. 2-0. 5). Cells were then rinsed once time with sterile water and once with 1X LiAc/TE (made from a 10X LiAc stock solution containing 1M lithium acetate pH 7. 5 and a 10X TE stock solution containing 0. 1M Tris-HCl, 10mM EDTA pH 7. 5), following by resuspending in 100µl of 1X LiAc/TE. 5-10µg of DNA (linearised or circular) and 50µg of salmon sperm carrier DNA (Stratagene) were added to the cells and incubated at room temperature for 10 minutes. 240µl of PEG/LiAC/TE solution (made from a 10X LiAc stock solution, a 10X TE stock solution and a 50% PEG 4000 stock solution) containing 40% PEG was added to the cells and the mixture was incubated at 30°C for 30 minutes. 42µl of dimethyl sulfoxide (DMSO) was added prior to heat-shock at 42°C. Cells were then rinsed one with sterile water and plated on selective agar plate.

2.2 Molecular methods

2. 2. 1 Standard recombinant DNA techniques

Escherichia coli XL1-Blue strain was used in this study. Calcium-mediated chemical transformation method or electroporation using Biorad™ *E. coli* pulser

was used to transform *E. coli* with plasmid DNA. DNA preparation and purification were performed using kits from Qiagen.

2.3 Cell biology and microscopy

2. 3. 1 Cell fixation

Cells were gown in appropriate media till mid exponential phase (OD_{595} = 0. 3-0. 5), fixed with 3.7% formaldehyde at growing temperature for 10 minutes to 30 minutes. Fixed cells were then rinsed thrice with phosphate-buffered saline (PBS) and were ready for staining.

2. 3. 2 Nuclei, F-actin and cell wall staining

To stain the cell wall, aniline blue, a dye that specifically bind 1,3-β-glucan, were added to the formaldehyde-fixed cells to a final concentration of 0. 5 μg/ml. To stain DNA and F-actin, fixed cells were permeabilized with PBS solution containing 1% Triton-X 100 for 1 minute, following by thrice rinsing with PBS. 1μg/ml 4',6-diamidino-2-phenylindole (DAPI) were added to the cell suspension to visualize the nuclei. Alexa-488 conjugated phalloidin at final concentration of 100ng/ml were used to stain the F-actin.

2. 3. 3 Immunofluorescence staining

Fixed cells were first rinsed once with PBS solution containing 1. 2M sorbitol and then suspended in 140µl PBS solution containing 1. 2M sorbitol. 60µl protoplasting enzyme mixture containing 5mg/ml lysing enzyme (Sigma) and 3mg/ml zymolyase (made in PBS contaning 1. 2 M sorbitol) were added to the cell suspension. The protoplast process were monitored under light microscope every 1-5 minutes by mixing small amount of cell suspension with sodium dodecyl sulfate (SDS) on glass slides. The protoplast process was stopped by adding 1ml of PBS solution containing 1% Triton-X 100. Protoplasted cells were harvested by centrifugation and rinsed three times with PBS and once with PBAL blocking solution (PBS + 10% BSA, 100 mM lysine hydrochloride, 50 µg/ml carbenicillin and 1 mM sodium azide), following by one hour blocking in PBAL solution. Then cell suspension was incubated with primary antibodies for three hours at room temperature or overnight at 4^{o}C. After three times washed with PBAL, cells were incubated in dark with flurochrome-conjugated secondary antibodies for one hour at room temperature. Before observed under microscope, stained cells were rinsed thrice with PBS. Anti-GFP, anti-Cdc4p antibodies and anti-TAT1 antibodies were used at a dilution of 1:1000, 1:200 and 1:100 respectively to individually visualized GFP tagged protein, Cdc4p and microtubules. Flurochrome-conjugated secondary antibodies were used at a dilution of 1:200.

2. 3. 4 Fluorescence microscopy

Staining with DAPI, aniline blue, Alexa 488-conjugated phalloidin and antibodies was carried out as described above. Images were captured using an Olympus IX71 microscope with a Plan Apo 100x/1.45 NA oil lens, equipped with a camera (CoolSnap ES; Photometrics) and MetaMorph software (version 6.2r6; MDS Analytical Technologies)..

2. 3. 5 Time-lapse microscopy

Live cell imaging methods were performed as described in Tran et al 2004 (Tran et al., 2004). Cells were gown in appropriate media till mid exponential phase. 1ml cells were then harvested by centrifugation and resuspended in 5µl -10µl fresh medium. 1µl cells were then mounted onto a concaved glass slide with 1. 2% agarose pad (prepared with the appropriate media), covered gently with a coverslip. Prior to imaging, the edge of coverslip were sealed with VALAP (vaseline/lanolin/paraffin, 1:1:1) to prevent evaporation. Septum formation and GFP-Atb2p time-lapse images were captured by using an Olympus 1X71 microscope. Time-lapse images of Rlc1p-GFP and Cdc15p-GFP were acquired by a Zeiss LSM 510 confocal microscope equipped with 100X1. 3NA PlanApo objective lens. Temperature was controlled by using an objective heater (objective heater controller, Bioptech). Movies were assembled into Qicktime format by metaphorgh software.

2. 3. 6 Confocal microscopy

To image GFP fluorescence, cells were observed on a Zeiss LSM 510 confocal microscope equipped with a 63X/1. 4NA PlanApo objective lens. Images were collected in 3D mode with 0. 6 μm step size and were shown by maximal projection. All confocal images were analyzed and processed by LSM image browser and Metamorph software.

2. 3. 7 Fluorescence recovery after photobleaching (FRAP) assay

cdc16-116 Rad24p-GFP cells were grown at 24^{o}C and shifted up to 36 ^{o}C for 1 hour. Cells were stained with aniline blue and mounted as described above. Photobleaching was performed on a Zeiss LSM 510 laser scanning confocal microscope, equipped with a 63X/1. 4NA PlanApo objective lens. An Argon/Krypton laser with ~ 10 mW at 488 nm was used for imaging (0. 05% power) and photobleaching (100% power). A long pass 505 nm filter was used for visualizing cells. Images were collected in 3D mode with 0. 6μm step size and 2. 5 minutes intervals. The fluorescence intensities of cells before and after photobleaching were measured by using the MetaMorph imaging software. Background fluorescence were corrected by deducing background signals from measured intensities. The $t_{1/2}$ recovery was determined graphically from the bleach recovery plots as the time corresponding to 50% intensity recovery.

3. Polarity Determinants Tea1p, Tea4p, and Pom1p Inhibit Division Septum Assembly at Cell Ends in Fission Yeast

3. 1 Introduction

Correct positioning of the cell division plane is crucial for cellular integrity and function. The mechanisms positioning the plane of cell division have been intensively studied in prokaryotic and eukaryotic cells (Balasubramanian et al., 2004; Glotzer, 2004). Recent studies have provided evidence for overlapping stimulatory and inhibitory mechanisms involved in cleavage plane specification in *Caenorhabditis elegans* (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003; Glotzer, 2004; Motegi et al., 2006). Whether such overlapping mechanisms coexist in other organisms has not been addressed. In addition, detailed molecular mechanisms underlying the stimulatory and inhibitory mechanisms involved in division site selection are not well understood.

In *S. pombe*, the actomyosin ring is assembled at the medial region of the cell upon entry into mitosis and is positioned overlying the region that was occupied by the interphase nucleus (Chang et al., 1996; Sohrmann et al., 1996). This mechanism ensures medial assembly of the division septum. Positioning of the actomyosin ring upon entry into mitosis depends on the anillin-related protein Mid1p / Dmf1p (Chang et al., 1996; Sohrmann et al., 1996). The polo-family protein kinase Plo1p is required for nuclear export of Mid1p upon entry into mitosis and is also important for division

site placement (Bahler et al., 1998; Paoletti and Chang, 2000). Division site selection also depends on the cell end localized polarity factor Pom1p (Bahler and Pringle, 1998).

In this chapter, we present evidence for an inhibitory mechanism that prevents division septum assembly at cell ends. This mechanism, which we refer to as tip-occlusion, requires the function of cell end localized proteins Tea1p, Tea4p, and Pom1p (tip complex). The tip-complex might regulate function of the FER / CIP (FCH) domain protein Cdc15p (Carnahan and Gould, 2003; Fankhauser et al., 1995) to achieve tip-occlusion.

3. 2 Results

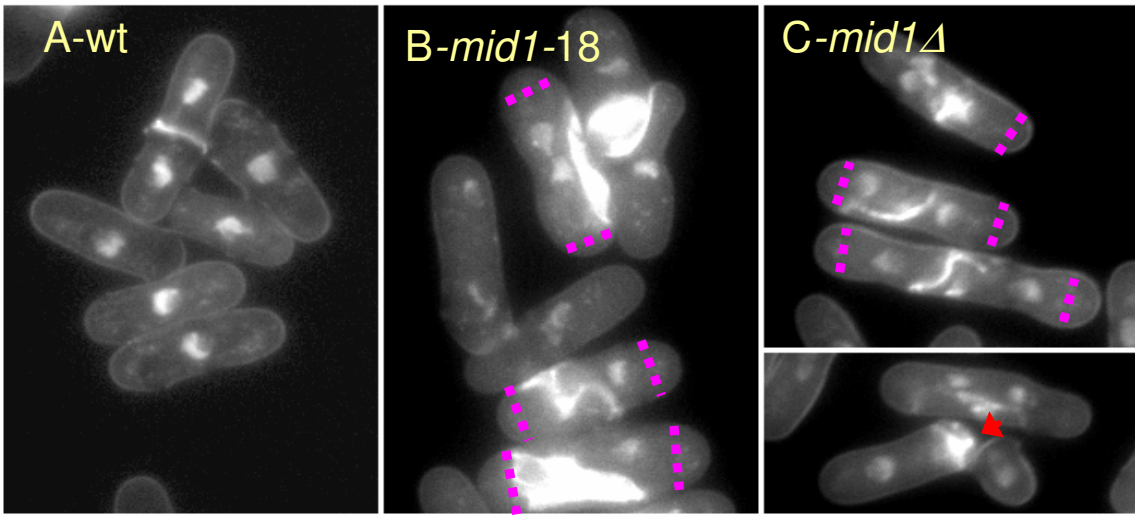
3. 2. 1 Division septa in mid1-defective cells are occluded from cell ends

In order to gain a better understanding of the role of Mid1p in division site selection we reinvestigated the phenotype of the *mid1*-18 (a temperature-sensitive allele) and *mid1*Δ mutants. As previously described (Balasubramanian et al., 1998; Chang et al., 1996; Sohrmann et al., 1996), division septa were misplaced and /or tilted with a small proportion traversing the long axis of *mid1*-18, but not wild-type cells cultured at 36°C (Figure 1 A and B). Interestingly, aniline blue staining of cell wall β-glucans revealed that the division septa rarely assembled at the hemispherical part of the cell ends in *mid1*-18 and *mid1*Δ cells (Figure 1B and C; hemispherical ends demarcated

Figure 1. Tip-occlusion in *mid1* mutant cells

Cells of the indicated genotypes (listed from A-C) were shifted to 36°C for 3. 5 hours, fixed and stained with DAPI and aniline blue to visualize nuclei and septa respectively. The red arrowhead indicates a tip-septum in *mid1*Δ that persists from a previous cytokinesis event. (D) Quantitation of the septal phenotypes in wild type, *mid1*-18 and *mid1Δ* cells. Cells of the indicated genotypes were shifted to 36°C for 3. 5 hours, fixed and stained with DAPI and aniline blue to visualize nuclei and division septa, respectively. Cells were observed by fluorescence microscopy. At least 500 cells were counted. Scale bar, 5 μm.

Figure 1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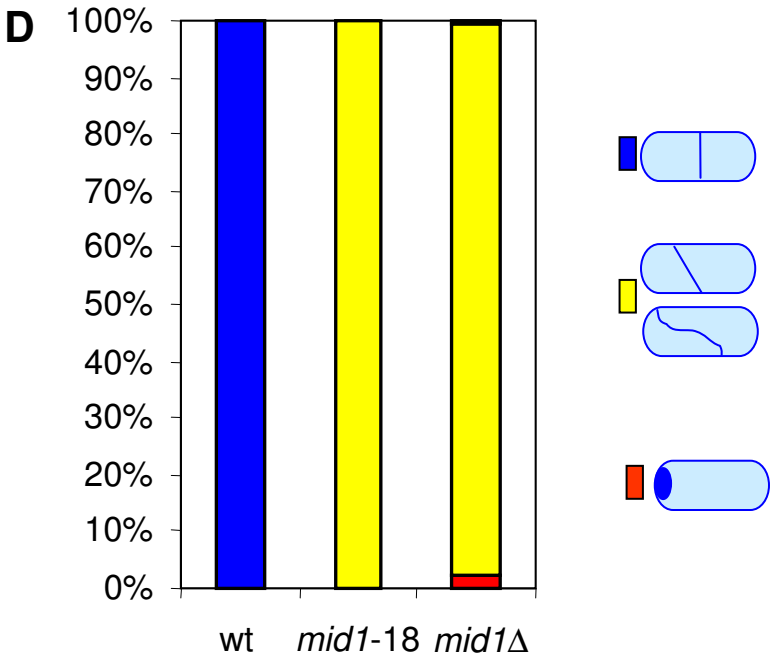
B-mid1-18
C-mid1Δ


D
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
wt
mid1-18
mid1Δ

with a dotted line and within 2 μm from cell ends). Almost 100% *mid1*-18 cells assembled division septa away from the cell ends. Less than 5% *mid1*Δ cells deposit septum material at the hemispherical part of cell ends (Figure1 D). Septal deposits observed at the cell ends in these cells appeared to have arisen from incomplete resolution of septa from the previous cell cycle (marked with an arrowhead in figure 1C). Tip-occlusion of division septa was also observed in *mid1*-366, *mid1*-42, *mid1*-C1 and *mid1*-C2 (data not shown). These observations suggested that although Mid1p is important for septum positioning, additional inhibitory mechanisms, which we term as tip-occlusion, prevent septum assembly at the hemispherical cell ends.

3. 2. 2 Polarity factors Tea1p, Tea4p, and Pom1p are required for tip-occlusion

We hypothesized that some cell end localized factors might be responsible for tip-occlusion. Although several cell end-localized polarity determinants have been identified, none of them, with the exception of Pom1p (Bahler and Pringle, 1998), have been described to affect division site selection (Figure 2A and J). We therefore tested whether loss of cell end localized molecules in conjunction with loss of Mid1p leads to deposition of the division septa at the cell ends. To this end, we created double mutants lacking *mid1* and one of six cell end localized polarity factors, *tea1 (*kelch-repeat protein), *tea2 (*plus end directed kinesin), *tea4/ wsh3* (SH3-domain protein), *pom1* (Dyrk-family protein kinase), *mod5* (Tea1p receptor), and *bud6* (related to actin-binding protein AIP3) (Arellano et al., 2002; Bahler and Pringle, 1998; Browning et al., 2000; Glynn et al., 2001; Martin et al., 2005; Mata and Nurse,

1997; Snaith and Sawin, 2003; Tatebe et al., 2005). These polarity mutants were chosen since cells lacking Pom1p, a key regulator of this polarization apparatus, misplace the division site.

Double mutants of the genotypes *mid1*-18 *tea1*Δ, *mid1*-18 *pom1*Δ (Bahler and Pringle, 1998), and *mid1*-18 *tea4*Δ were inviable at 36°C, a temperature at which all the single mutants could proliferate (data not shown). A synthetic lethal interaction was also detected at lower temperatures, such as at 24°C, when null mutants of *mid1* (*mid1*Δ) were combined with tip-complex mutants (data not shown). Interestingly, division septa were detected at the hemispherical cell ends in the vast majority of *mid1*-18 *tea1*Δ, *mid1*-18 *tea4*Δ, and *mid1*-18 *pom1*Δ cells (Figure 2B, C, D, J). Two major defects were observed in these double mutants (Figure 2J). First, cells were found to assemble one or two septa at the extreme cell ends (Figure 2 B, C, D; marked with green arrows). Second, division septa that appeared to be anchored to one or both cell ends and traversed the long axis were observed (Figure 2 B,C,D; marked with green asterisk). Such phenotypes were not detected in any of the single mutants, with the exception of *pom1*Δ, and were less frequently detected in *mid1*-18 *bud6*Δ, *mid1*-18 *tea2*Δ, and *mid1*-18 *mod5*Δ double mutants (Figure 2A,E, and Figure 2J). Collectively, these experiments suggested that Tea1p, Tea4p and Pom1p function to prevent division septum assembly at the cell ends in cells defective for Mid1p. The weak effect observed with *tea2*Δ, *bud6*Δ, and *mod5*Δ might suggest that these proteins are not critical for tip-occlusion, but might enhance this process. Tea1p and

Figure 2. Tip-complex proteins inhibit cell division at cell ends.

Cells of the indicated genotypes (listed from A-F) were shifted to 36°C for 3. 5 hours, fixed and stained with DAPI and aniline blue to visualize nuclei and septa respectively. Green arrows identify cells with septa at tips, while green asterisks identify cells with septa that span the long axis. (G) Time course analysis of septum assembly. *mid1*-18 and *mid1*-18 *tea1*Δ cells were grown at 24°C, synchronized by incubation in hydroxyurea (HU) for 6 hours, and shifted to 36°C following removal of HU. Cells were fixed at various time points and stained with aniline blue. The graph shows the percentage of cells with tip-associated septa. (H-I). Membrane organization in *mid1*-18 and *mid1*-18 *tea1*Δ cells. Cells of the indicated genotypes were shifted to 36°C, and stained for membrane sterols with filipin. Red asterisks identify tip-anchored membrane invaginations and septa. Scale bar, 5 μm. (J) Quantitation of the septal phenotypes in various septum positioning and polarity mutants. Cells of the indicated genotypes were shifted to 36^{o}C for 3. 5 hours, fixed and stained with DAPI and aniline blue to visualize nuclei and division septa, respectively. Cells were observed by fluorescence microscopy. At least 500 cells were counted. (K). Quantitation of septal phenotypes in synchronous *mid1*-18 and *mid1*-18 *tea1*Δ cells. Cells were synchronized at 24°C by incubation in hydroxyurea (HU) for 6 hours and shifted to 36°C following removal of HU. At the indicated time points, cells were fixed and stained with aniline blue to visualize septa.

Figure 2

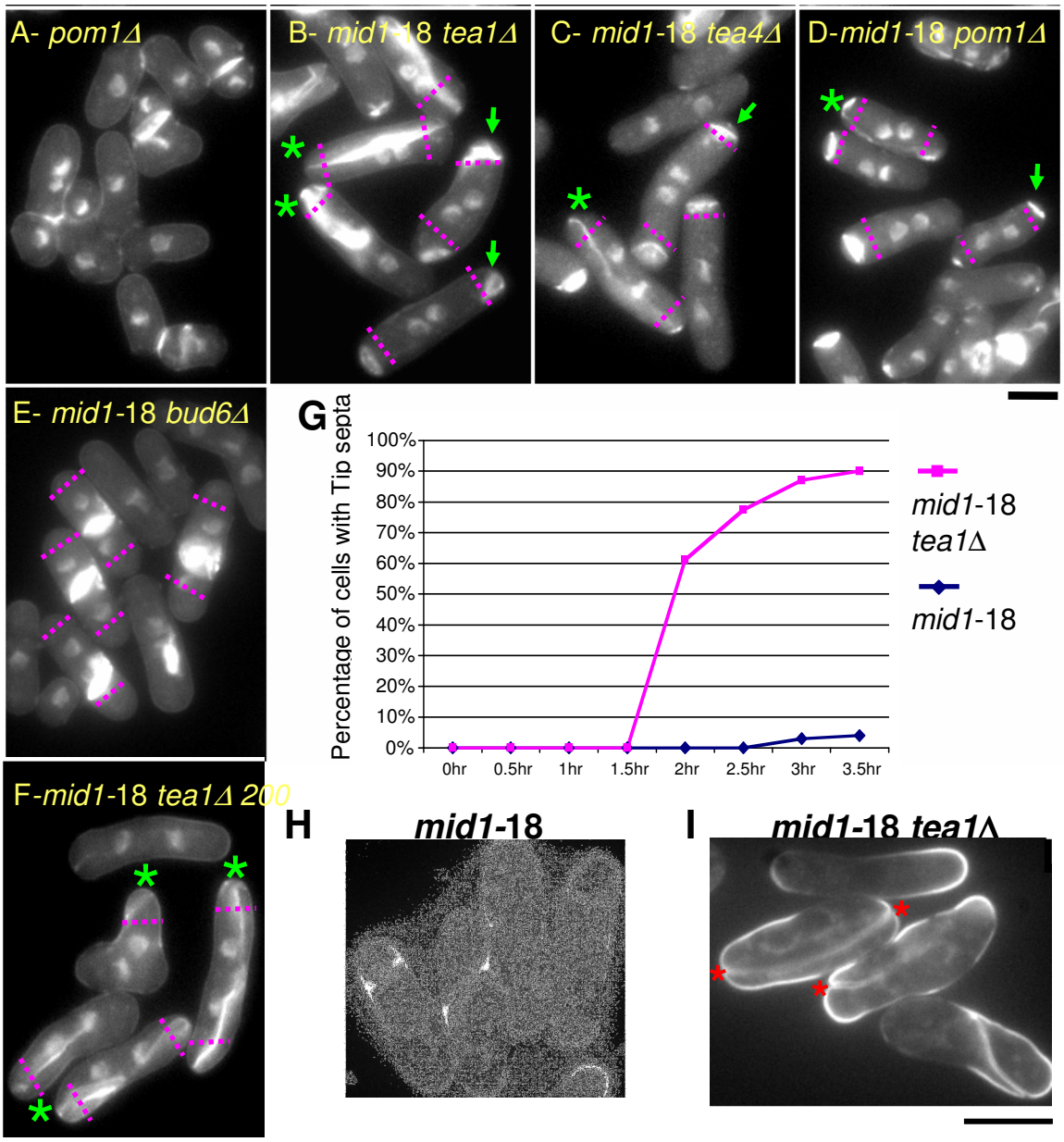
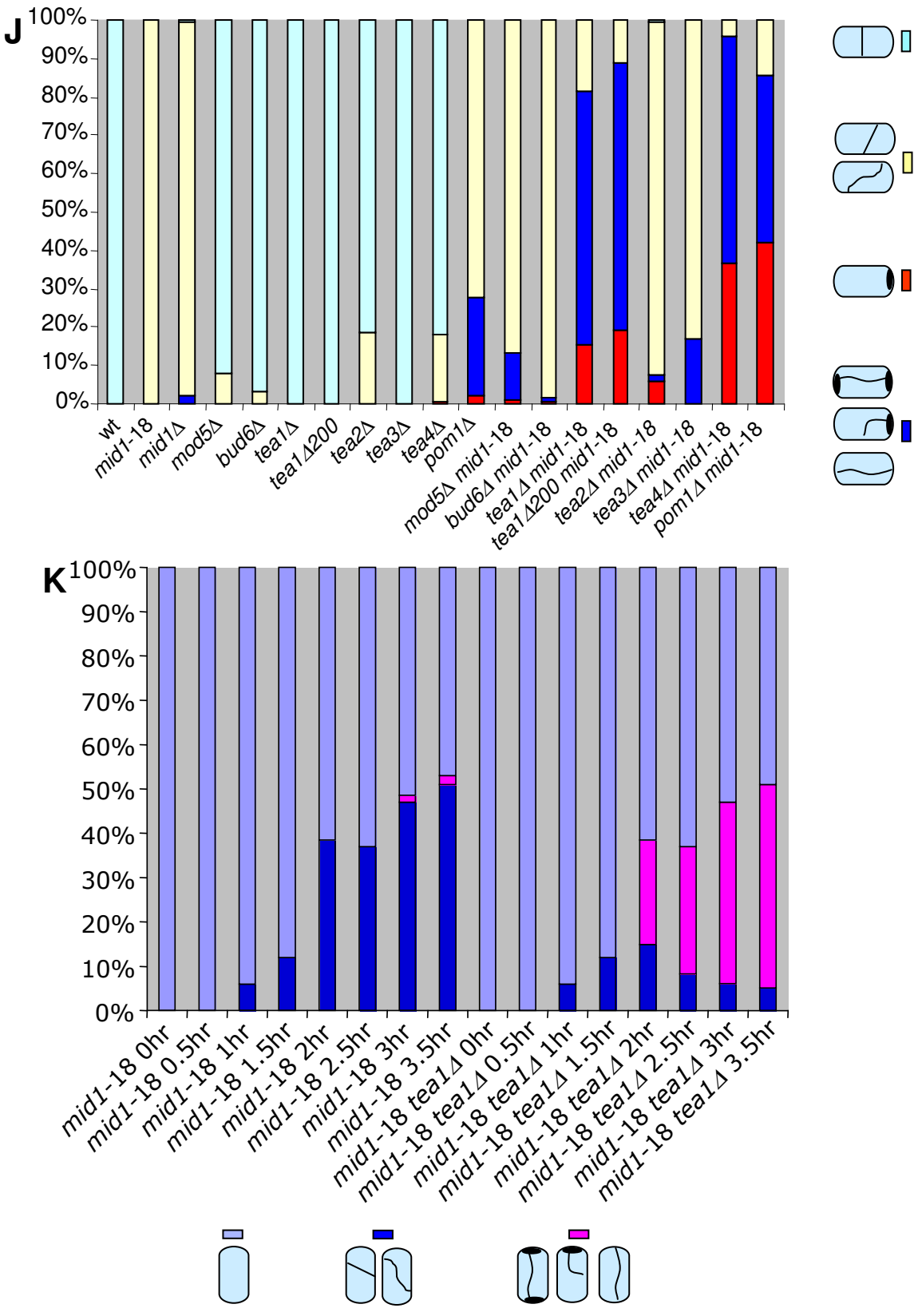

A- pom1Δ
B- mid1-18 tea1Δ
C- mid1-18 tea4Δ
D-mid1-18 pom1Δ
E- mid1-18 bud6Δ
F-mid1-18 tea1Δ 200
G
Percentage of cells with Tip septa
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0hr
0.5hr
1hr
1.5hr
2hr
2.5hr
3hr
3.5hr
mid1-18 tea1Δ
mid1-18
H
mid1-18
I
mid1-18 tea1Δ

Figure 2


J
100%
90%
80%
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30%
20%
10%
0%
wt
mid1-18
mid1Δ
mod5Δ
bud6Δ
tea1Δ
tea1Δ200
tea2Δ
tea3Δ
tea4Δ
pom1Δ
mod5Δ mid1-18
bud6Δ mid1-18
tea1Δ mid1-18
tea1Δ200 mid1-18
tea2Δ mid1-18
tea3Δ mid1-18
tea4Δ mid1-18
pom1Δ mid1-18
K
100%
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60%
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mid1-18 0hr
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mid1-18 1.5hr
mid1-18 2hr
mid1-18 2.5hr
mid1-18 3hr
mid1-18 3.5hr
mid1-18 tea1Δ 0hr
mid1-18 tea1Δ 0.5hr
mid1-18 tea1Δ 1hr
mid1-18 tea1Δ 1.5hr
mid1-18 tea1Δ 2hr
mid1-18 tea1Δ 2.5hr
mid1-18 tea1Δ 3hr
mid1-18 tea1Δ 3.5hr

Tea4p are detected at the cell ends as well as at the growing ends of microtubules (Martin et al., 2005; Mata and Nurse, 1997). Additional experiments with an allele of Tea1p that fails to localize to cell ends (Figure 2 F) suggested that the cell-end localized Tea1p might be more important for tip-occlusion.

It was possible that some of the tip-septa observed in *mid1*-18 *tea1*Δ cells might have persisted from a previous cytokinesis event. To test this idea, synchronous cultures of *mid1*-18 and *mid1*-18 *tea1*Δ were shifted to 36°C and the number of cells with tip-septa was quantitated (Figures 2G, K). Over 85% of *mid1*-18 *tea1*Δ cells assembled tip-septa after shift to the restrictive temperature, while tip-septa were not detected in *mid1*-18 cells. These and other studies described in subsequent sections established that the septa were indeed assembled at the cell ends in *mid1*-18 *tea1*Δ cells.

In order to ascertain if the septa at the cell ends formed as a result of membrane invagination and septum synthesis therein (as opposed to an excessive deposition of septum material around the cell ends, without any physical connection to the cell ends), *mid1*-18 and *mid1*-18 *tea1*Δ cells were stained with the membrane-sterol binding dye filipin (Wachtler et al., 2003). Membrane invaginations were not observed at the cell ends in *mid1*-18 single mutants (Figure 2H), but were clearly visualized in *mid1*-18 *tea1*Δ cells (Figure 2I; invaginations marked with asterisks). These experiments indicated that the tip-associated septa are likely to have resulted from the constriction of an actomyosin ring at the vicinity of the cell end and concomitant formation of tip-anchored septa.

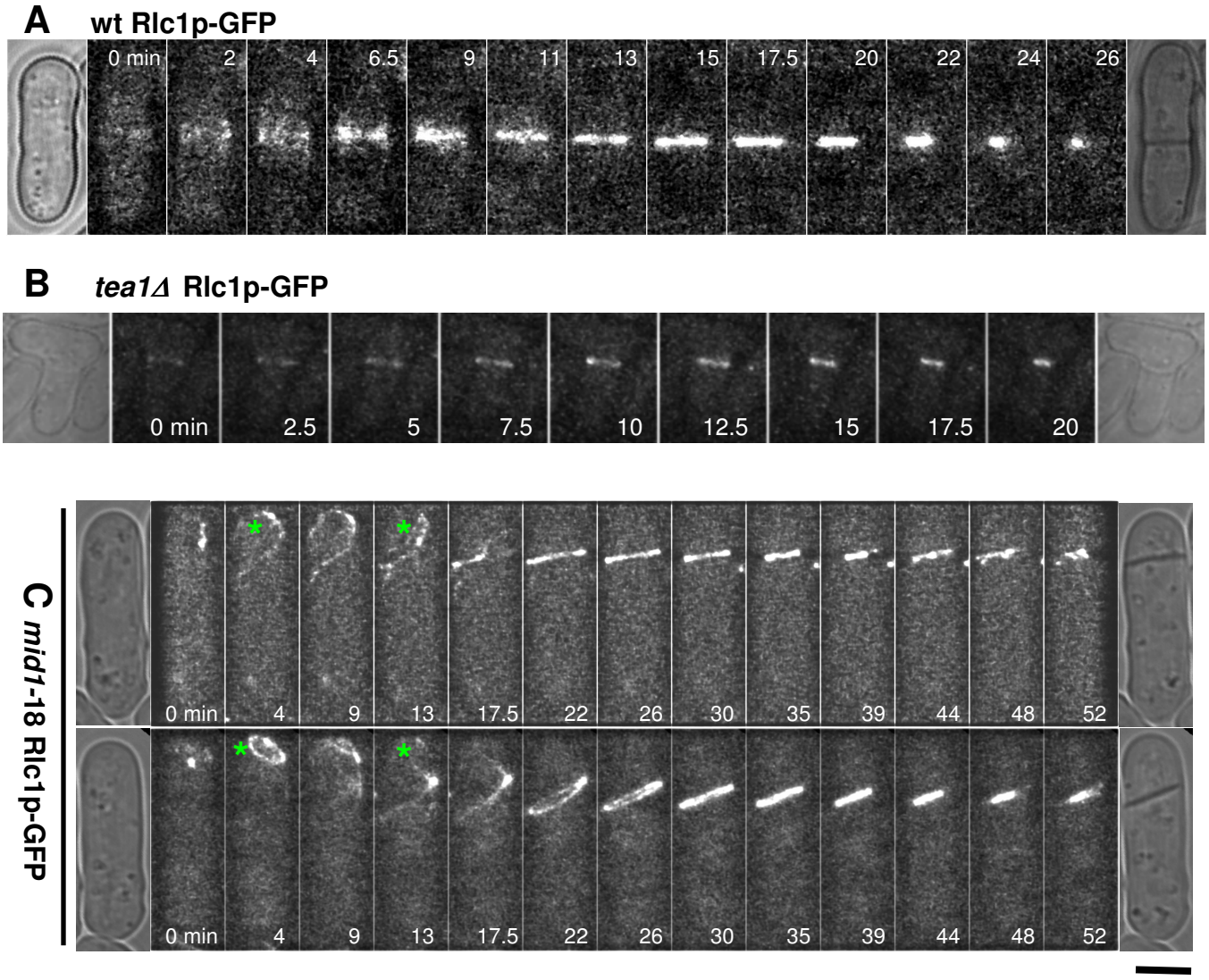
3. 2. 3 The tip-complex prevents division septum assembly at cell ends

In order to understand the mechanism of tip-occlusion, we first imaged the dynamics of the actomyosin ring in wild-type and mutant cells, using GFP-tagged myosin regulatory light chain (Rlc1p-GFP) as a marker. Wild-type (Figure 3A) and *tea1*Δ (Figure 3B) cells assembled medial actomyosin rings. Actomyosin rings were seen to assemble away from the medial region of the cell (in the vicinity of the cell ends; marked with green asterisks) in the *mid1*-18 and *mid1*-18 *tea1*Δ double mutants (Figure 3C, D). Actomyosin rings that assembled near the ends of *mid1*-18 cells (Figure 2C; cell end associated rings marked with asterisks) eventually migrated away from the ends and septation did not occur at the cell ends in these cells. In contrast, non-medial actomyosin rings in *mid1*-18 *tea1*Δ cells appeared to remain at the hemispherical cell ends and trailed the long axis (> 80%, n=26; three examples shown in figure 3D) leading eventually to its constriction in the long axis (Figure 3D; see the position of arrowheads, relative to cell ends). In some instances, particularly evident in *mid1*-18 *tea4*Δ cells (Figure 3E), and less frequently in *mid1*-18 *tea1*Δ cells (data not shown), actomyosin rings assembled at the cell ends and underwent constriction immediately without trailing the long axis of the cell, which might have resulted from a relatively more rapid compaction of the ring. Such a constriction event led to a tightly organized septum at the cell end. Collectively, these experiments suggested that under conditions of actomyosin ring assembly near the cell ends, tip-complex polarity factors prevent retention of the actomyosin rings and septum assembly at the cell ends.

Figure 3. Actomyosin ring retention at the cell ends in the absence of Mid1p and tip-complex proteins.

Wild type (A) and *tea1Δ* (B) cells expressing Rlc1p-GFP were imaged by confocal microscopy. Shown are wild-type and *tea1Δ* cells assembling actomyosin rings in the medial region of the cell. In the *mid1*-18 panel (C), asterisks denote assembly of actomyosin rings near cell ends, whereas the arrowheads in the *mid1*-18 *tea1*Δ panel (D) indicate ring constriction relative to the cell ends. *mid1*-18 *tea4Δ* cells (E) expressing Uch2p-GFP (nuclear marker) and Rlc1p-GFP were shifted to 36^{o}C for 2. 5 hours and imaged by confocal microscopy. Asterisks indicate the actomyosin ring at the cell end, which undergoes constriction. Scale bar, 5µm.

Figure 3


A
wt Rlc1p-GFP
0 min
2
4
6.5
9
11
13
15
17.5
20
22
24
26
B
tea1Δ Rlc1p-GFP
0 min
2.5
5
7.5
10
12.5
15
17.5
20
C mid1-18 Rlc1p-GFP
0 min
4
9
13
17.5
22
26
30
35
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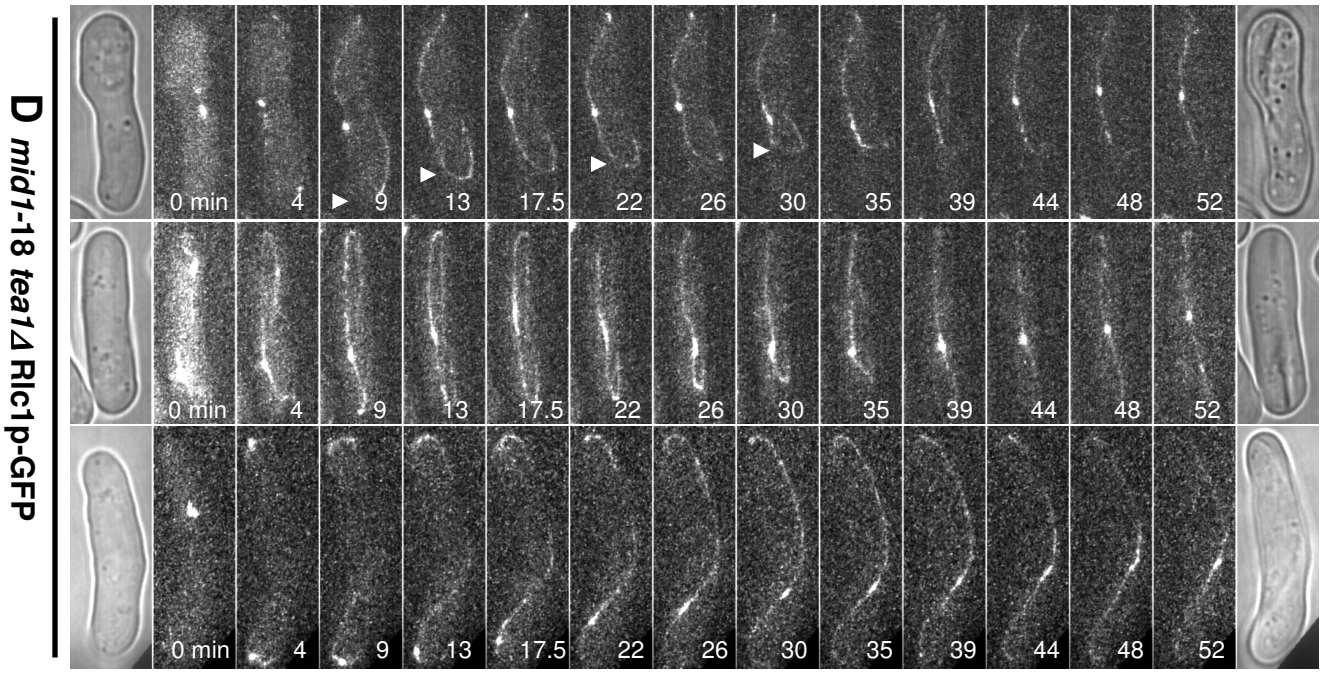
44
48
52

Figure 3


D mid1-18 tea1Δ Rlc1p-GFP
0 min
4
9
13
17.5
22
26
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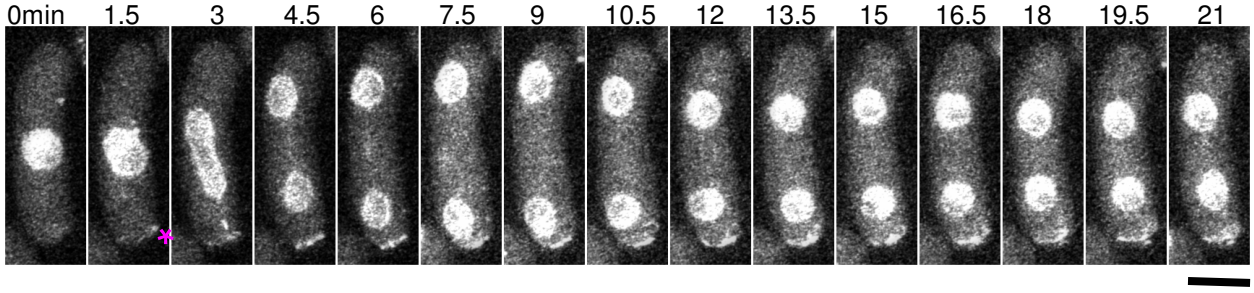
35
39
44
48
52


E mid1-18 tea4Δ
0min
1.5
3
4.5
6
7.5
9
10.5
12
13.5
15
16.5
18
19.5
21

In *S. pombe*, assembly of the division septum is linked to constriction of the actomyosin ring. Recent studies have shown that in the absence of septum assembly, fully formed actomyosin rings can migrate to different locations (Pardo and Nurse, 2003), suggesting that the division septum "anchors" the constricting actomyosin ring to the division plane. It was therefore possible that the migration of actomyosin rings away from cell ends was a result of tip-complex mediated inhibition of division septum assembly at cell ends in *mid1*-18 cells. Division septum assembly requires function of Cps1p (Liu et al., 2002), an integral membrane protein that localizes to the vicinity of the actomyosin ring. We therefore compared the localization of GFP-Cps1p to that of Rlc1p-GFP in *mid1*-18, and *mid1*-18 *tea1*Δ cells (Figure 4A and B). Whereas Rlc1-GFP was readily detected in cable-like structures at the cell ends in *mid1*-18 and *mid1*-18 *tea1*Δ cells, GFP-Cps1p was detected in cables at the cell ends in *mid1*-18 *tea1*Δ cells, but not in *mid1*-18 cells. Time-lapse imaging of GFP-Cps1p failed to provide evidence for the presence of Cps1p in cables in the vicinity of the cell ends in *mid1*-18 cells, although such cables were readily visualized in *mid1*-18 *tea1*Δ cells (Figure 4C and D).

We then characterized the formation of division septa in *mid1*-18 and *mid1*-18 *tea1*Δ cells by time-lapse microscopy. To this end, wild-type, *mid1*-18 (Figure 5A, B) and *mid1*-18 *tea1*Δ cells (Figure 5C) were grown in the presence of aniline blue, a β-glucan binding compound. Division septa did not assemble at the cell ends in *mid1*-18 cells. In the majority of *mid1*-18 *tea1*Δ cells (9/12), however, division septa preferentially initiated at the cell ends and subsequently spanned the long axis (three examples shown in Figure 5C). In one case, a tip-assembled septum that did not trail the long axis was

Figure 4. Investigation of the localization of Rlc1p and Cps1p at cell ends in *mid1*-18 and *mid1*-18 *tea1*Δ cells.

(A-B). Panel A shows representative images of Rlc1p-GFP and GFP-Cps1p fluorescence in cable-like structures at the cell ends in *mid1*-18 and *mid1*-18 *tea1*Δ cells cultured at 36°C, while their quantitation is shown in panel B. At least 100 cells were counted for the presence of tip-localized Rlc1p cables and Cps1p cables. (C-D) Analysis of GFP-Cps1p localization by time-lapse microscopy in *mid1*-18 and *mid1*-18 *tea1*Δ cells. *mid1*-18 and *mid1*-18 *tea1Δ* cells expressing GFP-Cps1p were shifted to 36^{o}C for 3 hours and imaged by confocal microscopy. GFP-Cps1p was detected in actomyosin cables at the cell ends in *mid1*-18 *tea1*Δ, but not in wild-type and *mid1*-18 cells. Scale bar, 5μm.

Figure 4

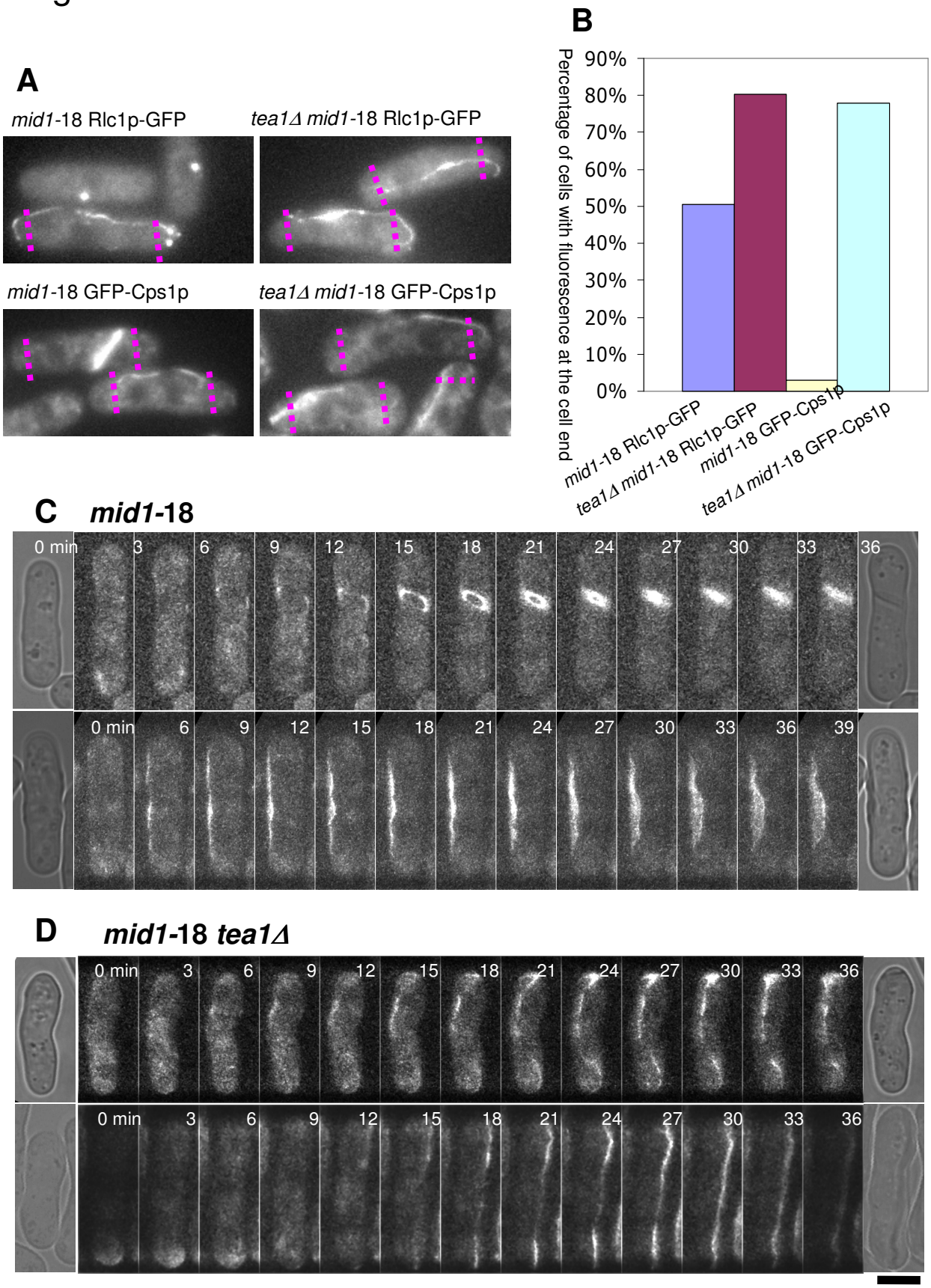
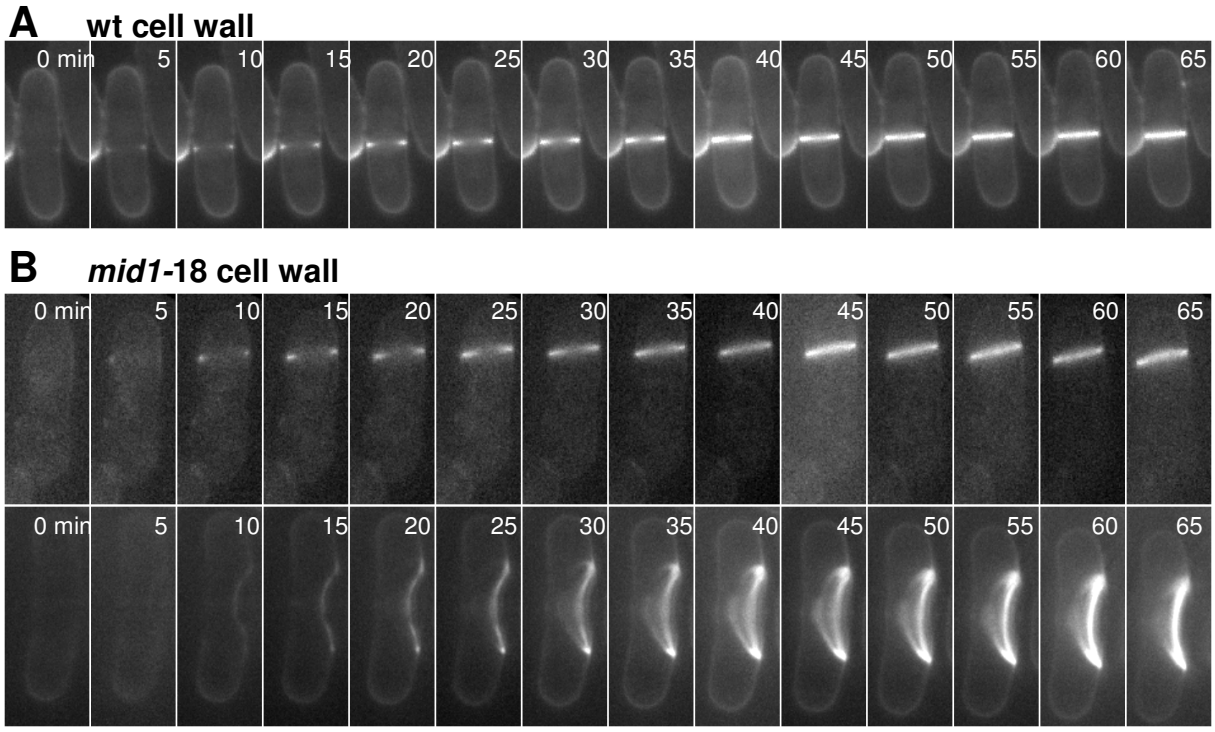
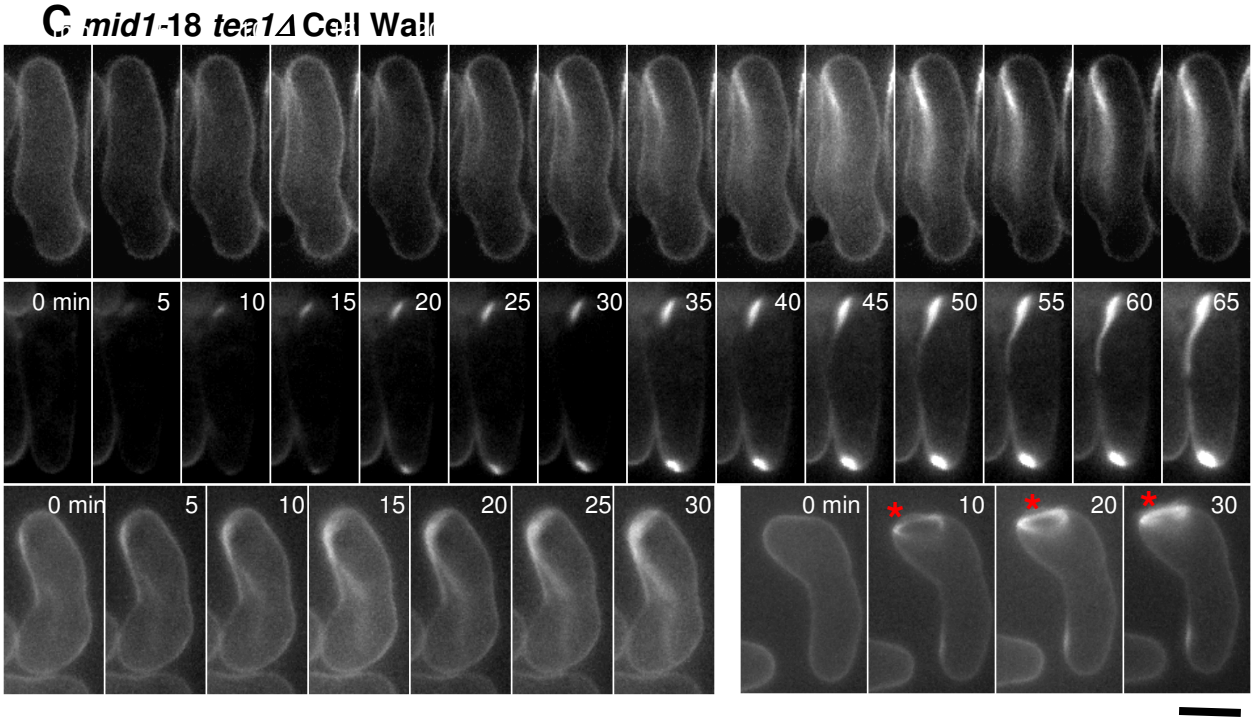

A
mid1-18 Rlc1p-GFP
tea1Δ mid1-18 Rlc1p-GFP
mid1-18 GFP-Cps1p
tea1Δ mid1-18 GFP-Cps1p
B
Percentage of cells with fluorescence at the cell end
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
mid1-18 Rlc1p-GFP
tea1Δ mid1-18 Rlc1p-GFP
mid1-18 GFP-Cps1p
tea1Δ mid1-18 GFP-Cps1p
C mid1-18
0 min 3 6 9 12 15 18 21 24 27 30 33 36
0 min 6 9 12 15 18 21 24 27 30 33 36 39
D mid1-18 tea1Δ
0 min 3 6 9 12 15 18 21 24 27 30 33 36
0 min 3 6 9 12 15 18 21 24 27 30 33 36

Figure 5. Septum assembly at the cell ends in the absence of Mid1p and tip-complex proteins.

Wild type (A), *mid1*-18 (B) and *mid1*-18 *tea1*Δ (C) cells were grown at 24°C and shifted to 36°C for 3 hours. Aniline blue was added to the growth medium and cells were then imaged. Note that division septa did not assemble at the cell ends in *mid1*-18 mutant cells, and assembled at the medial region, as expected, in wild-type cells. *mid1*-18 *tea1*Δ cells assembled aberrant septa at the cell ends. A cell in which the septum does not appreciably trail the long axis is indicated with a red asterisk. Elapsed time is shown in minutes. Scale bar, 5µm.

Figure 5


A wt cell wall
0 min 5 10 15 20 25 30 35 40 45 50 55 60 65
B mid1-18 cell wall
0 min 5 10 15 20 25 30 35 40 45 50 55 60 65
0 min 5 10 15 20 25 30 35 40 45 50 55 60 65


C mid1-18 tea1Δ Cell Wall
0 min 5 10 15 20 25 30 35 40 45 50 55 60 65
0 min 5 10 15 20 25 30
0 min 10 20 30

observed (Figure 5C; cell marked with red asterisk). These experiments revealed that the septa that assemble along the long axis of *mid1*-18 *tea1*Δ cells are preferentially initiated at the cell ends. Collectively, time-lapse imaging revealed that although actomyosin rings are initiated at the cell ends in *mid1*-18 and *mid1*-18 *tea1*Δ cells, localization of Cps1p to actomyosin cables at the cell ends and assembly of the division septa at cell ends are inhibited in the presence of the tip-complex.

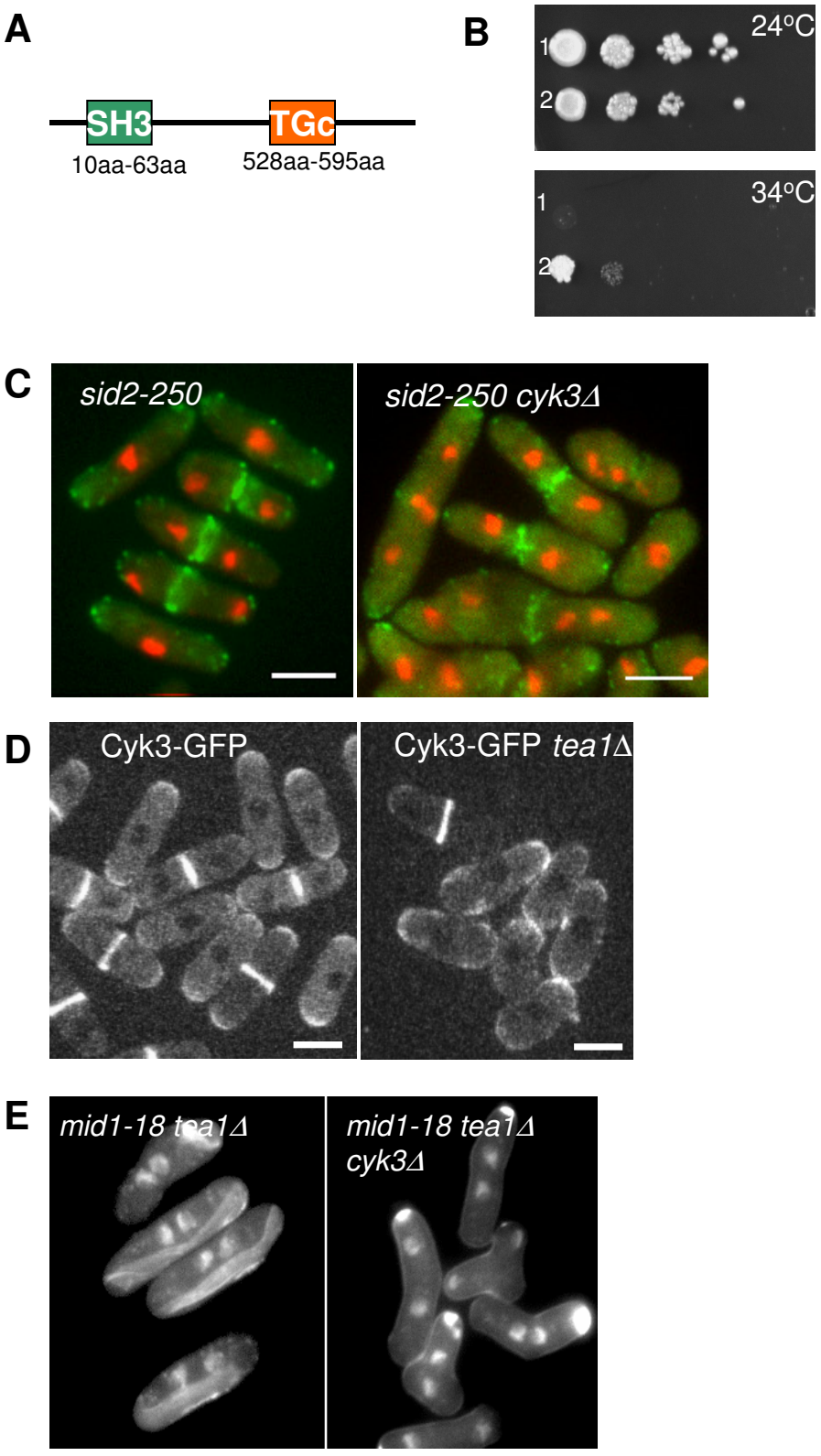
3. 2. 4 Cyk3p, a candidate target of tip-complex

To gain molecular insight into the process of tip occlusion, we carried out a genetic screen to identify proteins that upon overproduction rescued the lethality resulting from simultaneous loss of Mid1p and the tip-complex. Cyk3p, a 886 amino-acid protein with an SH3 and a transglutaminase domain (Figure 6A; encoded by SPAC9G1. 06c) was able to suppress the *mid1*-18 *tea1*Δ mutant for colony formation when expressed from a multi copy plasmid, while control plasmids were unable to do so (Figure 6B). Cells lacking *cyk3* were viable (data not shown). However, a role for Cyk3p in actomyosin ring integrity was observed upon abrogation of the cytokinesis checkpoint in *sid2*-250 mutants (Figure 6C). Figure 6C shows the accumulation of multinucleate *cyk3*Δ *sid2*-250 cells at 25°C, whereas multinucleate cells were not observed in the two parental single mutants under similar conditions. *cyk3* mutants displayed synthetic genetic interactions (data not shown) with mutants defective in Imp2p and Cdc4p, two proteins important for cytokinesis, (Demeter and Sazer, 1998) further suggesting a role for Cyk3p in normal actomyosin ring assembly / function.

Figure 6. Tip-complex might negatively regulate the tip-localized actomyosin ring protein Cyk3p.

(A) Cyk3, an 886 amino acid protein, contains SH3 (10aa-63aa) and Tgc domains (528aa-595aa). (B) Expression of Cyk3p from the multicopy plasmid pALKS suppresses the lethality of *tea1Δ mid1*-18 at 36°C. *tea1Δ mid1*-18 with empty pALKS (1) and pALKS-cyk3 (2) were grown at 24°C and spotted on YES plates with tenfold series dilution and assayed for colony formation at 24°C and 36°C for 4 days. (C) *sid2*-250 *cyk3Δ* double mutants accumulate multiple nuclei at 24°C. (D) Cyk3p-GFP localizes to cell tips and medial ring in wild-type and *tea1*Δ cells. (E) *mid1*-18 *tea1Δ cyk3Δ* assembles reduced tip septa. *mid1*-18 *tea1*Δ and *mid1*-18 *tea1*Δ *cyk3*Δ cells were shifted to 36°C for 3. 5 hrs and stained with DAPI and anilin blue. Note that the triple mutant makes qualitatively less septum and makes significantly less at the cell ends. Scale bar, 5μm.

Figure 6


A
SH3
TGc
10aa-63aa
528aa-595aa
B
24°C
1
2
34°C
1
2
C
sid2-250
sid2-250 cyk3Δ
D
Cyk3-GFP
Cyk3-GFP tea1Δ
E
mid1-18 tea1Δ
mid1-18 tea1Δ
cyk3Δ

Cyk3p localized to growing ends of the cell in interphase and was detected in the actomyosin ring and the cell ends in mitotic wild-type cells (Figure 6D). Cyk3p localization at cell ends was unaffected in *tea1*Δ (Figure 6D), *tea4*Δ, and *pom1*Δ mutants (data not shown), although in all cases more protein was detected at the growing end. Given that Cyk3p localization was independent of Tea1p and that tip-occlusion was still observed in *mid1*-18 *cyk3*Δ cells (data not shown), it appeared that tip-localized Cyk3p might be a target of tip occlusion. To test if this was the case, we created triple mutants defective in Mid1p, Tea1p and Cyk3p (*mid1*-18 *tea1*Δ *cyk*3Δ). Strikingly, strong reduction of septal deposits at cell ends was observed in these strains compared with *mid1*-18 *tea1*Δ cells (Figure 6E). Similarly reduced tip-septa were also observed in triple mutants of the genotypes *mid1*-18 *tea4*Δ *cyk3*Δ and *mid1*-18 *pom1*Δ *cyk3*Δ (data not shown). The reduction of septal material in *mid1*-18 *tea1*Δ *cyk3*Δ, *mid1*-18 *tea4*Δ *cyk3*Δ and *mid1*-18 *pom1*Δ *cyk3*Δ might be result of a partial requirement for Cyk3p in cytokinesis. The effect of Cyk3p overexpression could lead to a reduction of the rate of cytokinesis through other mechanisms, since these cells make normal actomyosin rings and septa. However, given that tip-septa still could be observed at the cell ends in *mid1*-18 *tea1*Δ *cyk*3Δ, Cyk3p is not the sole target of tip-complex.

3. 2. 5 Compromising Cdc15p function restores tip-occlusion in *mid1*-18 *tea1*Δ cells

We then sought to gain a molecular understanding of the mechanism by which the tip complex prevents actomyosin ring retention and division septum assembly at the cell

ends. We focused our attention on the FER / CIP (FCH) domain containing protein Cdc15p, which is essential for actomyosin ring maintenance, Cps1p localization, and division septum assembly (Carnahan and Gould, 2003; Fankhauser et al., 1995). Cdc15p has been shown to localize to cell ends during interphase and to the cell ends and the actomyosin ring during cytokinesis (Figure 7A, B, C (Carnahan and Gould, 2003; Fankhauser et al., 1995). We first assessed if cell end localization of Cdc15p was dependent on the tip complex. Cdc15p was able to localize to the cell ends, both in the presence and absence of tip complex proteins (Figure 7A and B; data shown for *tea1*Δ; not shown for *pom1*Δ and *tea4*Δ). Localization of both N and C-terminally tagged Cdc15p was unaffected in *tea1*Δ cells (Figures 7A, B).

Cdc15p is essential for actomyosin ring maintenance and septum assembly and localizes to actomyosin rings and cell ends. We therefore considered the possibility that the retention of actomyosin rings and assembly of septa at the cell ends, in *mid1*-18 *tea1*Δ cells, might depend on Cdc15p. If this were the case, *mid1*-18 *tea1*Δ cells compromised for *cdc15* function should not be able to assemble septa at cell ends. To test this idea, we sought to create a strain of *mid1*-18 *tea1*Δ in which Cdc15p function was compromised. However, the construction of such a strain was complicated by the fact that Cdc15p is essential for cell viability and that all conditional-alleles abolish the ability of cells to maintain actomyosin rings and assemble septa.

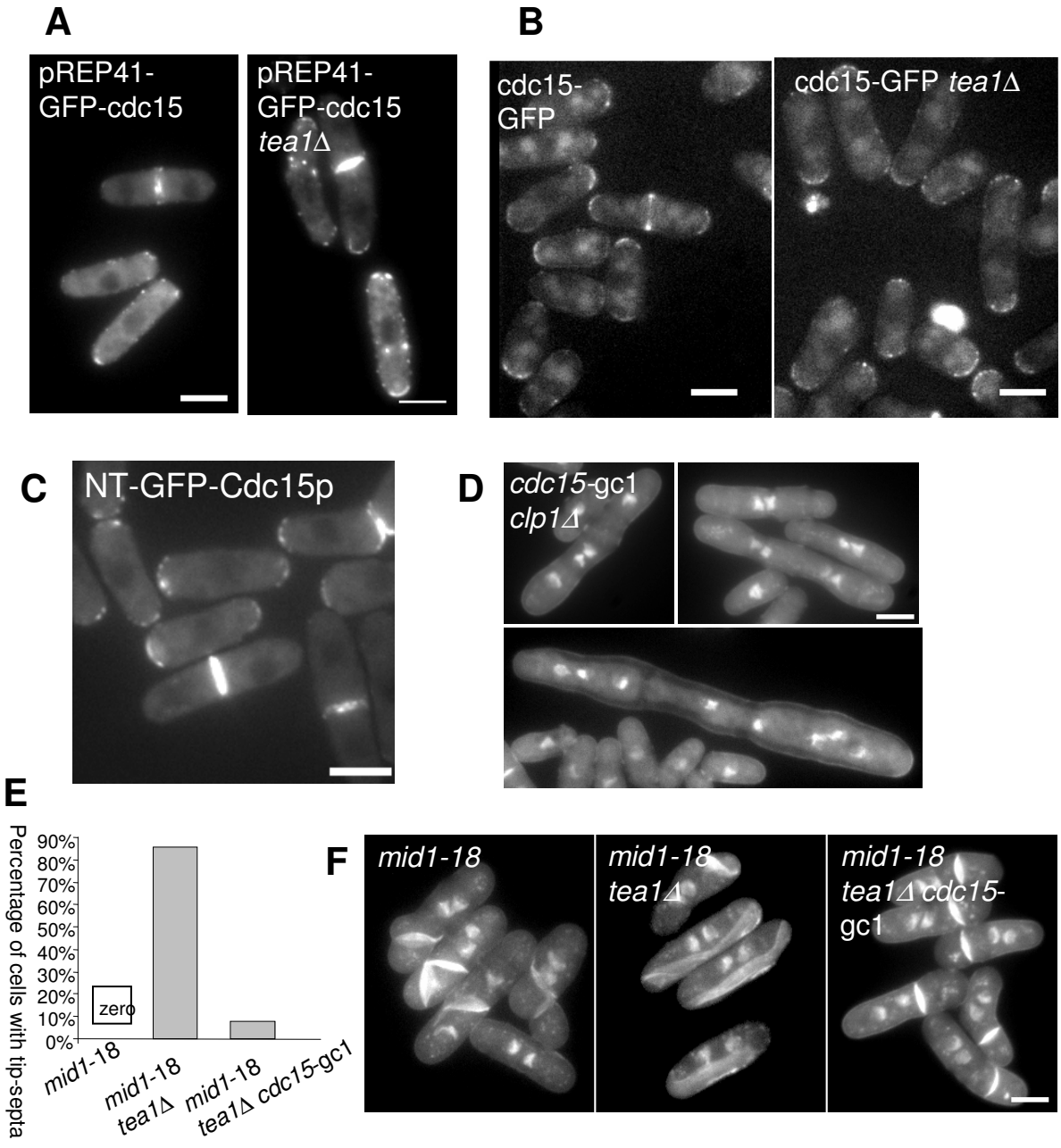
We had independently established that fusion of the green fluorescent protein to the C-terminus of Cdc15p compromised its function in cell division, thereby generating a

hypomorphic allele of *cdc15* (referred to as *cdc15*-gc1). In wild-type asynchronous cultures, approximately 15% cells contain actomyosin rings, whereas an asynchronous culture of *cdc15*-gc1 contains 30% cells with actomyosin rings, indicative of a compromise in the rate of actomyosin ring assembly and / or division septum assembly.

Figure 7. The localization of Cdc15p is independent of Tea1p, and partial loss of Cdc15p function restores tip-occlusion in *mid1*-18 *tea1*Δ.

(A-B) Wild type and *tea1*Δ cells expressing N (A) or C (B) terminally GFP tagged Cdc15p were grown at 30°C and imaged by fluorescence microscopy. (C) N-terminally GFP-tagged Cdc15p is fully functional and localizes to the cell ends and the actomyosin ring. nmt1-GFP-Cdc15p cells were grown in YES medium at 30°C and imaged by fluorescence microscopy. (D) Fusion of GFP to the C-terminus of Cdc15p generates a partially compromised protein and generates the hypomorphic allele, *cdc15*-gc1. The *cdc15*-gc1 *clp1*Δ strain, but not the two parental strains accumulated cells with > 2 nuclei (23 out of 415 cells) and even up to 8 nuclei. (E-F). Restoration of tip-occlusion in *mid1*-18 *tea1*Δ *cdc15*-gc1 cells. *mid1*-18, *mid1*-18 *tea1*Δ, and *mid1*-18 *tea1*Δ *cdc15*-gc1 cells were shifted to 36°C for 4 hours, fixed and stained with DAPI and aniline blue to visualize nuclei and septa respectively. Quantitation of cells with tip-localized septa is shown in panel E and representative images are shown in panel F. Scale bar, 5μm.

Figure 7

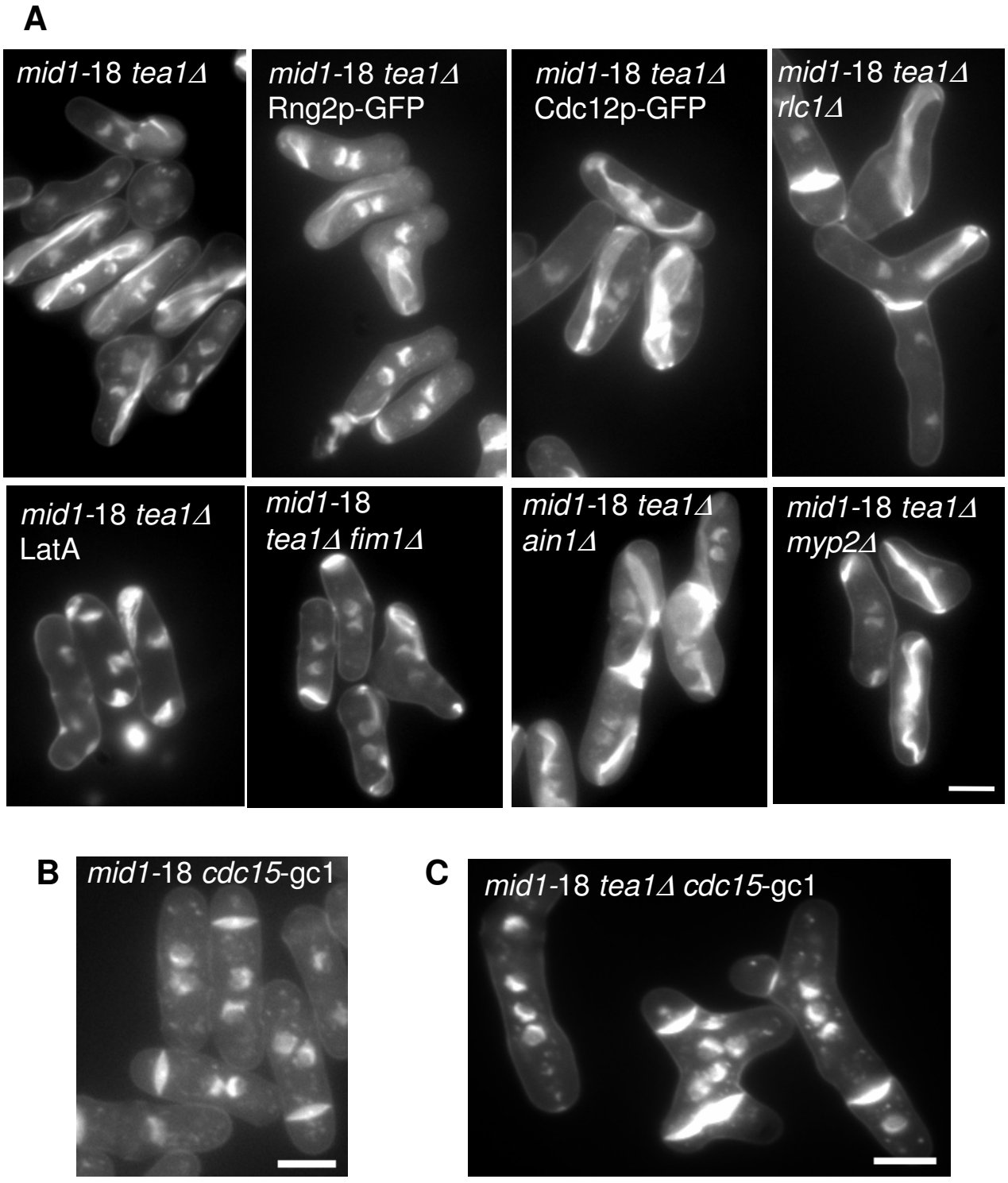

A
pREP41-GFP-cdc15
pREP41-GFP-cdc15 tea1Δ
B
cdc15-GFP
cdc15-GFP tea1Δ
C
NT-GFP-Cdc15p
D
cdc15-gc1 clp1Δ
E
Percentage of cells with tip-septa
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
zero
mid1-18
mid1-18 tea1Δ
mid1-18 tea1Δ cdc15-gc1
F
mid1-18
mid1-18 tea1Δ
mid1-18 tea1Δ cdc15-gc1

This effect is more pronounced, upon removal of the cytokinesis checkpoint protein Clp1p (Mishra et al., 2004). The cytokinesis checkpoint ensures maximal viability of cells compromised for the actomyosin ring and the septum machinery. The *cdc15*-gc1 *clp1*Δ strain, but not the two parental strains accumulated cells with > 2 nuclei (23 out of 415 cells) and even up to 8 nuclei (Figure 7D). With the availability of this *cdc15*-allele, we created a strain of the genotype *mid1*-18 *tea1*Δ *cdc15*-gc1 to assess the role of Cdc15p in septum assembly at cell ends. At the restrictive temperature, as described previously, over 85% of *mid1*-18 *tea1*Δ cells accumulated extensive tip-septa. Interestingly, assembly of septa at cell ends was observed in less than 8 % of the *mid1*-18 *tea1*Δ *cdc15*-gc1 triple mutant and, as expected, was not detected in the *mid1*-18 single mutant (Figure 7E and F). These experiments established that partial loss of Cdc15p function was sufficient to prevent septum assembly at cell ends in *mid1*-18 *tea1*Δ cells, although septum assembly elsewhere was not affected. Suppression of tip-septation was also observed when the HA epitope was placed downstream of Cdc15p (data not shown). However, compromise of function of several other actomyosin ring components (Rlc1p, Fim1p, Ain1p, Myp2p, Rng2p, Cdc12p, F-actin), by deletion of the corresponding genes, by introduction of epitopes at the N or C-termini, or by treatment with drugs, did not restore tip-occlusion in *mid1*-18 *tea1*Δ cells (Figure 8A). Introduction of *cdc15*-gc1 did not correct the septum-positioning defect of *mid1*-18 cells, but corrected the orientation of septa in *mid1*-18 and *mid1*-18 *tea1*Δ cells (Figure 8B). Introduction of *cdc15*-gc1 did not rescue the lethality of *mid1*-18 *tea1*Δ cells, due to the accumulation of multiple nuclei in the same compartment of the cell (Figure 8C).

Figure 8. Restoration of tip-occlusion in *mid1*-18 *tea1*Δ by *cdc15*-gc1, but not by several other cytokinesis mutants.

(A) Tip-occlusion is not restored in *mid1*-18 *tea1*Δ cells, upon loss of various cytokinesis regulatory proteins. Mutant cells of the indicated genotypes were shifted to 36°C for 3. 5 hours and fixed and stained with DAPI and aniline blue. For LatA treatment, *mid1*-18 *tea1Δ* were treated with 0. 2 μm LatA before shift up to 36°C. Note that the phenotype of all these mutant cells resembled that of *mid1*-18 *tea1Δ*. (B) *cdc15*-gc1 corrected the orientation, but not the position, of division septa in *mid1*-18 cells. (C) *mid1*-18 *tea1Δ* *cdc15*-gc1 cells were shifted to 36°C for 6 hours and stained as above. Note that most cells accumulated 4 nuclei in the same compartment, despite restoration of tip-occlusion, due to abnormal patterns of growth and septation. Scale Bar, 5μm.

Figure 8

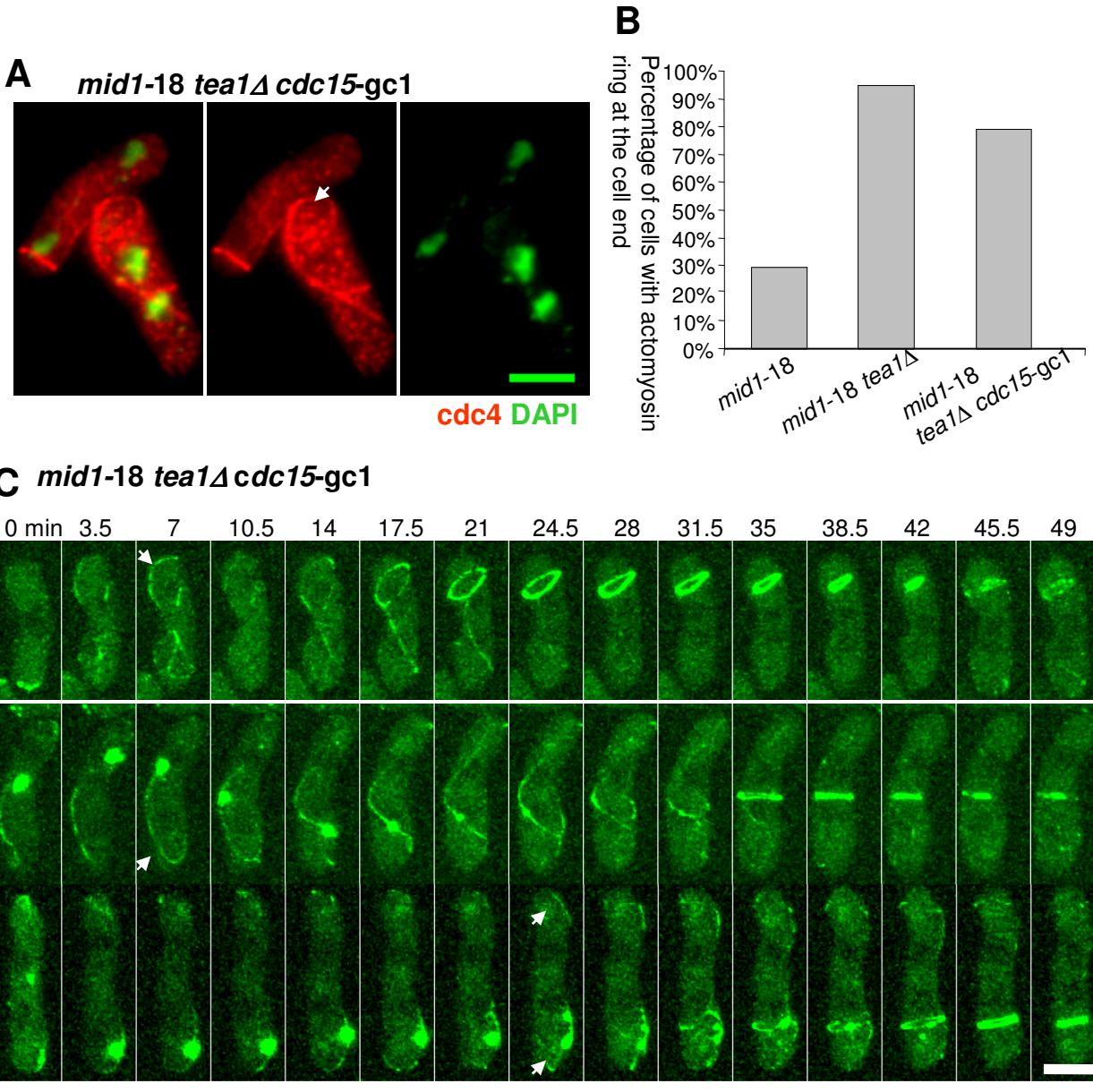

A
mid1-18 tea1Δ
mid1-18 tea1Δ Rng2p-GFP
mid1-18 tea1Δ Cdc12p-GFP
mid1-18 tea1Δ rlc1Δ
mid1-18 tea1Δ LatA
mid1-18 tea1Δ fim1Δ
mid1-18 tea1Δ ain1Δ
mid1-18 tea1Δ myp2Δ
B
mid1-18 cdc15-gc1
C
mid1-18 tea1Δ cdc15-gc1

Since the septum orientation defect of *mid1*-18 single mutants was corrected upon introduction of *cdc15*-gc1, we considered two possible explanations for the near-complete elimination of tip-septa in *mid1*-18 *tea1*Δ *cdc15*-gc1 cells. First, it was possible that actomyosin rings were assembled away from the cell ends in the *mid1*-18 *tea1*Δ *cdc15*-gc1 triple mutant (equivalent to partial suppression of the defect of *mid1*-18 in ring placement). Second, it was possible that actomyosin rings assembled near the cell ends in these triple mutants, as in the case of *mid1*-18 and *mid1*-18 *tea1*Δ cells. However, unlike in *mid1*-18 *tea1*Δ cells, and as in the case of the *mid1*-18 single mutant, septum assembly at the ends might be compromised (equivalent to suppression of the defect of *tea1*Δ in tip occlusion). To distinguish between these possibilities, cells of the genotypes *mid1*-18, *mid1*-18 *tea1*Δ and *mid1*-18 *tea1*Δ *cdc15*-gc1 were shifted to the restrictive temperature, fixed and stained with antibodies against the myosin light chain, Cdc4p. Actomyosin rings were readily observed at the cell ends in *mid1*-18 *tea1*Δ *cdc15*-gc1 (Figure 9A and B; marked with an arrowhead), suggesting that the deposition of septa away from the cell ends in these strains was not due to assembly of rings away from cell ends. Rather it appeared that actomyosin rings were not retained at the cell ends in *mid1*-18 *tea1*Δ *cdc15*-gc1 cells. To test if this was the case, we studied the dynamics of the actomyosin ring in *mid1*-18 *tea1*Δ *cdc15*-gc1 cells (Figure 9C). Consistent with the previous data, actomyosin rings assembled near the cell ends (marked with arrowheads in figure 9C) in all cells imaged ($n > 12$) although these rings were not retained therein. Constriction of the actomyosin ring and septum assembly ensued following migration of the actomyosin rings away from the cell ends in *mid1*-18 *tea1*Δ *cdc15*-gc1 cells. These experiments established that compromise of Cdc15p function was sufficient to restore

Figure 9. *mid1*-18 *tea1*Δ *cdc15*-gc1 assemble actomyosin rings at the cell ends, but septate after migration of actomyosin rings away from the cell ends.

mid1-18, *mid1*-18 *tea1*Δ, and *mid1*-18 *tea1*Δ *cdc15*-gc1 cells were shifted to 36°C for 2. 5 hours, fixed and stained with DAPI and Cdc4p antiserum to visualize nuclei and actomyosin rings respectively. A merged image of actomyosin rings and nuclei of *mid1*-18 *tea1*Δ *cdc15*-gc1 cells is shown in panel A, in which the arrowhead indicates the presence of a ring at the cell end. Panel B shows quantitation of actomyosin rings at the cell ends in *mid1*-18, *mid1*-18 *tea1*Δ and *mid1*-18 *tea1*Δ *cdc15*-gc1 cells. Panel C shows time-lapse imaging of *mid1*-18 *tea1*Δ *cdc15*-gc1 cells at 36°C. The arrowhead indicates the presence of an actomyosin ring at the cell end. Note that the Cdc15p-GFP itself served as a marker for the actomyosin ring. Elapsed time is shown in minutes. Scale bar, 5μm.

Figure 9


A
mid1-18 tea1Δ cdc15-gc1
cdc4 DAPI
B
Percentage of cells with actomyosin ring at the cell end
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
mid1-18
mid1-18 tea1Δ
mid1-18 tea1Δ cdc15-gc1
C
mid1-18 tea1Δ cdc15-gc1
0 min
3.5
7
10.5
14
17.5
21
24.5
28
31.5
35
38.5
42
45.5
49

tip-occlusion in *mid1*-18 *tea1*Δ cells. The orthogonal orientation of septa in *mid1*-18 *cdc15*-gc1 and *mid1*-18 *tea1*Δ *cdc15*-gc1 might indicate the ability of the actomyosin ring (and thereby septa) to assume an orthogonal orientation upon delays in septum assembly.

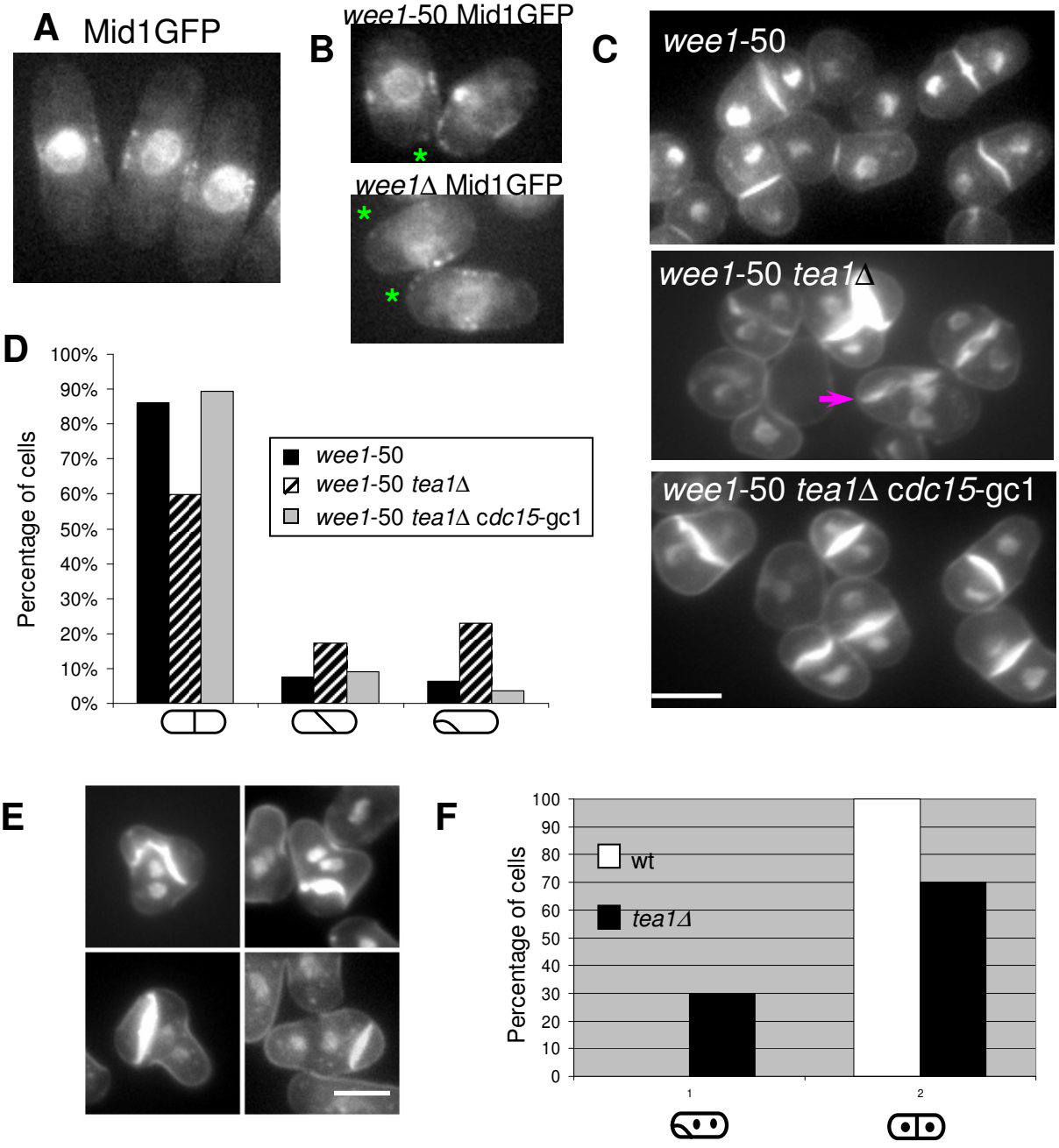
3. 2. 6 Tip-occlusion is important for fidelity of cytokinesis in smaller cells and for resumption of tip growth in cells lacking Mid1p

We then sought to identify physiological conditions in which tip-occlusion played a role in division site placement. The position of the division site in wild-type cells is dictated by the medial-band of Mid1p (Figure 10A), whose width corresponds with the diameter of the nucleus (Paoletti and Chang, 2000). We therefore reasoned that in smaller cells, where the cell ends constitute a larger fraction of the cell cortex, tip-occlusion might play a role in division site placement. To test if this was the case, we quantitated the position of the division site in *wee1*-50 mutants (Nurse, 1975), *wee1*Δ, *wee1*-50 *tea1*Δ, and *wee1*Δ *tea1*Δ cells. Cells lacking Wee1p divide at a cell length of 7-10 μm in which Mid1p localizes into a broader zone, including at cell ends (Figure 10B; marked with green asterisks). Strikingly, *wee1*-50 *tea1*Δ cells displayed an additive deleterious interaction (data not shown) and, unlike in the *wee1*-50 single mutant (Figure 10C and D), a high proportion of *wee1*-50 *tea1*Δ cells displayed a septum that ran along the long axis (pink arrowhead in Figure 10C). As in the case with *mid1*-18 *tea1*Δ, tip occlusion was restored in *wee1*-50 *tea1*Δ cells upon introduction of *cdc15*-gc1 (Figure 10C and D). Growth of *tea1*Δ cells in medium containing a poor nitrogen source such as proline, to reduce cell

Figure 10. Physiological roles for tip-occlusion and a model for tip-occlusion.

(A-B) Mid1p-GFP localization in (A) wild-type and (B) *wee1* mutants. Red asterisks indicate mislocalized Mid1p cortical patches. (C-D) Tip-complex increases the fidelity of division site selection in smaller *mid1*$^{+}$ cells. Cells of the indicated genotypes were grown at 25°C, shifted to 36°C for 3 hrs, fixed and stained as described above. Quantitation of septal locations is shown in panel D. 500 cells were counted in each case. (E) Cells lacking the tip-complex, but containing Mid1p, display abnormal septa in poor media. *tea1Δ* cells were grown in medium with proline as the sole nitrogen source at 36°C for 12 hours, fixed and stained with aniline blue and DAPI to visualize septum and DNA. (F) Quantitation of septation patterns of wild-type and *tea1*Δ grown in medium with proline.

Figure 10


A Mid1GFP
B
wee1-50 Mid1GFP
wee1Δ Mid1GFP
C
wee1-50
wee1-50 tea1Δ
wee1-50 tea1Δ cdc15-gc1
D
Percentage of cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
wee1-50
wee1-50 tea1Δ
wee1-50 tea1Δ cdc15-gc1
E
F
Percentage of cells
100
90
80
70
60
50
40
30
20
10
0
wt
tea1Δ
1
2

size at division, also resulted in septum positioning defects (Figure 10E and F). Thus, tip-occlusion might play a role in positioning of the division site in the presence of Mid1p, for example, when rings are placed near cell ends. Additional experiments performed under conditions that allow actomyosin ring assembly in interphase established that tip-occlusion also functions in interphase (data not shown).

3. 2. 7 Physiolgical analysis of tip-occlusion

In order to understand the basis of lethality in cells lacking Mid1p and the tip-complex, we compared the growth patterns of *mid1*-18 *tea1*Δ and *mid1*-18 cells grown at 36°C. Despite the mis-positioning of the division septum, *mid1*-18 cells were able to initiate tip growth following improper cytokinesis (Figure 11A). In contrast, tip growth and cell elongation were not initiated following improper cytokinesis in *mid1*-18 *tea1*Δ cells (Figure 11B; marked with arrows). Upon prolonged incubation, *mid1*-18 *tea1*Δ cells tended to swell on the sides and eventually lysed. These studies suggested that assembly of division septa at the hemispherical cell ends prevents subsequent tip elongation and polarized growth, thereby leading to lethality.

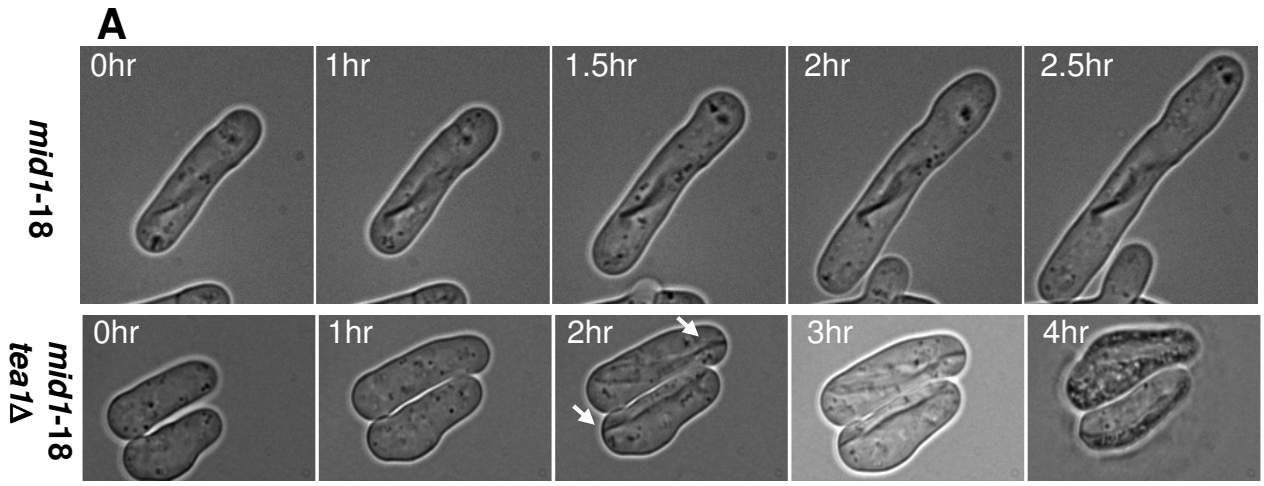
Alternatively, it was possible that Mid1p and the tip-complex played a redundant role in growth initiation following cytokinesis, and the lethality was attributable to growth defects that occur in the absence of both proteins. To differentiate between these possibilities, we created triple mutant strains defective in *mid1*, *tea1*, and *cdc7* genes. Cdc7p is a protein kinase essential for septum assembly (Fankhauser and Simanis, 1994).

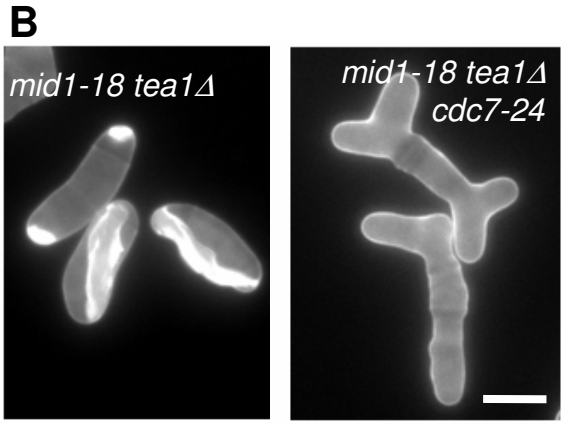
These cells were shifted to 36°C for 2 or 4 hrs. Interestingly, at 36°C (restrictive conditions for *cdc7*), *tea1*Δ *mid1*-18 *cdc7*-24 triple mutant cells were able to resume

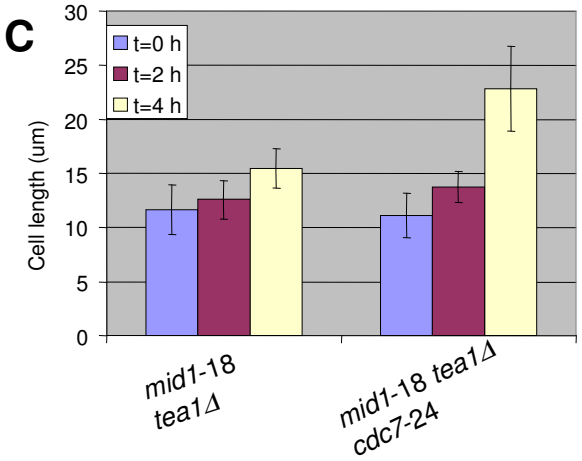
Figure 11. Physiolgical analysis of tip-occlusion.

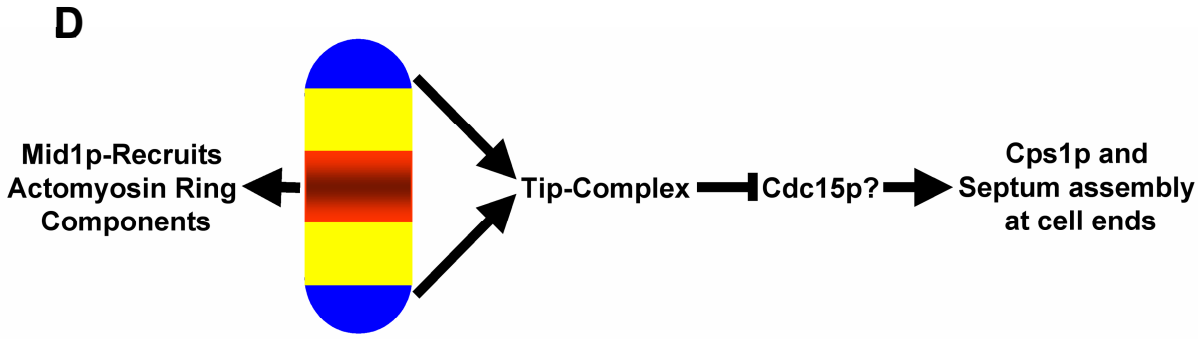
(A) The growth patterns of *mid1*-18 *tea1*Δ and *mid1*-18 cells grown at 36°C. *mid1*-18 cells and *mid1*-18 *tea1*Δ cells were grown at 36°C on YES agar pad, and then examine by time-lapse microscopy. (B) Suppression of tip-elongation defect of *mid1*-18 *tea1*Δ in the absence of Cdc7p-kinase. Cells of indicated genotype were shifted to 36°C for 2 or 4 hrs, fixed and stained with aniline blue. (C) Quantification of cells length in *mid1*-18 *tea1*Δ and *mid1*-18 *tea1*Δ *cdc7*-24. (D) A model for tip occlusion. The cortical band of Mid1p recruits actomyosin ring components to the medial region of the cell. In tip-occlusion, the tip-complex prevents actomyosin ring retention, Cps1p localization, and septum assembly at the cell ends. Cdc15p is a candidate for regulation by the tip-complex to achieve tip-occlusion. Scale Bar, 5μm.

Figure 11


A
mid1-18
0hr
1hr
1.5hr
2hr
2.5hr
mid1-18 tea1Δ
0hr
1hr
2hr
3hr
4hr


B
mid1-18 tea1Δ
mid1-18 tea1Δ cdc7-24


C
t=0 h
t=2 h
t=4 h
Cell length (um)
0
5
10
15
20
25
30
mid1-18 tea1Δ
mid1-18 tea1Δ cdc7-24


D
Mid1p-Recruits Actomyosin Ring Components
Tip-Complex
Cdc15p?
Cps1p and Septum assembly at cell ends

growth following failed cytokinesis (Figure 11B and C; representative images are shown in panel B and cell length distributions are shown in panel C). Under similar conditions *mid1*-18 *tea1*Δ double mutant cells assembled division septa at the ends and ceased to grow (Figure 11B and C). These studies established that assembly of division septa at cell ends prevents further tip elongation.

3. 3 Discussion

In many cell types, establishment of the plane of cytokinesis is thought to occur either via stimulatory signals that deliver activators of cleavage furrow induction to the medial cortex, or by local inhibition of negative regulators of furrow formation at the medial cortex (Glotzer, 2004). While these mechanisms have usually been considered in isolation, results presented in this study, taken together with studies in nematodes (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003; Glotzer, 2004; Motegi et al., 2006), suggest that stimulatory and inhibitory mechanisms can occur simultaneously. Such dual regulation of cytokinesis using stimulatory and inhibitory mechanisms might ensure fidelity of cytokinesis under a variety of developmental and environmental conditions.

The stimulatory mechanism in fission yeast utilizes the function of the anillin-related Mid1p, which links the position of the interphase nucleus to the medial assembly of the actomyosin ring and the division septum (Balasubramanian et al., 1998; Chang et

al., 1996; Sohrmann et al., 1996). Mid1p assembles into a cortical band that serves to recruit several components of the actomyosin ring to the incipient division site (Balasubramanian et al., 1998; Celton-Morizur et al., 2004; Paoletti and Chang, 2000; Sohrmann et al., 1996). The inhibitory mechanism requires the tip-localized polarity factors Tea1p, Tea4p and Pom1p (tip-complex). This mechanism involving the tip-complex proteins ensures that the actomyosin ring and the division septa are occluded from the cell tips. Whereas the Mid1p based primary mechanism is usually sufficient for the fidelity of actomyosin ring positioning, tip-occlusion appears to function under specific conditions, such as in cells lacking Mid1p, in cells of reduced size, and possibly in cells with altered morphologies (Ge et al., 2005). While either of these two mechanisms is sufficient for cell viability, the absence of both mechanisms results in lethality since growth after cytokinesis is impeded due to the presence of the division septum at the cellular growth zones. While our study uncovers an essential role for the tip-complex in cells lacking Mid1p, Pom1p (and possibly other members of the tip-complex) also plays a role in regulating localization of Mid1p (Celton-Morizur et al., 2006; Padte et al., 2006). Thus, the tip-complex might have Mid1p-dependent and-independent roles in the regulation of cytokinesis.

How does the tip-complex prevent actomyosin ring anchoring and septum assembly at cell ends in cells lacking Mid1p? Tip complex proteins have been shown to be present in several sub-complexes (Feierbach et al., 2004). Our studies have established that Tea1p, Tea4p, and Pom1p are largely responsible for tip-occlusion and function in a single pathway (approximately 25% cells with misplaced septa in

double mutants, *tea1 pom1*, *tea4 pom1* and *pom1* mutants; data not shown) to this end. Given that the localization of the Pom1p-kinase depends on Tea1p and Tea4p (Bahler and Pringle, 1998; Tatebe et al., 2005), it is likely that tip-occlusion is controlled via Pom1p. Our genetic and physiological analysis have also shown that reduction of function of the FCH-protein Cdc15p is sufficient to prevent septum assembly at the cell ends (Figure 11 D). Since compromise of function of several other ring proteins does not restore tip occlusion in *mid1*-18 *tea1*Δ cells, it is likely that Cdc15p function is under negative regulation by the tip-complex. Cdc15p is a multi-domain phosphoprotein with an ability to interact with cell membranes (Takeda et al., 2004), the formin Cdc12p (Carnahan and Gould, 2003), and the Arp2/3 complex activators Wsp1p and Myo1p (Carnahan and Gould, 2003). Cdc15p is required for the maintenance of actomyosin rings (Takeda et al., 2004; Wachtler et al., 2006), for organization of membranes (Liu et al., 2002) and membrane proteins, such as Cps1p, during cytokinesis (Liu et al., 2002), and for septum assembly. We have shown that the tip-complex based inhibitory mechanism prevents assembly of Cps1p into actomyosin cables in the vicinity of the cell ends, thereby preventing division septum assembly at the cell ends. It is possible that Cdc15p in the vicinity of the cell ends might be modified by the tip-complex and rendered incapable of organizing membranes and Cps1p to actomyosin cables at cell ends. Since Cdc15p undergoes a dramatic turnover in the ring (in under 20 seconds in fluorescence recovery after photobleaching experiments; data not shown), actomyosin cables in the vicinity of the cell ends might accumulate modified Cdc15p incapable of organizing the septation machinery. A delay in septum assembly might increase the probability of the ring

assuming an orthogonal organization and away from cell ends. Understanding the molecular mechanism of regulation of Cdc15p and / or its associated factors by the tip-complex will require a thorough biochemical characterization of Cdc15p and the tip-complex.

Prevention of actomyosin ring attachment and septum assembly at the cell tips by tip-complex proteins is reminiscent of the function performed by the bacterial Min-proteins. In bacteria, MinCD proteins prevent assembly / polymerization of the cell division FtsZ rings at cell ends (Margolin and Bernander, 2004). Prevention of cytokinesis at cell ends, utilizing microtubule-based factors is also akin to the astral relaxation-like mechanisms, which have been identified in several eukaryotes (Balasubramanian et al., 2004; Glotzer, 2004). Further analysis of fission yeast tip-occlusion should thus provide valuable insight into the general mechanisms of cytokinesis.

4. Cortical Node-Independent Assembly of Actomyosin Rings in Fission Yeast

4. 1 Introduction

In fission yeast, an actomyosin ring is assembled early in mitosis and constricts following mitotic exit (Fankhauser et al., 1995; Marks et al., 1986; McCollum et al., 1995; Wu et al., 2006). Recent studies, using a combination of fluorescence imaging of live cells and computational approaches, have led to the proposal that actomyosin ring assembly is initiated from multiple cortical nodes, each containing two dimers of the formin-related protein Cdc12p, anillin-related protein Mid1p, type II myosins, the F/BAR domain protein Cdc15p, and the IQGAP-related protein Rng2p (Vavylonis et al., 2008; Wu et al., 2006). It has been shown that Mid1p plays a key role in the cortical anchoring of these nodes and that loss of Mid1p function eliminates the assembly of detectable nodes (Wu et al., 2006). Computational modeling experiments have revealed that forces generated by type II myosins combined with a search and capture mechanism help compact nodes placed within a 8 μm zone into a tightly organized actomyosin ring. Although this proposed mechanism is very attractive, a simple prediction that arises from these studies is that cells containing fewer or no nodes should either assemble abnormal actomyosin rings or should be unable to assemble actomyosin rings.

In this part, we have characterized the process of actomyosin ring assembly in cells lacking Mid1p and thereby lacking detectable nodes. We find that cells lacking Mid1p and detectable nodes assemble normal-looking actomyosin rings, albeit with a delayed kinetics. Thus a Mid1p and membrane-anchored node independent mechanism is sufficient for the assembly of properly organized actomyosin rings.

4. 2 Result

4. 2. 1 Myosin Membrane-anchored nodes are not detected in cells lacking Mid1p

In *S. pombe*, Wu et al. have shown that Mid1p assembles first into nodes and recruits other proteins (including type II myosin and its light chain Rlc1p, formin Cdc12p, F-BAR protein Cdc15p, and IQGAP-related Rng2p) into nodes (Motegi et al., 2004; Motegi et al., 2000; Paoletti and Chang, 2000; Wu et al., 2006). Shown in Figure 12 A are examples of Rlc1p-3GFP nodes in wild-type cells and their absence in *mid1Δ* cells, consistent with studies of Wu et al. Furthermore, as shown by Wu et al, we have found that Cdc15p organizes into a few node-like structures (15 ± 7 nodes / cell; n=17; Fig. 16 A), and that the number and intensity of these structures is increased (45 ± 18 nodes / cell; n=22; Figure 12 A) upon treatment with Latrunculin-A (LatA), which prevents actin polymerization (Ayscough et al., 1997). We have also been able to observe colocalization of Cdc15p-GFP nodes with Rlc1p-mCherry nodes in LatA treated cells (Figure 12 B). As shown by Wu et al, we have found that organization

of Cdc15p into nodes in untreated and LatA treated cells depended on Mid1p function. Although we have been able to detect Rlc1p and Cdc15p in cortical nodes, we have been unable to detect the formin Cdc12p in cortical nodes in unperturbed cells, consistent with studies by Chang and colleagues (Chang, 1999; Yonetani et al., 2008). Nevertheless, as described by Wu et al (Wu et al., 2006) , we did detect Cdc12p nodes upon LatA treatment (Figure 12A). Again, organization of Cdc12p nodes in LatA treated cells depended on Mid1p function. Approximately 51% of Cdc12p nodes (n = 270), generated by LatA treatment, co-localized with Rlc1p nodes (Figure 12C). We have also found that Rlc1p nodes are not detected in mutants defective in the fission yeast Polo-kinase, Plo1p (data not shown), which is a known regulator of fission yeast Mid1p (Bahler et al., 1998). Collectively, these studies established that some actomyosin ring components (such as Rlc1p; Cdc15p) are detected in nodes in unperturbed cells, while others (such as Cdc12p) are detected in nodes only upon LatA treatment. Furthermore, our studies (as well as Wu et al. , 2006 and Motegi et al. , 2004) have established that organization of cortical nodes depends on Mid1p-function.

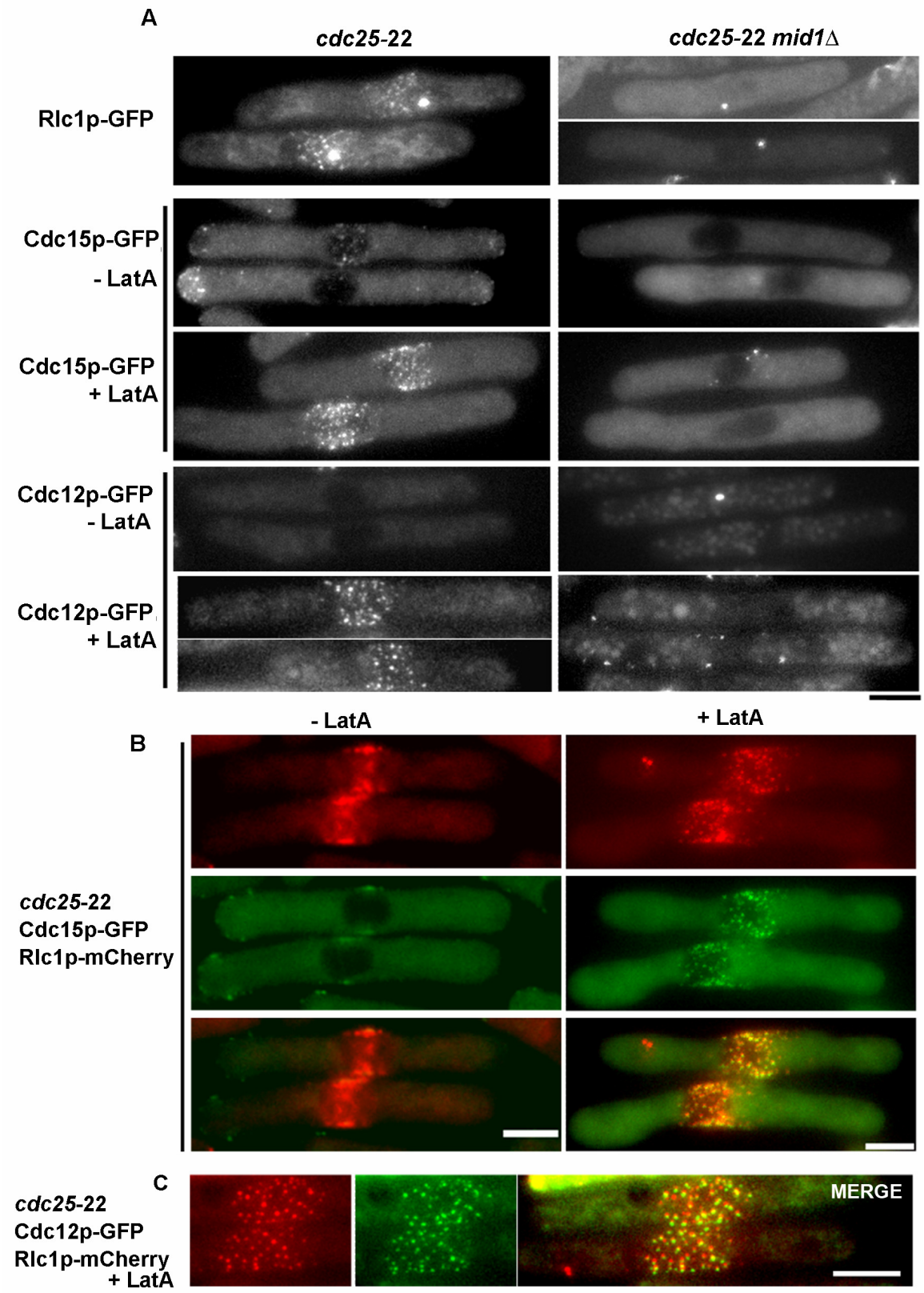
4. 2. 2 Cells lacking Mid1p and detectable nodes assemble normal actomyosin rings upon inhibition of division septum synthesis

Cells defective for Mid1p and Plo1p, and thereby lacking detectable nodes, have been shown to assemble abnormally organized (generally not orthogonal) actomyosin rings

Figure 12. Membrane associated nodes of Rlc1p, Cdc15p and Cdc12p are not detected in cells lacking Mid1p.

(A) *cdc25*-22 and *cdc25*-22 *mid1*Δ cells expressing Rlc1p-GFP, Cdc15p-GFP, or Cdc12p-GFP were arrested at the G2/M boundary by incubation at the restrictive temperature of 36ºC. Cells were then shifted to the permissive temperature of 24ºC and images were captured 30-45 minutes after shift-down. In some instances, as indicated, Latrunculin A (LatA) was added to the culture to facilitate visualization of the cortical nodes of Cdc15p and Cdc12p. (B and C) *cdc25*-22 cells expressing Rlc1p-mCherry and Cdc15p-GFP (panel B) or Rlc1p-mCherry and Cdc12p-GFP (panel C) were cultured as described in A, treated with LatA and images were captured. Scale bars, 5μm.

Figure 12


A
cdc25-22
cdc25-22 mid1Δ
Rlc1p-GFP
Cdc15p-GFP
- LatA
Cdc15p-GFP
+ LatA
Cdc12p-GFP
- LatA
Cdc12p-GFP
+ LatA
- LatA
+ LatA
B
cdc25-22
Cdc15p-GFP
Rlc1p-mCherry
C
cdc25-22
Cdc12p-GFP
Rlc1p-mCherry
+ LatA
MERGE

(Bahler et al., 1998; Balasubramanian et al., 1998; Chang et al., 1996; Sohrmann et al., 1996), which at first glance is consistent with the predictions from the computational and imaging studies of Wu et al. , and Vavylonis et al (Vavylonis et al., 2008; Wu et al., 2006). We have found that the abnormal rings in *mid1* and *plo1* mutant cells (lacking detectable nodes) become stabilized due to septum assembly prior to ring-compaction. Shown in Figure 13A are examples of F-actin distribution in wild-type, cells of three different mutants alleles of *mid1* (*mid1*-18, *mid1*-ΔNES, and *mid1*Δ), and *plo1*-1 cells. Unlike in mitotic wild-type cells, actomyosin rings in mitotic mid1 and *plo1*-1 mutant cells are detected at various angles (Figure 13A) (Bahler et al., 1998). To eliminate any confusion in scoring phenotypic effects that might arise from the assembly of division septum along improperly organized rings, we inactivated Cps1p, a 1,3 β-glucan synthase involved in septum assembly (Liu et al., 1999), in cells defective in Mid1p and Plo1p function. To our surprise, *cps1*-191 *mid1*-18, *cps1*-191 *mid1*-ΔNES, *cps1*-191 *mid1*Δ and *cps1*-191 *plo1*-1 cells, like wild-type cells assembled orthogonal actomyosin rings (Figure 13B and C). Over 85 % of *cps1*-191 *mid1*-18, *cps1*-191 *mid1*-ΔNES, *cps1*-191 *mid1*Δ and *cps1*-191 *plo1*-1 cells, held at the restrictive temperature for *cps1*-191, contained orthogonally organized normal-appearing actomyosin rings. Rings in *cps1*-191 *mid1*-18, *cps1*-191 *mid1*-ΔNES, *cps1*-191 *mid1*Δ and *cps1*-191 *plo1*-1 cells were positioned at abnormal sites, consistent with a role for Mid1p and Plo1p in positioning of the actomyosin ring.

It remained formally possible that inactivation of Cps1p function led to the restoration of medial nodes, by unidentified mechanisms. To test this possibility *cps1*-191 and *cps1*-

191 *mid1*Δ cells were fixed and stained with antibodies against tubulin and the actomyosin ring protein Cdc4p (Figure 13D). Cdc4p was readily detected in medial nodes in 30 / 54 *cps1*-191 cells with short prometaphase-like spindles, while the rest contained fully formed actomyosin rings. Such organization of Cdc4p was not observed in *cps1*-191 *mid1*Δ cells (36 / 53 contained no observable structures and 17 / 53 contained disorganized cables), ruling out the possibility that normal ring assembly in *cps1*-191 *mid1*Δ cells is due to the restoration of medial nodes in these cells. Collectively, these observations established that Mid1p and detectable nodes are not required for assembly of orthogonal actomyosin rings. These observations also established that the non-orthogonal rings become fixed at these improper angles due to septum assembly prior to actomyosin ring compaction in *mid1* and *plo1* mutants.

To gain further insight into the mechanism of orthogonal ring assembly in the absence of Mid1p (and thereby in the absence of nodes) we imaged ring assembly in *cps1*-191 *mid1*-18 expressing Rlc1p-GFP as a marker of the actomyosin ring. As previously reported by others and us (Bahler et al., 1998; Huang et al., 2007), assembly of F-actin and myosin II containing bundles initiated at non-medial sites that were typically located near the cell ends in these cells lacking Mid1p function (Figure 13E). Interestingly, in late anaphase, these actin and myosin II containing bundles rapidly organized into orthogonal ring structures (Figure 13E). These studies established that in the absence of Mid1p (and all detectable nodes) actomyosin bundles assemble initially from random locations (typically near the cell ends) and at various angles and that they eventually compact into orthogonal actomyosin rings.

Figure 13. Orthogonal actomyosin rings assemble with high efficiency in *mid1* and *plo1* mutants.

(A and B) Cells of the indicated genotypes were shifted to 36ºC, fixed and stained with Alexa 488-phalloidin to visualize F-actin rings. (C) Quantitation of cells with orthogonal rings. At least 500 cells were scored for each genotype (D) Nodes are not restored in *cps1*-191 *mid1*Δ mutants. *cps1*-191 and *cps1*-191 *mid1*Δ cells were fixed and stained with TAT-1 antibodies (tubulin), Cdc4p antibodies, and DAPI (nuclei). (E) Randomly oriented myosin II bundles organize eventually into orthogonal myosin II rings in *cps1*-191 *mid1*-18 cells. *cps1*-191 *mid1*-18 cells expressing Rlc1p-GFP were imaged by confocal microscopy at 36^{o}C. Elapsed time is shown in minutes. Scale bars, 5μm.

.

Figure 13

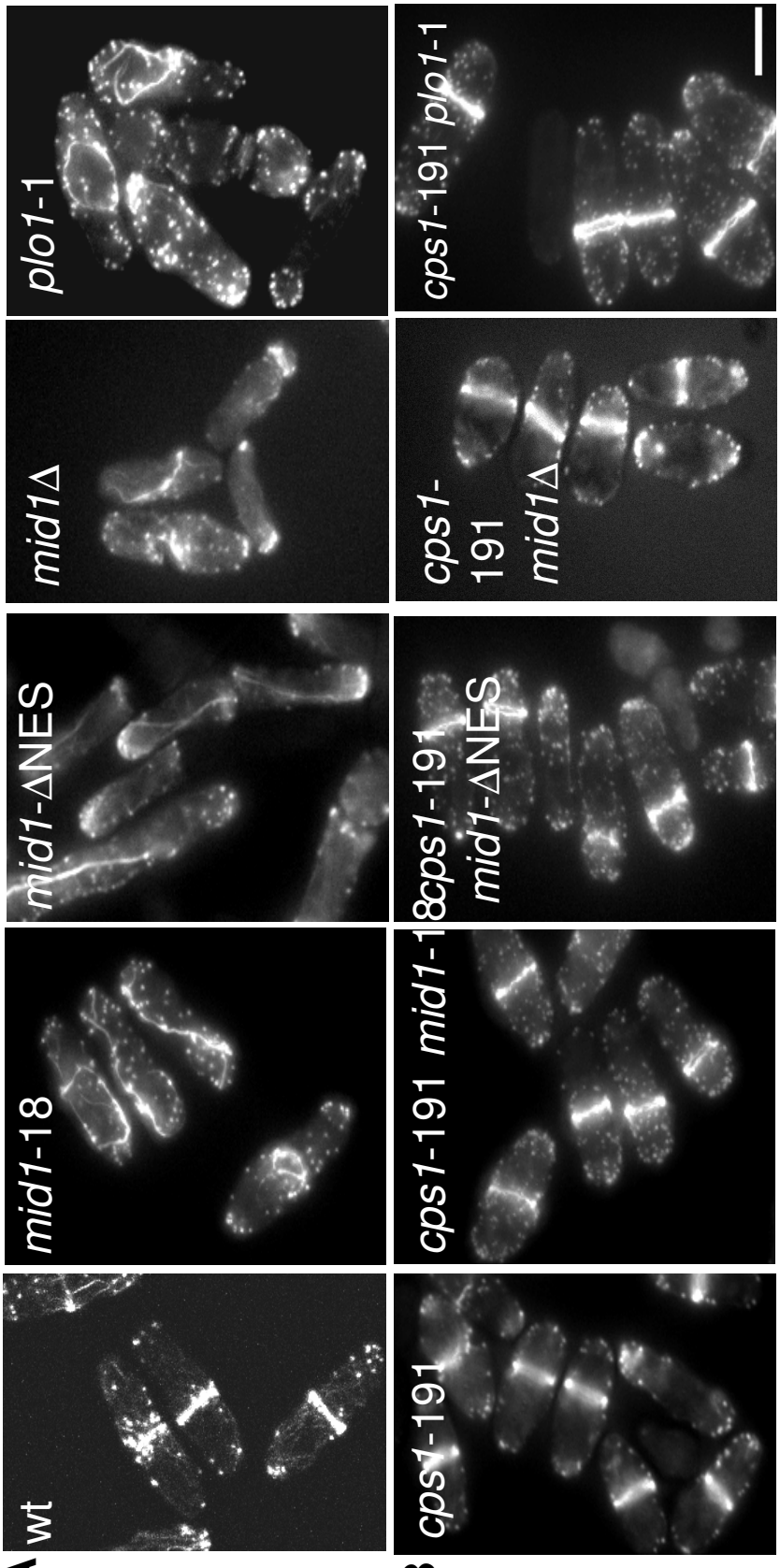
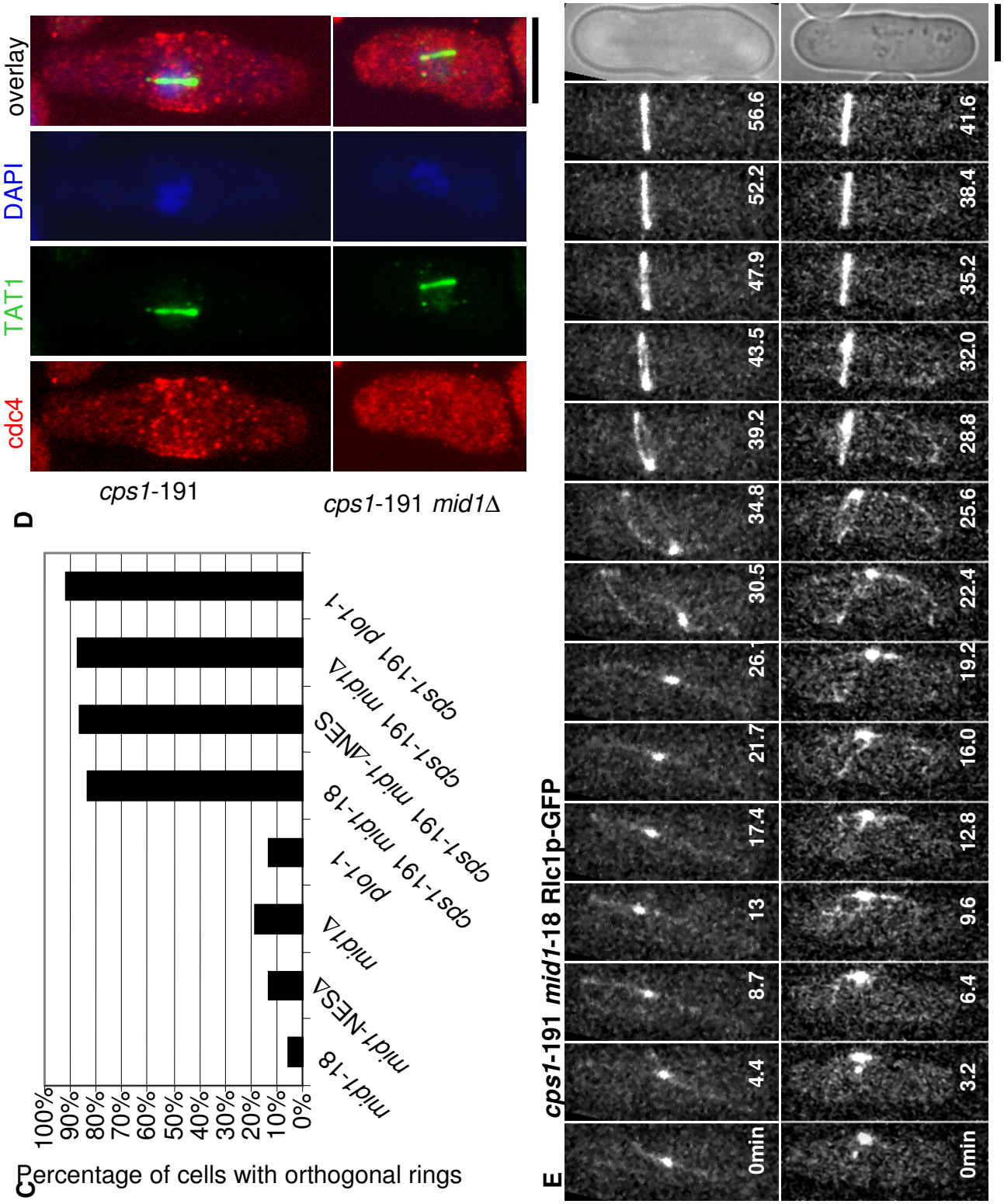

A
wt
mid1-18
mid1-ΔNES
mid1Δ
plo1-1
B
cps1-191
cps1-191 mid1-18
cps1-191 mid1-ΔNES
cps1-191 mid1Δ
cps1-191 plo1-1

Figure 13


D
overlay
DAPI
TAT1
cdc4
cps1-191
cps1-191 mid1Δ
C
Percentage of cells with orthogonal rings
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
mid1-18
mid1-NESΔ
mid1Δ
plo1-1
cps1-191 mid1-18
cps1-191 mid1-ΔNES
cps1-191 mid1Δ
cps1-191 plo1-1
E
cps1-191 mid1-18 Rlc1p-GFP
0min
4.4
8.7
13
17.4
21.7
26.1
30.5
34.8
39.2
43.5
47.9
52.2
56.6
0min
3.2
6.4
9.6
12.8
16.0
19.2
22.4
25.6
28.8
32.0
35.2
38.4
41.6

4. 2. 3 Mid1p and membrane-bound nodes play an important role in the kinetics of actomyosin ring assembly in early stages of mitosis

Although normal actomyosin rings were eventually assembled in *cps1*-191 *mid1*Δ cells, the assembly process was significantly slower, suggesting that Mid1p and the nodes might play an important role in the kinetics of actomyosin ring assembly. To better characterize ring assembly in *cps1*-191 *mid1*Δ cells we imaged dynamics of actomyosin ring assembly and mitosis simultaneously in cells expressing Rlc1p-GFP and Pcp1p-GFP (a marker of the spindle pole body) (Rajagopalan et al., 2004). In *cps1*-191 mutants (Figure 14A), actomyosin ring assembly was initiated soon after separation of SPBs, indicative of prometaphase stage of the cell cycle. Organization of an actomyosin ring was completed in less than 10 minutes (9. 7 ± 1. 4 minutes; n=7 cells) from the time of SPB separation. By contrast, although actomyosin cable assembly was initiated soon after SPB separation in *cps1*-191 *mid1*Δ cells (Figure 14B), organization of normal actomyosin rings was accomplished only after full separation of the SPBs to the opposite ends of the cell, indicative of a post-anaphase stage of the cell cycle. Starting at the point of SPB separation, organization of normal actomyosin rings took at least two times longer (32. 3 ± 19. 5 minutes; n=8 cells) in *cps1*-191 *mid1*Δ cells.

To further investigate the timing of organization of actomyosin rings in *cps1*-191 *mid1*Δ cells, we fixed and stained *cps1*-191 and *cps1*-191 *mid1*Δ cells with antibodies against tubulin and Cdc4p. Consistent with the time-lapse studies fully formed actomyosin rings were detected in *cps1*-191 cells with intermediate length and elongated spindles as well

Figure 14. Mid1p and associated medial nodes are required for the organization of actomyosin rings in early mitosis.

(A and B) *cps1*-191 and *cps1*-191 *mid1*Δ cells expressing Rlc1p-GFP (myosin II ring) and Pcp1p-GFP (SPBs) were imaged by time-lapse microscopy. The SPBs are marked with pink asterisks. Elapsed time is shown in minutes. Scale bars, 5μm (C) *cps1*-191 and *cps1*-191 *mid1*Δ cells were fixed and stained with antibodies against tubulin and Cdc4p. Representative images of cells with intermediate length and elongated spindles as well as those with post-anaphase arrays (PAA) are shown. Scale bars, 5μm. (D) Quantitation of orthogonal rings and randomly oriented cables in *cps1*-191 and *cps1*-191 *mid1*Δ cells at various stages of mitosis. At least 50 cells were scored in each category.

Figure 14

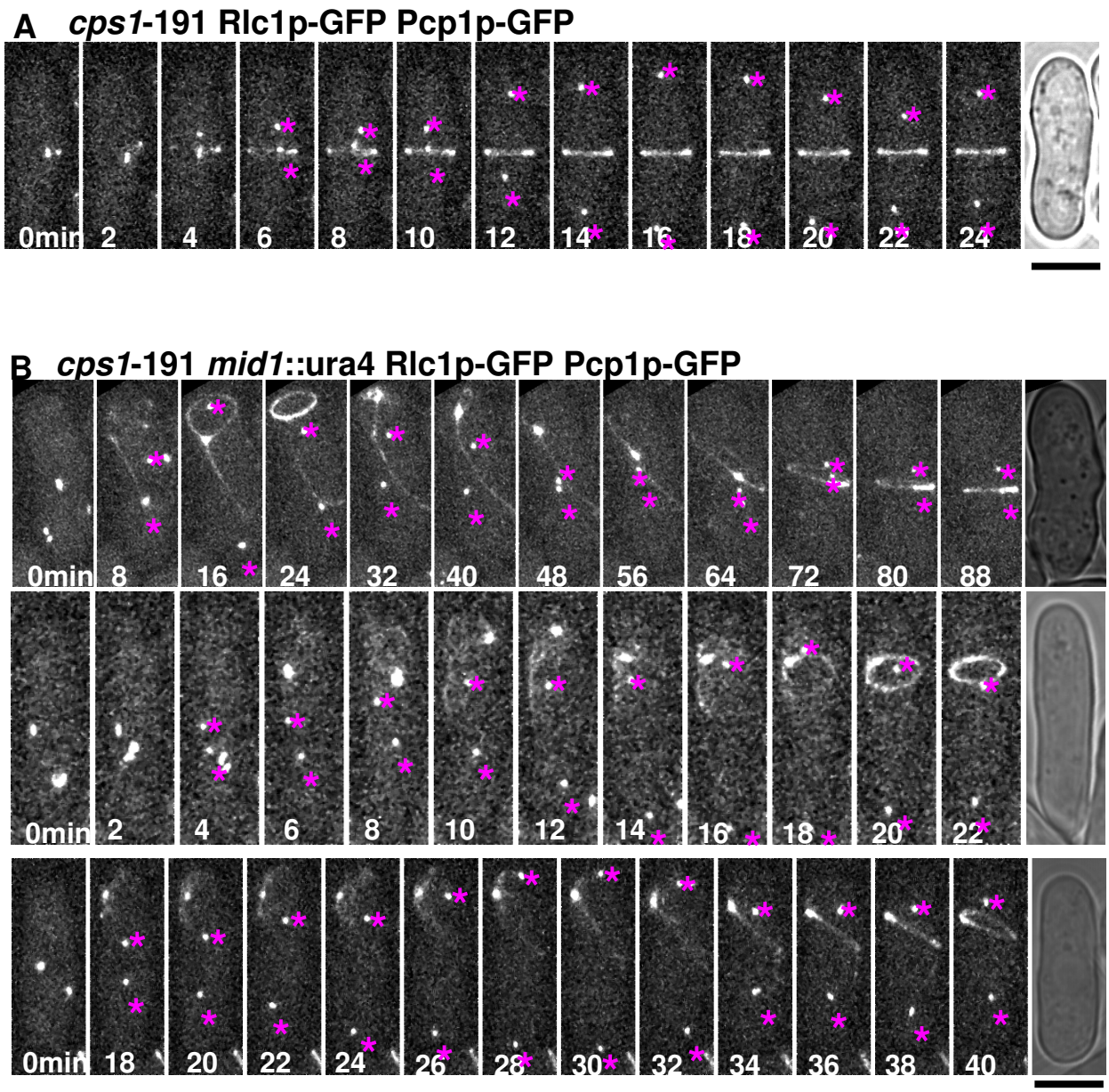
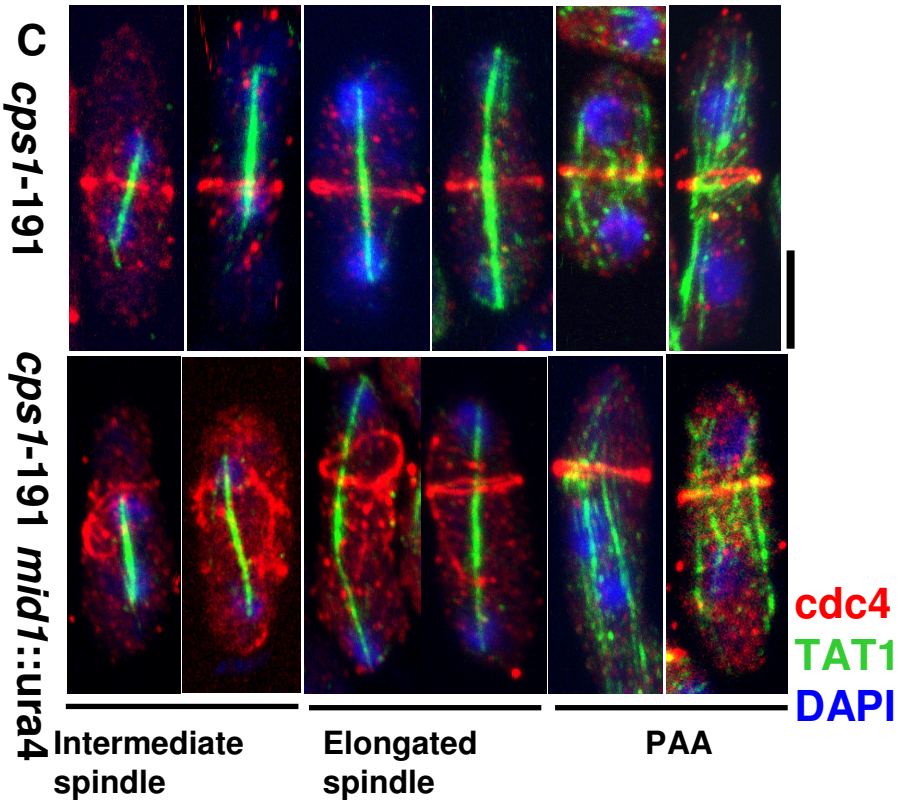
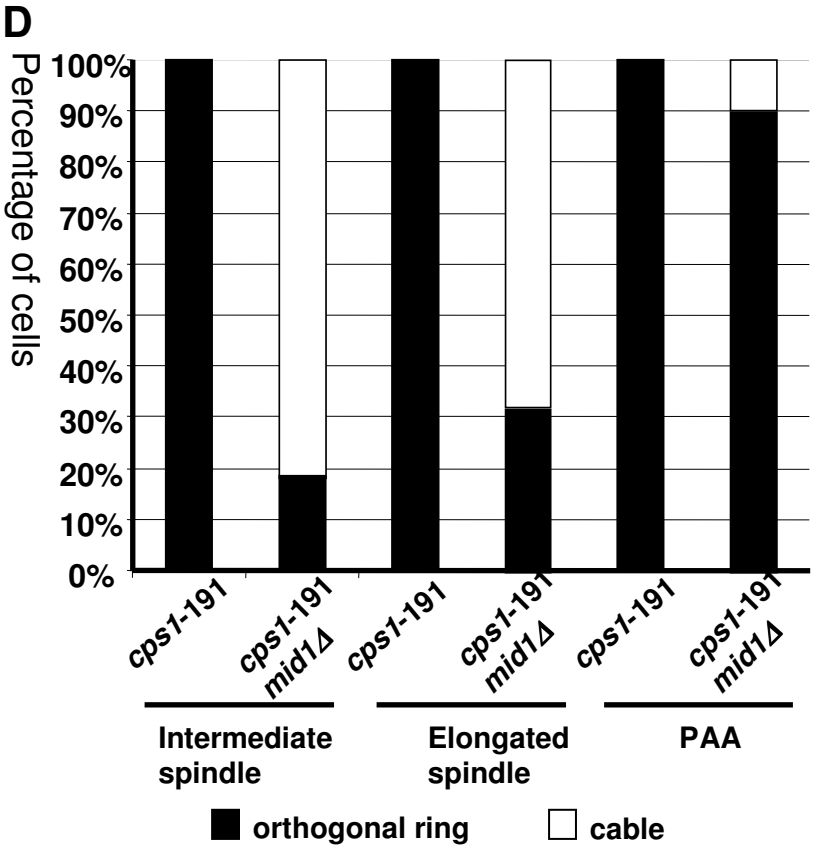

A cps1-191 Rlc1p-GFP Pcp1p-GFP
0min 2 4 6 8 10 12 14 16 18 20 22 24
B cps1-191 mid1::ura4 Rlc1p-GFP Pcp1p-GFP
0min 8 16 24 32 40 48 56 64 72 80 88
0min 2 4 6 8 10 12 14 16 18 20 22
0min 18 20 22 24 26 28 30 32 34 36 38 40

Figure 14


C
cps1-191
cps1-191 mid1::ura4
cdc4
TAT1
DAPI
Intermediate spindle
Elongated spindle
PAA


D
Percentage of cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
cps1-191
cps1-191 mid1Δ
cps1-191
cps1-191 mid1Δ
cps1-191
cps1-191 mid1Δ
Intermediate spindle
Elongated spindle
PAA
orthogonal ring
cable

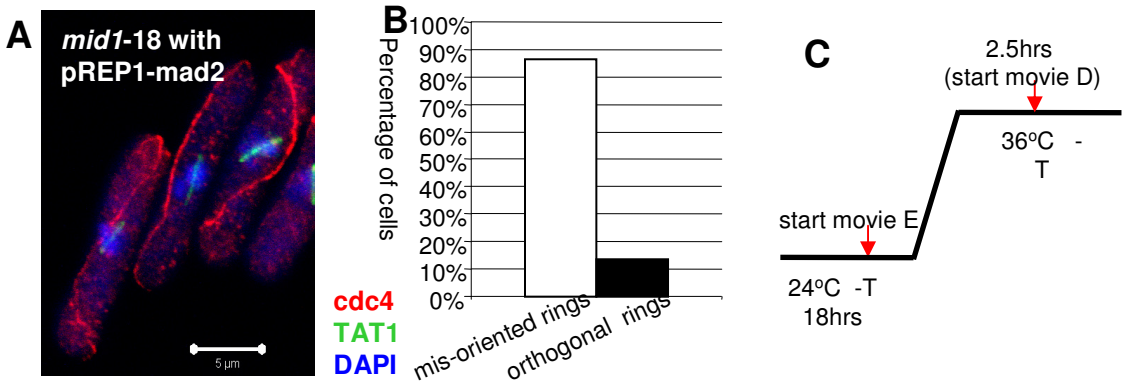
as in cells with post anaphase microtubular arrays (Figure 14C and D). By contrast, in the *cps1*-191 *mid1*Δ cells, fully formed actomyosin rings were observed in less than 20% cells with intermediate length spindles and in less than 35% cells with elongated spindles (Figure 14C and D). Fully formed actomyosin rings, however, were detected in nearly 90% of *cps1*-191 *mid1*Δ cells (Figure 14C and D). Taken together, these experiments suggested that Mid1p and membrane anchored nodes play an important role in organizing actomyosin rings in early mitosis, but that other mechanisms might operate in later stages of mitosis and that these pathways are sufficient to organize actomyosin rings.

To independently test if Mid1p was important for ring assembly in early mitotic cells, we arrested *mid1*-18 cells expressing Rlc1p-GFP at metaphase by over production of the mitotic checkpoint protein Mad2p (He et al., 1997). We carried out three different experiments. In the first, we induced expression of Mad2p and then shifted cells to the restrictive temperature for *mid1*-18, fixed and stained these cells with antibodies against Cdc4p (actomyosin ring), tubulin (mitotic spindle) and DAPI (DNA). In this experiment, we found that over 85% of metaphase arrested *mid1*-18 cells contained non-orthogonal actomyosin cables (Figure 15 A and B). In the second experiment, we induced expression of Mad2p and then shifted cells to the restrictive temperature for *mid1*-18 and imaged the process of actomyosin ring assembly (Figure 15 C and D). We found that actomyosin cables and bundles assembled efficiently in metaphase arrested *mid1*-18 cells. However, these actomyosin cables failed to become organized into rings even upon incubation for nearly 90 minutes (Figure 15 D). In the third experiment, we induced expression of Mad2p, allowed actomyosin ring assembly at the permissive temperature

Figure 15. Mid1p and cortical nodes are important for orthogonal ring assembly in early mitosis.

(A) *mid1*-18 cells expressing Rlc1p-GFP were arrested in metaphase by over production of the mitotic checkpoint protein Mad2p, expressed under control of the thiamine-repressible *nmt1* promoter. Cells were grown in the absence of thiamine for 18 hours at 24ºC, shifted to 36ºC for 3. 5 hours, fixed and stained with TAT-1 antibodies (tubulin), Cdc4p antibodies (rings) and DAPI (nuclei). (B) Quantitation of orthogonal rings and mis-oriented rings in metaphase arrested *mid1*-18 cells (C) Schematic representation of imaging experiments in panels D and E. (D) Time-lapse imaging of myosin II ring / cable assembly in metaphase arrested *mid1*-18 cells. 0-min refers the time when the imaging was initiated (2. 5 hours after shift to 36ºC). (E) Time-lapse imaging of unraveling of a preformed medial myosin II ring in a metaphase arrested *mid1*-18 cell upon shift to the restrictive temperature. Scale bars, 5μm.

Figure 15


A
mid1-18 with
pREP1-mad2
5 µm
B
Percentage of cells
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
cdc4
TAT1
DAPI
mis-oriented rings
orthogonal rings
C
2.5hrs
(start movie D)
36ºC -
T
start movie E
24ºC -T
18hrs

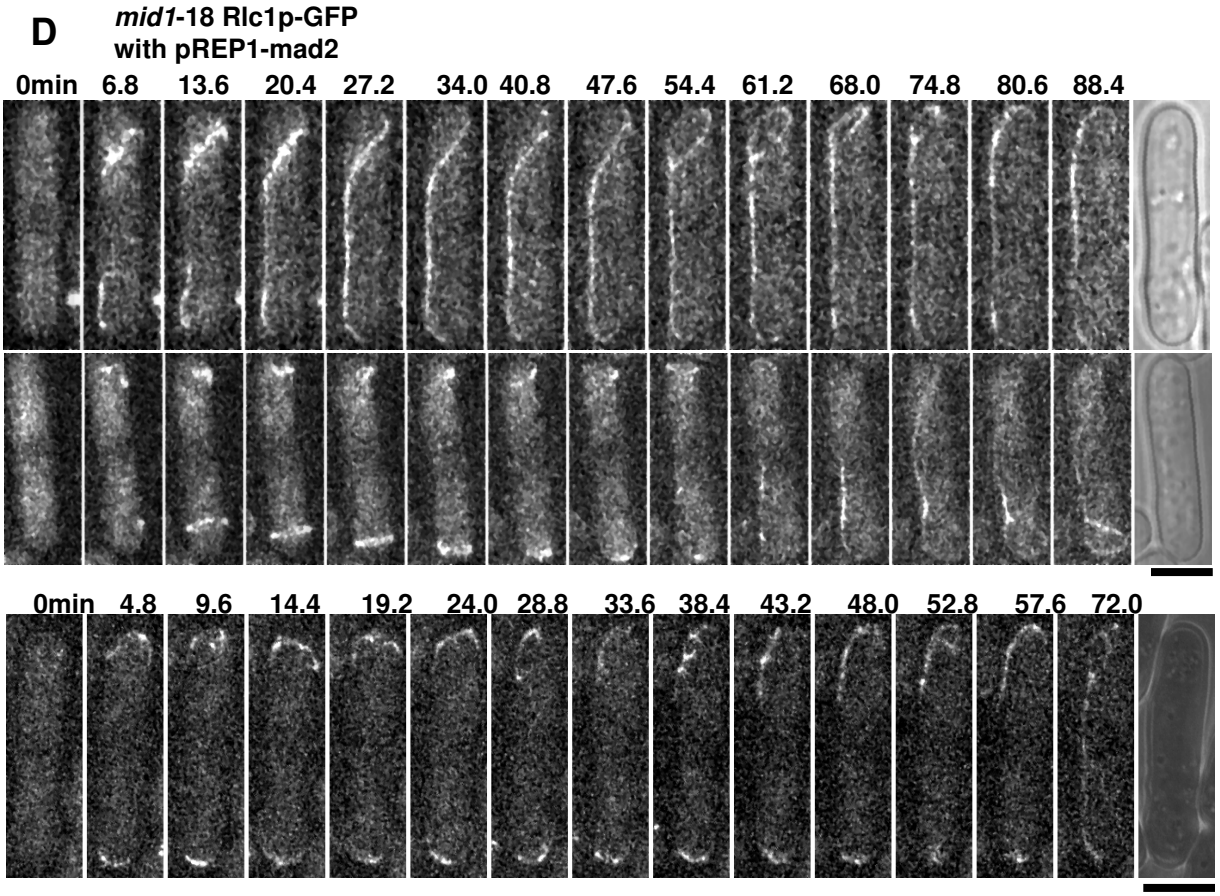
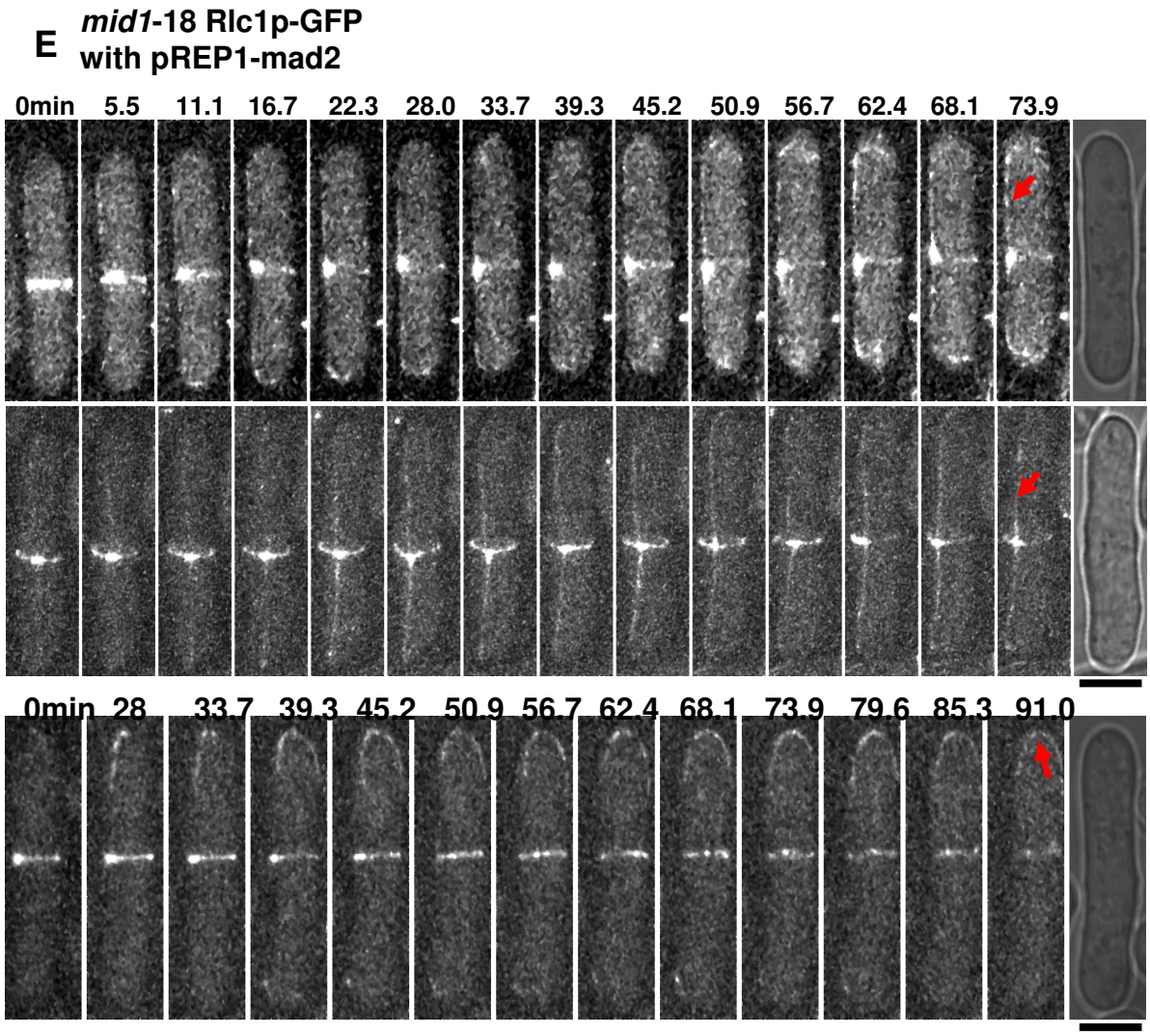

D
mid1-18 Rlc1p-GFP
with pREP1-mad2
0min 6.8 13.6 20.4 27.2 34.0 40.8 47.6 54.4 61.2 68.0 74.8 80.6 88.4
0min 4.8 9.6 14.4 19.2 24.0 28.8 33.6 38.4 43.2 48.0 52.8 57.6 72.0

Figure 15


E
mid1-18 Rlc1p-GFP
with pREP1-mad2
0min 5.5 11.1 16.7 22.3 28.0 33.7 39.3 45.2 50.9 56.7 62.4 68.1 73.9
0min 28 33.7 39.3 45.2 50.9 56.7 62.4 68.1 73.9 79.6 85.3 91.0

for *mid1*-18 and subsequently shifted cells to the restrictive temperature (Figure 15 C and E). In this experiment, we found that shift of metaphase-arrested *mid1*-18 with a preexisting proper medial actomyosin ring (assembled at the permissive temperature for *mid1*-18 cells) to the restrictive temperature led to unraveling and disassembly of this preexisting ring (Figure 15 E; shown with red arrows). These observations suggested that Mid1p (and possibly the cortical nodes) were important for organization of actomyosin rings in early mitosis and that Mid1p function might be continuously required for the medial maintenance of the actomyosin ring until septation. The fact that metaphase-arrested *mid1*-18 cells failed to organize orthogonal rings even after a 90 minute duration without septation ruled out the possibility that prevention of septation in *cps1*-191 *mid1*$^-$ double mutants was solely responsible for actomyosin ring organization. Further, this result suggested that some aspect of regulation occurring after metaphase is responsible for the organization of orthogonal rings in *cps1*-191 *mid1*$^-$ cells.

To further assess if Mid1p and the cortical nodes were important for ring assembly in metaphase arrested cells, we arrested *nda3*-KM311 and *nda3*-KM311 *mid1*Δ cells at prometaphase by shift to the restrictive temperature of 18ºC. *nda3*-KM311 is a cold-sensitive mutant allele of the sole gene encoding the fission yeast β-tubulin (Hiraoka et al., 1984). *nda3*-KM311 cells arrest in prometaphase (due to the inability of cells to assemble a mitotic spindle) with a stable orthogonal actomyosin ring (Chang et al., 1996). Interestingly, we found that nearly 59% of *nda3*-KM311 *mid1*Δ cells contained mis-oriented rings, while the rest contained orthogonal rings (Figure 16 A-C; Rlc1p and Sid4p are shown in panel A, while F-actin is shown in panel C). By contrast, rings in the *nda3*-

KM311 cells were always orthogonal (data not shown). These results further supported the notion that the function of Mid1p and cortical nodes was important for organization of orthogonal actomyosin rings in early mitosis. The slightly higher proportion of orthogonal rings observed in *nda3 mid1*Δ cells might be due to the prolonged block of *nda3*-KM311 cells at the restrictive temperature (6 hours), which might lead to leakage of these cells past the prometaphase block.

4. 2. 4 Activation of the SIN is sufficient for the assembly of orthogonal actomyosin rings in the absence of Mid1p and membrane anchored nodes

Our experiments have led to the conclusion that in the absence of Mid1p and the nodes, a second pathway can allow organization of actomyosin rings with a high efficiency. We sought to identify elements of this second pathway. Since organization of proper actomyosin rings occurred after passage through anaphase we considered the possibility that the septation initiation network (SIN) might play a role in organization of actomyosin rings in the absence of Mid1p and the membrane anchored nodes. The SIN is a signaling module that is activated upon passage through anaphase and plays a key role in maintenance and organization of actomyosin rings (Krapp et al., 2004; McCollum and Gould, 2001; Simanis, 2003; Wolfe and Gould, 2005). Previous studies have shown that ectopic activation of the SIN led to the formation of actomyosin rings even in interphase cells (Cerutti and Simanis, 1999). We therefore tested if activation of SIN in interphase arrested *mid1*-18 and *mid1*Δ cells would lead to the formation of orthogonal actomyosin rings. This experiment was performed since it bypasses the normal requirement for entry

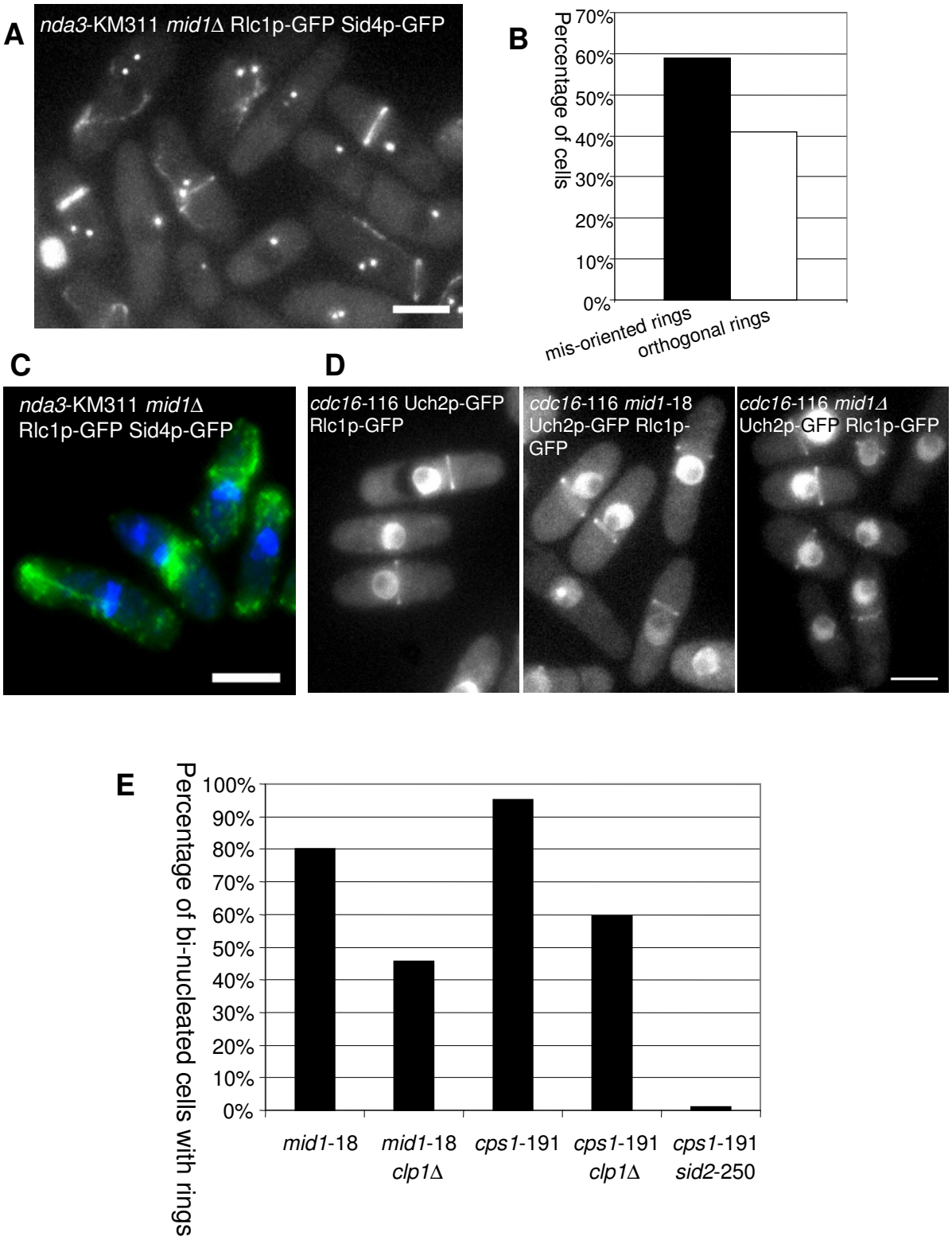
into mitosis for actomyosin ring assembly, which in turn is dependent on Mid1p function. We shifted *cdc16*-116, *cdc16*-116 *mid1*-18 and *cdc16*-116 *mid1*Δ cells expressing Rlc1p-GFP and Uch2p-GFP (as a nuclear marker) to the restrictive temperature for *cdc16*-116 mutants. Cdc16p is a GTPase activating protein for the Spg1p-GTPase, a key component of the SIN (Furge et al., 1998). Therefore *cdc16*-116 mutants maintain Spg1p in the GTP bound form, leading to the persistent activation of the SIN. Interestingly, actomyosin rings in *cdc16*-116, *cdc16*-116 *mid1*-18 and *cdc16*-116 *mid1*Δ were all found to be orthogonal in orientation (Figure 16 D). These studies established that activation of SIN led to the organization of orthogonal actomyosin rings in the absence of Mid1p and membrane anchored nodes.

The fact that actomyosin bundle and ring assembly was significantly compromised in cells lacking Mid1p and Clp1p (a positive regulator of the SIN) further established that ring assembly in the absence of Mid1p and the cortical nodes depended on the SIN (Figure 16 E). Additional genetic studies (Liu et al. , 1999; and figure 16 E) showed inability of *cps1*-191 *sid2*-250 and a reduced ability of *cps1*-191 *clp1*Δ cells to assemble and maintain actomyosin rings. These genetic studies provided further evidence that organization of actomyosin bundles in *mid1* mutants and their reorganization in the *cps1*-191 background depended on the function of the SIN.

Figure 16. Upon activation of the Septation initiation network, Mid1p and cortical nodes are not required for orthogonal ring assembly.

(A) *nda3*-KM311 *mid1*Δcells expressing Rlc1p-GFP and Sid4p-GFP were cultured at 18^{o}C for 6 hours and imaged by florescence microscopy. The SPBs are marked with pink asterisks. (B) Quantitation of orthogonal rings and mis-oriented rings in *nda3*-KM311 *mid1*Δ cells. (C) Prometaphse arrested *nda3*-KM311 *mid1*Δ cells were fixed and stained with Alexa 488-phalloidin and DAPI to visualize the F-actin cables and rings and the nuclei. (D) Upon SIN activation orthogonal actomyosin rings assemble in the absence of Mid1p and cortical nodes. Cells of the indicated genotypes expressing Rlc1p-GFP and Uch2p-GFP were arrested in interphase by treatment with hydroxyurea (12mM) for 6 hours at 24ºC, shifted to 36ºC to inactivate Cdc16p function in the presence of HU, and images captured. (E) Quatitation of presence of actomyosin rings / cables in binucleate cells of *mid1*-18, *mid1*-18 *clp1Δ*, *cps1*-191, *cps1*-191 *clp1*Δ and *cps1*-191 *sid2*-250. Cells of the indicated genotypes were cultured at 25ºC, and were then shifted to 36^{o}C for 3. 5 hours, fixed and stained with phalloidin and DAPI to visualize actomyosin rings / bundles and the nuclei respectively. At least 200 cells were scored for each genotype. Scale bars, 5μm.

Figure 16


A
nda3-KM311 mid1Δ Rlc1p-GFP Sid4p-GFP
B
Percentage of cells
70%
60%
50%
40%
30%
20%
10%
0%
mis-oriented rings
orthogonal rings
C
nda3-KM311 mid1Δ Rlc1p-GFP Sid4p-GFP
D
cdc16-116 Uch2p-GFP Rlc1p-GFP
cdc16-116 mid1-18 Uch2p-GFP Rlc1p-GFP
cdc16-116 mid1Δ Uch2p-GFP Rlc1p-GFP
E
Percentage of bi-nucleated cells with rings
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
mid1-18
mid1-18 clp1Δ
cps1-191
cps1-191 clp1Δ
cps1-191 sid2-250

4. 3 Discussion

Collectively our studies with two mutants lacking nodes (*mid1*Δ and *plo1*-1) have established that the accumulation of several components of the actomyosin ring into nodes is not essential for organization of orthogonal actomyosin rings. The fact that cells with no detectable nodes assemble normal rings suggests that although the nodes play an important role in expediting the process of actomyosin ring assembly, the presence of actomyosin ring components in medially placed nodes is not a prerequisite for assembly of actomyosin rings. It is likely that activation of the SIN pathway might lead to actomyosin contractility, which in turn might allow organization of actomyosin bundles / cables into a ring structure.

How then do Mid1p and the membrane-bound nodes participate in actomyosin ring assembly? Previous studies using electron microscopy of myosin SI decoration of F-actin filaments have shown that in early mitosis the actomyosin ring is composed of two parallel bundles of F-actin organized together with other components of the ring structure (Kamasaki et al., 2007). It is possible that such an arrangement is achieved by actin filament cross-linking, rather than by myosin II based contractility, which depends on anti-parallel organization of actin filaments. Consistent with the idea that myosin II may not be fully active in early mitosis, Lord and colleagues have shown that the localization of the UCS-domain protein Rng3p, an activator of fission yeast myosin II (Lord and Pollard, 2004), occurs only after completion of anaphase, during ring constriction. Finally, actomyosin ring constriction (via activation of myosin II based contractility) occurs concomitant with activation of SIN in anaphase B (Sparks et al., 1999).

Collectively, previous studies have shown that at the point of assembly of actomyosin rings, myosin II might not be fully active. It is therefore possible that Mid1p and membrane-associated nodes might play a key role in the medial maintenance of moderately non-contractile actomyosin bundles. However, activation of myosin II based contractility after anaphase, when SIN is activated and Rng3p is localized to the actomyosin ring might be sufficient for the organization of orthogonal actomyosin rings in the absence of Mid1p and detectable membrane-bound nodes. The Mid1p and membrane-anchored node mediated maintenance of actomyosin bundles in the medial cortex might be of importance only in the proper spatial coordination of cytokinesis. The Mid1p and membrane-anchored node dependent medial maintenance of actomyosin rings and bundles might not involve the formin Cdc12p, since it is detected in nodes only upon Latrunculin A treatment (Yonetani et al., 2008). This maintenance mechanism might instead depend on membrane-associated actomyosin ring proteins such as Mid1p and Cdc15p (Celton-Morizur et al., 2004; Takeda et al., 2004; Wachtler et al., 2006). Computational modeling and further cell biological analyses of actomyosin ring assembly in *cps1*-191 *mid1*Δ cells should provide additional insights into the general mechanism of cytokinesis.

5. Assembly of Microtubules and Actomyosin Rings in the Absence of Nuclei and Spindle Pole Bodies

5. 1 Introduction

Whether nuclei, chromosomes, and centrosomes are essential for cleavage furrow and microtubule assembly is an actively debated topic in cell biology. For long term, the nucleus and the centrosomes (spindle pole bodies; SPBs in yeast) are believed to play key roles in the organization of various cellular structures, such as the actomyosin ring and microtubules. However, more and more evidences of various models contradicted this long-lasting idea (Bucciarelli et al., 2003; Carazo-Salas and Nurse, 2006; Daga et al., 2006; Faruki et al., 2002; Khodjakov et al., 2000; Picard et al., 1988; Sluder et al., 1986; Zhang and Nicklas, 1995; Zhang and Nicklas, 1996). The ability to generate cells lacking nuclei and SPBs is key to the elucidation of the role of the nucleus and the SPB in various cellular processes.

In this chapter, we described a novel genetic method to efficiently exclude nuclei and SPBs from the fission yeast cells. We used this approach to examine the role of nuclei and centrosomes in cytoskeleton assembly. Our results supported the idea that organizations of microtubules and actomyosin rings / furrows can be occurred independent of chromosomes and centrosomes.

5. 2 Result

5. 2. 1 An efficient genetic method to generate anucleate cells

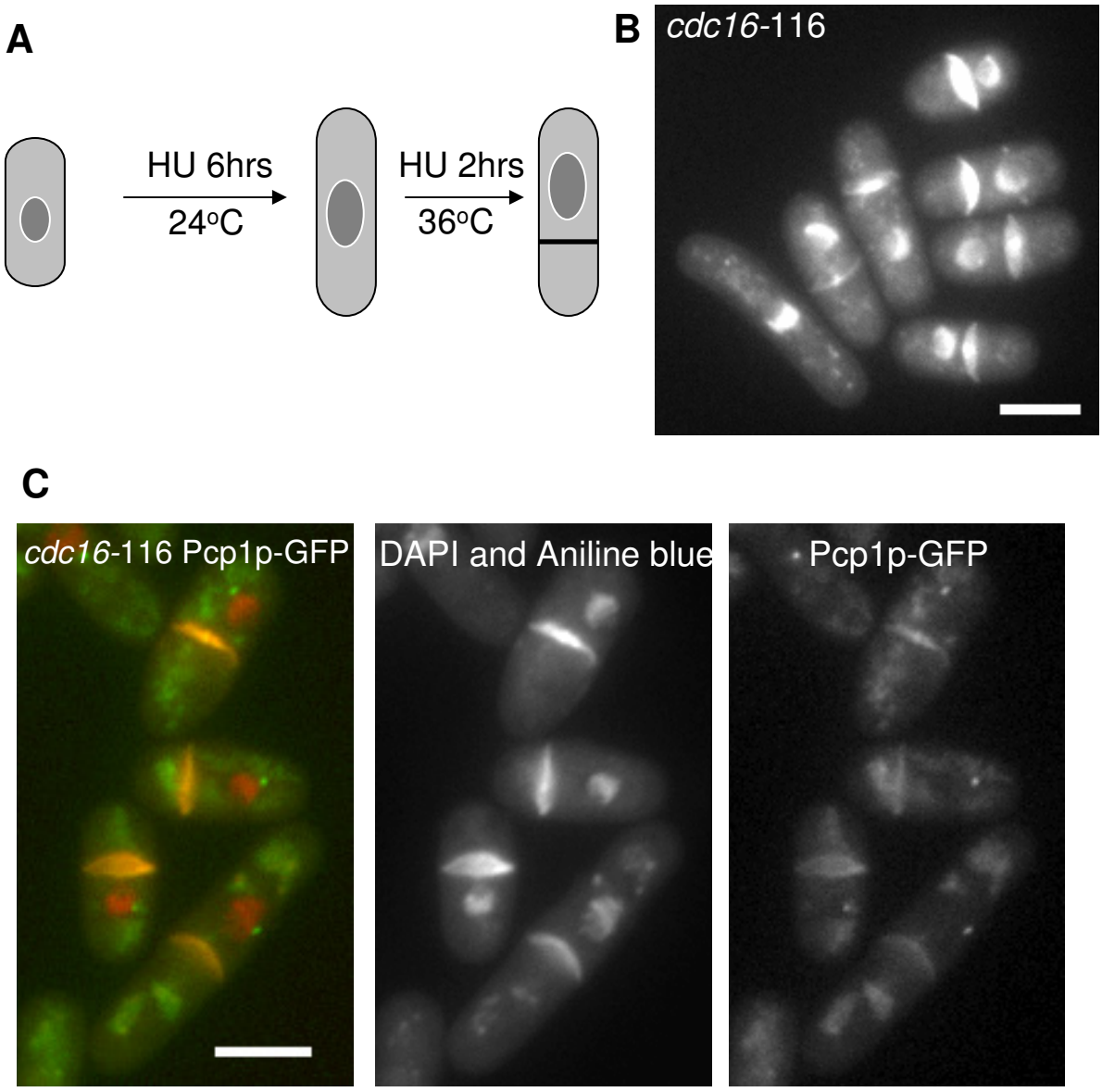
Recently, a method has been described to produce anucleate fission yeast cells (Carazo-Salas and Nurse, 2006; Daga and Chang, 2005; Daga et al., 2006). This method employs centrifugation to misplace the nucleus in asynchronously growing cells. The ensuing cytokinesis divides the cell into one binucleate and one anucleate daughter cell. However, formation of anucleate cells occurred at low frequencies when the centrifugation method was used. As an alternative, we made use of the temperature sensitive *cdc16*-116 mutant to generate a high number of cells lacking nuclei and SPBs.

The general strategy of this method is outlined in Figure 17A. It utilizes the ability of *cdc16* mutant cells to form division septa in interphase, when the cell contains only one nucleus. To enrich the population of cells in interphase, we treated cells with 12 mM hydroxyurea (a drug that prevents DNA synthesis) at the permissive temperature of 24^{o}C. After six hours of incubation with HU, the majority of cells were blocked in interphase due to activation of the S-phase checkpoint. Cells were shifted to the restrictive temperature 36^{o}C in the presence of HU to inactivate the function of *cdc16*, leading to formation of septa. As a result, such cells were divided into two compartments, one with a single nucleus, and the other without a nucleus (Figure 17B). The compartment with a

Figure 17. A genetic method to generate anucleate cells.

(A) Outline of the strategy to generate anucleate cells. *cdc16*-116 mutant cells were treated with HU for 6 hours at 24°C to arrest cells in interphase. In the presence of HU, cells were shifted to 36°C to inactive Cdc16p, leading to the formation of septa even when the cells were in interphase. The formation of septa divides the cells into two compartments, one of which lacks a nucleus. (B) *cdc16*-116 mutant cells, treated with HU as described above, were fixed and stained with DAPI and aniline blue to visualize nuclei and septa, respectively. Shown are the un-cleaved septa formed in interphase cells dividing the cell into two compartments, one of which lacks a nucleus. (C) Anucleate cells do not contain SPBs. *cdc16*-116 cells expressing Pcp1p-GFP were shifted to the restrictive temperature in the presence of HU, fixed and stained with DAPI and aniline blue to visualize nuclei and septa, respectively. Pcp1p-GFP (green) is associated with nuclei (red) and is not observed in anucleate compartments. Scale, 5 μm.

Figure 17


A
HU 6hrs
24°C
HU 2hrs
36°C
B
cdc16-116
C
cdc16-116 Pcp1p-GFP
DAPI and Aniline blue
Pcp1p-GFP

nucleus was usually larger than the one lacking a nucleus. By this method, we increased the percentage of anucleate cells in the population to 56. 2%. Among septated cells, 94. 6% of cells contained an anucleate compartment.

In fission yeast vegetative cells, the SPB is tightly associated with the nucleus. To examine whether anucleate compartments in *cdc16*-116 mutant cells contain SPBs, we visualized SPBs using the SPB marker Pcp1p-GFP, a calmodulin-binding protein, which is an essential component of SPB (Flory et al., 2002; Rajagopalan et al., 2004). In wild type cells, Pcp1p-GFP associates with SPBs throughout the cell cycle (Flory et al., 2002). *cdc16*-116 cells expressing Pcp1p-GFP were treated with HU and then shifted up as described. We found that Pcp1p-GFP associated with nuclei, and Pcp1p-GFP signal was not detected in the anucleate compartment (Figure 17C). This indicates that SPBs are absent in anucleate compartments in *cdc16*-116 mutant cells.

5. 2. 2 FRAP revealed distinct nucleate and anucleate compartments in *cdc16*-116 cells upon cytokinesis induced in interphase

Although our method generates high numbers of anucleate cells devoid of SPBs, we noted that during repeated rounds of cytokinesis, the nucleate and anucleate compartments remained attached after septation. To determine if the cytoplasm of the anucleate compartment is discontinuous from that of the attached nucleate compartment we performed fluorescence recovery after photobleaching (FRAP) studies. *cdc16*-116 cells expressing Rad24p-GFP, a 14-3-3 protein which resides largely in the cytoplasm

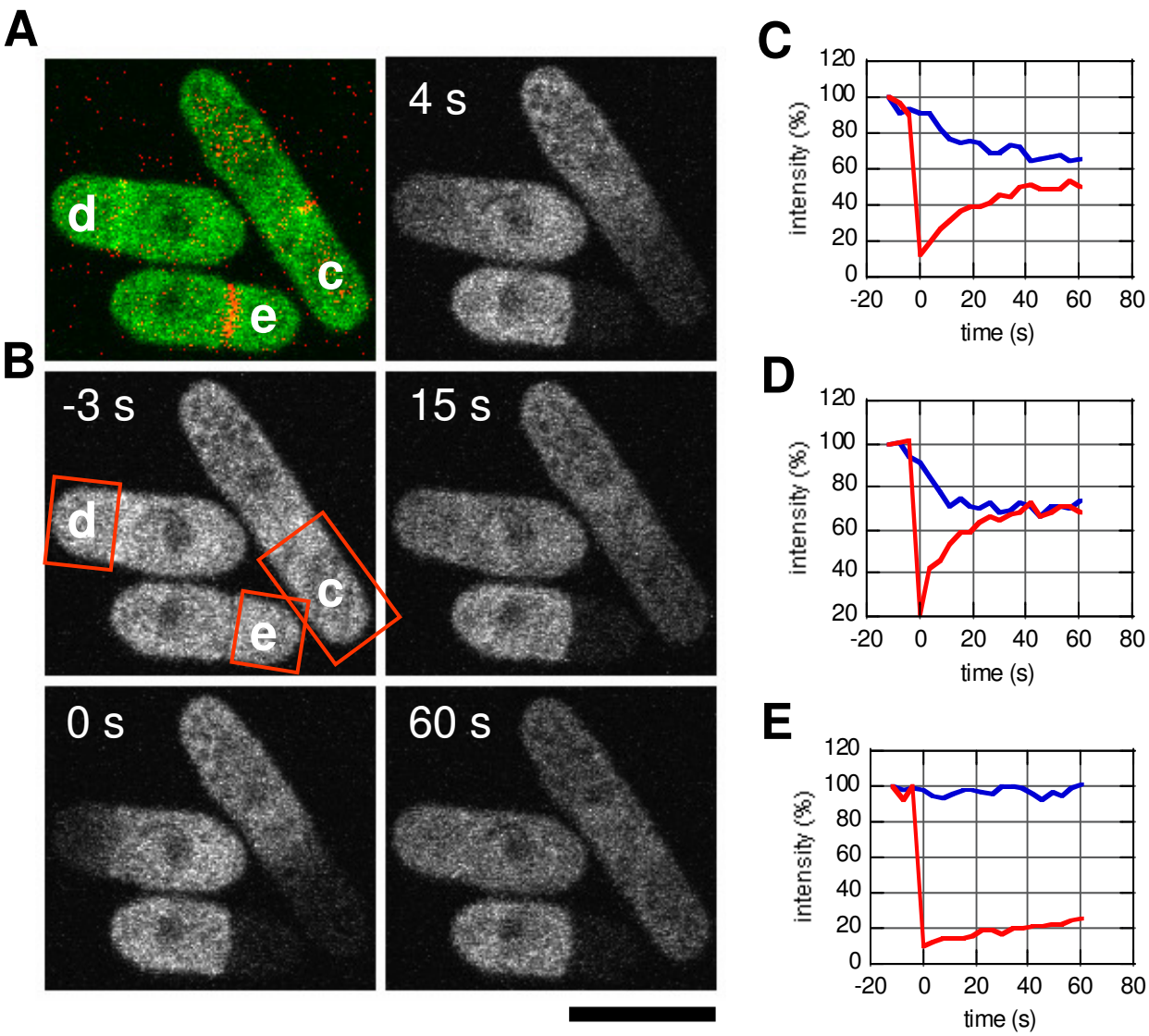
(Mishra et al., 2005), were used in these studies. Cells at different stages of septation are clearly distinguished by the cell wall staining with aniline blue (Figure 18A). We performed FRAP on cells early in septation (Figure 18B, cell d), half-way through septation (Figure 18B, cell c), and cells that had completed septation (Figure 18B, cell e). In cells which were in the process of septation, fluorescence recovery (recovery half-time $\tau_{1/2}$: ~ 8 s) was observed in the bleached half, and the corresponding fluorescent decay was observed in the unbleached half (Figure 18B, C and D). In contrast, in attached cells that had completed septation, no evidence of fluorescence recovery was observed (Figure 18B and E). To establish the limit of our error in scoring cells with completed septa, we performed FRAP on 408 cells which showed completed septa as judged by aniline blue staining as well as by Differential interference contrast (DIC) imaging. Only 4 of 408 cells showed partial recovery after bleaching. This represents a ~ 1% error rate in our method of scoring completely septated cells. The four cells that did show weak fluorescence recovery out of 408 photobleached cells might represent cells that were in the process of completion of septum assembly in which the division septum resembled a complete septum when visualized by anilin blue staining. Taken together, our data indicated that septation in *cdc16*-116 cells produced attached, but distinct, nucleate and anucleate cells. Thus, *bona fide* anucleate cells could be reliably generated for subsequent investigations of the role of nuclei and SPBs in various cellular processes.

5. 2. 3 Dynamic microtubules in anucleate cells

Figure 18. FRAP reveals distinct anucleate cells in the *cdc16*-116 mutant.

(A) Merged pseudo-colors image of *cdc16*-116 cells expressing Rad24p-GFP (green) stained with the cell wall tracer aniline blue (red). Temperature sensitive *cdc16*-116 cells were treated with hydroxyurea (HU) for 6 h to arrest cells at S phase, and shifted to the non-permissive temperature of 36°C for 2 h. The three cells shown are in different stages of septation, ranging from early septation (cell c) and mid-septation (cell d) with shared cytoplasm between the two daughter cell halves, to completed septation with a distinct cell wall separating the two daughter cell halves (cell e). (B) Fluorescence recovery after photobleaching (FRAP) was performed on cells at various stages of septation. Cell regions c, d, and e are regions that were photobleached (red dotted boxes). Shown is a time-lapse montage of the behavior of Rad24p-GFP within 60 s after photobleaching. (c, d, e) Normalized fluorescence recovery curves of cell c, d, and e, respectively. Cell c and d showed complete recovery within ~ 60 s after photobleaching ($\tau_{1/2}$ ~ 8 s). The unbleached cell halves (blue lines) showed fluorescence decay as a mirror image to the fluorescent recovery of the bleached cell halves (red lines). Cell e showed no significant recovery in the bleached half, and no decay in the unbleached half. Scale, 5 μm.

Figure 18


A
B
C
D
E
d
c
e
4 s
-3 s
15 s
0 s
60 s
intensity (%)
time (s)
120
100
80
60
40
20
0
-20
0
20
40
60
80

We asked if fission yeast cells could organize the microtubule cytoskeleton in the absence of nuclei and SPBs. There are three major microtubule organizing centers (MTOCs) in fission yeast – the SPBs, the interphase MTOCs (iMTOCs), and the mitotic equatorial MTOCs (eMTOCs). The SPBs and iMTOCs are associated with the nuclear envelope (Tran et al., 2001; West et al., 1998), and the eMTOCs are associated with the actomyosin ring (Heitz et al., 2001). It has been reported that *cdc16*-116 cells blocked at S phase and subjected to non-permissive temperature failed to make eMTOCs (Heitz et al., 2001).

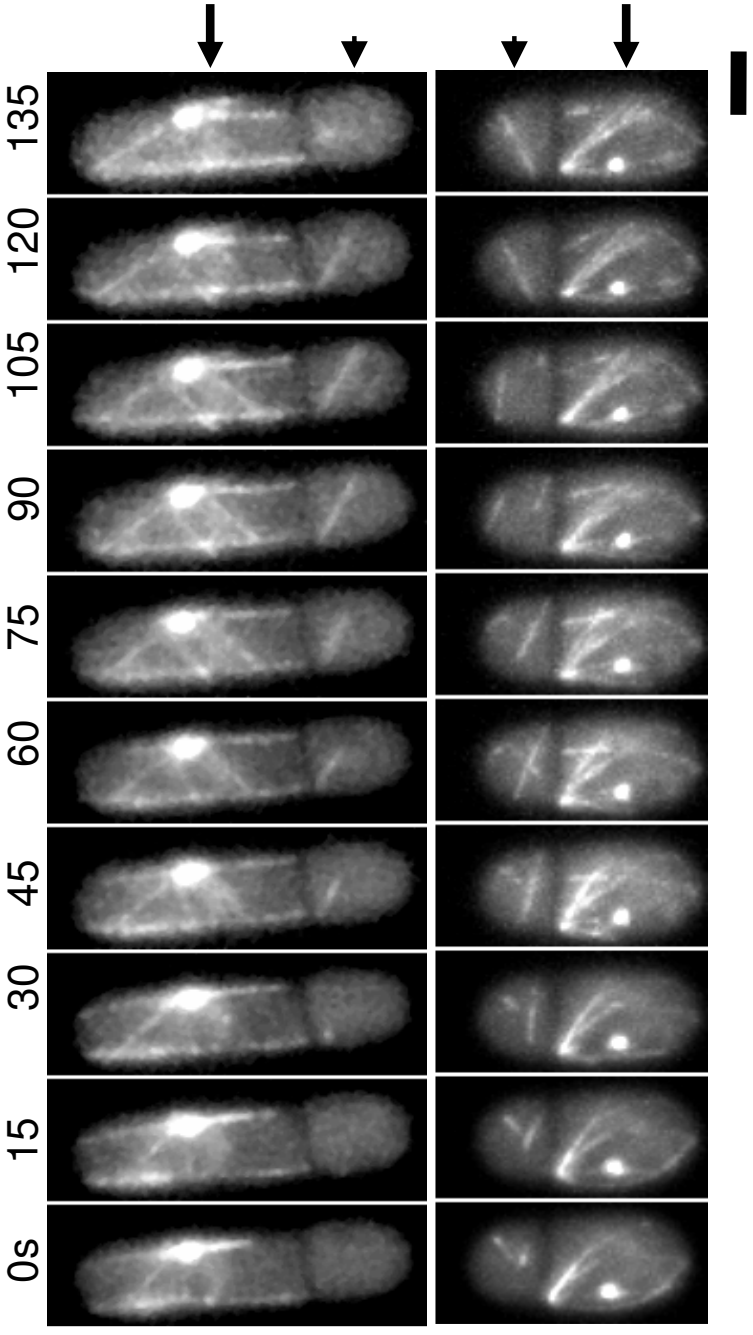
We imaged anucleate cells expressing GFP-α-tubulin to visualize microtubules. To identify the nucleate compartments, the SPB marker Pcp1p-GFP, was also expressed in these cells. In nucleate cells, we observed multiple robust and dynamic microtubule bundles (Figure 19, arrows indicates the compartments with nuclei). These microtubule bundles exhibited growth and shrinkage phases reminiscent of wild-type behavior. Interestingly, dynamic cytoplasmic microtubules were also present in anucleate compartments (Figure 19, arrowheads indicate the anucleate compartments). The behavior of anucleate microtubules resembled that of wild-type microtubules. This result is consistent with the recently published data (Carazo-Salas and Nurse, 2006; Daga et al., 2006). These two groups used a high-speed centrifugation method to generate anucleated cells, in which SIN is not activated. Thus, activated SIN signaling might not be required for microtubules assembly in anucleated cells. Taken together, these experiments indicate that microtubules can self-assemble in the absence of nuclei and SPBs.

Figure 19. Dynamic cytoplasmic microtubules are present in anucleate compartments.

Shown are two time-lapse montages of *cdc16*-116 Pcp1p-GFP cells expressing GFP-Atb2p (α-tubulin). The larger nucleate cells, indicated by arrows, have multiple robust and dynamic microtubule bundles. In contrast, the smaller anucleate cells, indicated by arrowheads, have fewer, yet dynamic microtubules.

Scale, 5 μm.

Figure 19



5. 2. 4 Actomyosin ring assembly in anucleate cells

We then examined if the actomyosin ring can assemble in anucleate cells. *cdc16*-116 cells expressing the nuclear envelope marker Uch2p-GFP (Li et al., 2000b) were treated as above to generate anucleate cells, and were stained with phalloidin to visualize F-actin (Figure 20A). Strikingly, nucleate and anucleate compartments were found to contain F-actin rings and/or cables. We observed F-actin ring structures in 4. 5% (9 out of 200) of anucleate cells. This percentage is statistically higher than the 1% error rate ($p<0.05$), and we therefore conclude that the F-actin ring structures are indeed organizing in the anucleate cells. Figure 20A shows examples of HU-arrested *cdc16*-116 cells, of which 6 contain rings of F-actin in both the nucleate and anucleate compartments, and 3 contain rings only in the anucleate compartment. F-actin rings in anucleate cells appeared normally organized, although occasionally we also observed cables of F-actin that were not integrated into the F-actin ring (Figure 20A). To analyze if the rings organized in anucleate cells also contained type II myosin, *cdc16*-116 cells expressing Uch2p-GFP and Rlc1p-GFP (Naqvi et al., 2000), an actomyosin ring protein related to type II myosin regulatory light chains, were treated as above and subjected to live cell imaging. In nucleate cells, actomyosin rings were organized and subsequently underwent constriction. These rings in the nucleate compartments did not assemble at the geometric center of the cell as shown previously for the division septa in interphase *cdc16*-116 cells (Minet et al., 1979). Consistent with the presence of F-actin rings in anucleate compartments, ~ 10% (8 out of 77 cells) of anucleate cells were observed to form rings containing Rlc1p-GFP. This percentage is statistically higher than the 1% error rate ($p<0.05$), and we therefore

conclude that the Rlc1p-GFP ring structures are indeed assembled in the anucleate cells. Some of these rings were assembled in the anucleate compartment following assembly of an additional septum in the adjacent nucleate compartment as shown in time lapse images. The rings in anucleate cells also assembled at random locations (Figure 20A). These anucleate-cell rings were qualitatively less intense compared to those in the nucleate cells and failed to constrict. Instead, they disassembled approximately 20-50 minutes after assembly (Figure 20B). These rings also appeared less robust and might be unstable, given that upon fixation only 9 out of 200 cells were found to contain F-actin rings, whereas in live cell time lapse imaging experiments 8 out of 77 cells were found to be capable of assembling Rlc1p-GFP rings. Thus, although the continued presence of nuclear-associated structures such as SPBs, chromosomes, and/or additional unidentified structures might be important for actomyosin ring constriction and division septum assembly, these structures are strictly not required for ring assembly.

Figure 20. Assembly of cell division structures in anucleate cells.

(A) F-actin ring assembly. Shown is a collage of fixed *cdc16*-116 cells expressing the nuclear membrane marker Uch2p-GFP stained with the F-actin specific dye Alexa 488-phalloidin. F-actin rings / cables present in the anucleate compartments are indicated with arrows, while asterisks identify the nucleate compartments. Scale, 10µm. (B) Actomyosin rings in anucleate cells are unstable and do not constrict. Shown is a time-lapse montage of a live *cdc16*-116 cell expressing the nuclear membrane marker Uch2p-GFP and the actomyosin ring marker Rlc1p-GFP. The connected but distinct cells have a nucleate half and an anucleate half. During time point 0 min to 25 min, a newly formed actomyosin ring was organized in the nucleate cell (arrow, 0 min). This ring underwent complete constriction within ~ 30 min. In contrast, although the anucleate cell organized an actomyosin ring (arrow, 40 min), this ring failed to undergo constriction during ~ 60 min of observation. Scale, 5 µm.

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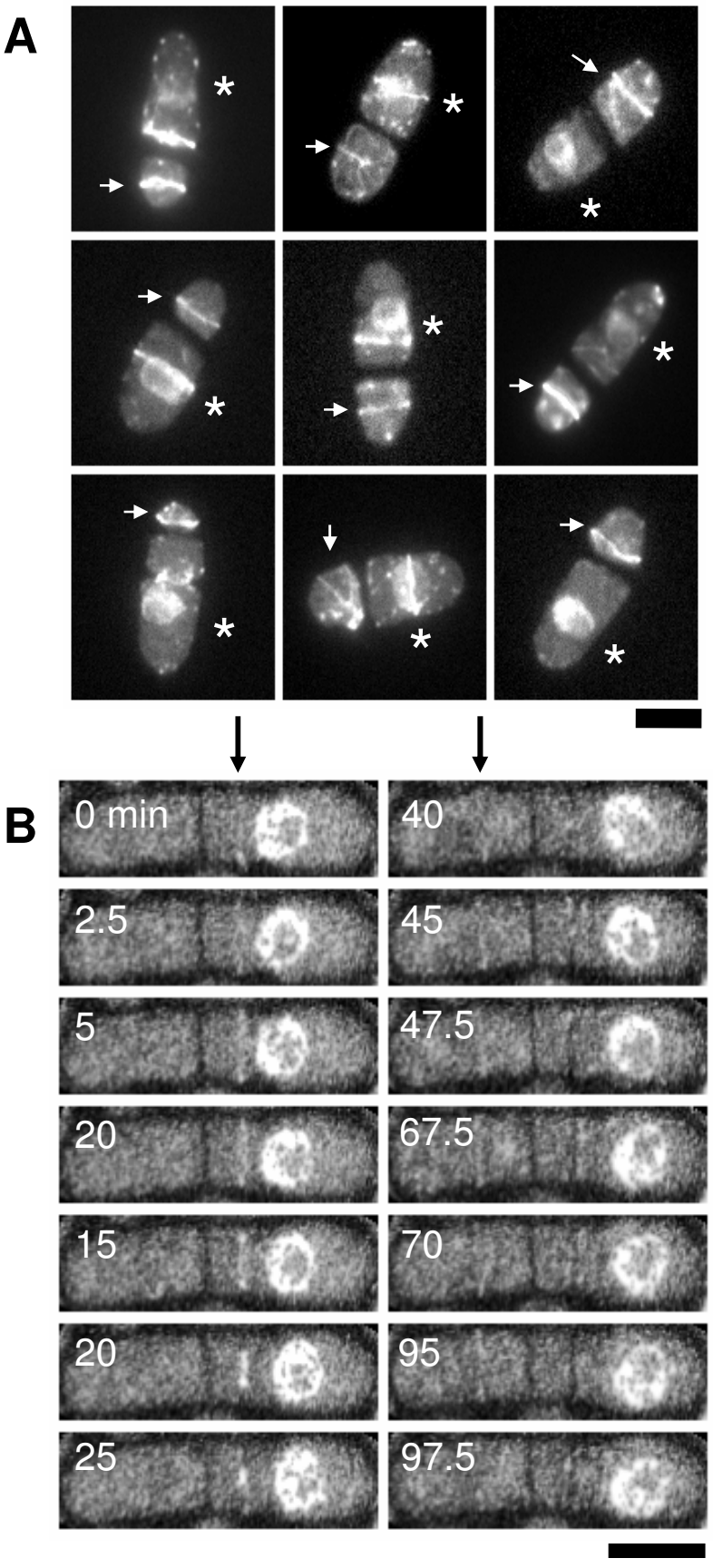
Figure 20



5. 3 Discussion

In this part we have described an efficient method for generation of anucleate fission yeast cells and used this approach to show that the assembly of actomyosin rings and microtubules can occur in the absence of nuclei and SPBs. Clearly, primary determinants such as the centrosomes, the nucleus, the spindle, or the chromosomes, are still essential for efficient organization and proper regulation of the microtubules and actomyosin ring. However, once committed to form rings, such as upon activation of the SIN pathway, the primary determinants are not continuously required to organize the ring.

Actomyosin rings can infrequently assemble in cells lacking nuclei and SPBs. However, these actomyosin rings do not constrict or support septum assembly, but instead disassemble. Thus, although actomyosin ring assembly, upon SIN activation, is independent of nuclei and SPBs, actomyosin ring constriction and division septum assembly depend on continued presence of nuclei, SPBs and / or other nuclear associated organelles such as the endoplasmic reticulum.

In animal cells, assembly of the cleavage furrow actomyosin structures depends on signaling from centrosomes, chromosomes, astral microtubules or spindle mid-zone microtubules (Balasubramanian et al., 2004). Our study has presented evidence for organization of actomyosin rings in cells lacking nuclei and SPBs in fission yeast. Thus, it is possible that, at least in some cell types, actomyosin ring assembly can occur in the absence of chromosomes, nuclei and SPBs upon cytoplasmic stimulation of cytokinesis.

Recent studies have shown that Drosophila mutants lacking chromosomes assemble normal and functional cleavage furrows(Bucciarelli et al., 2003). Furthermore, during male meiosis of Drosophila, cleavage furrows assemble in the absence of astral microtubules (Bonaccorsi et al., 1998). In future, it will be interesting to assess if animal cells completely devoid of nuclei, astral microtubules, and centrosomes (the SPB equivalent in higher eukaryotes) are able to assemble actomyosin cleavage furrows.

6. Conclusion and Future Directions

Cytokinesis consists of three major steps. The first step is to define the position at which the contractile ring will form. The second step in cytokinesis is to assemble the cell division apparatus. The third step is the constriction of the cell division ring that leads to physical cleavage. My studies provide some interesting insights into the mechanisms of positioning and assembly of the division ring.

Understanding how cells select and organize their division plane is of fundamental importance in cell and developmental biology. Whether specification of the division plane is through positive regulation or negative regulation, or a combination of the two is under vigorous debate. In bacteria, the Min system negatively regulates the formation of the division plane at the cell poles (Lutkenhaus, 2007). In animal cells, there is still no accepted model for positioning the cleavage furrow. My study provides a new entry into this debate using fission yeast as a model. In fission yeast, Mid1p functions in the cell center to define the division site (Chang et al., 1996; Sohrmann et al., 1996). It acts as a positive regulator; loss of Mid1p function leads to aberrant positioning of the division plane. However, even though the division plane is misplaced, the *mid1* mutant cells are still viable. In chapter III, my study uncovers a phenomenon, which I termed "tip-occlusion" that prevents *mid1* mutant cells from the assembly of division septa at cell tips, and hence ensures viability of the *mid1* mutant. The Mid1p-dependent mechanism and tip-occlusion function in positive and negative ways, respectively, to determine the division site. It is remarkable that a

similar combinatorial principle, negative regulation of the division plane at cell poles (astral relaxation or polar relaxation) and the positive regulation of division plane in the cell center (central spindle stimulation and/or astral stimulation), also positions the division site in animal cells too (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003; Glotzer, 2004; Motegi et al., 2006). Thus, the interplay between positive medial signals and a negative polar signal appears to be a conserved mechanism that improves the fidelity of the division plane specification.

Although there is no agreed model for cleavage furrow positioning in animal cells, microtubules are established as a key factor in this process. In fission yeast, like in animal cells, the microtubule network influences the definition of the division plane at least at three levels. Firstly, the interphase array of microtubules position the interphase nucleus in the medial region of cells (Tran et al., 2001). Mid1p, the key player specifying the division site, shuttles between the nucleus and the membrane cortex in the vicinity of the nucleus (Paoletti and Chang, 2000). By positioning the nucleus in the center of the cells, microtubules define the broad band of Mid1p to the cell center. Secondly, the tip-complex is delivered by interphase microtubules to the cell poles where ring anchoring is prevented (Huang et al., 2007). Finally, during late anaphase, eMTOC microtubules are required to anchor the ring at the cell center by unidentified mechanism (Pardo and Nurse, 2003).

In animal cells, the negative signals that inhibit contractility are relayed by astral microtubules (Glotzer, 2004). However, the nature of such signals remains unknown.

In the future, it will be interesting to investigate the cytokinesis function of potential tip-complex homologs in animal cells. Although no Tea1p orthologues are obvious in higher eukaryotes, Tea1p may have sequence and functional homology to the ezrin-radixin-moesin family of metazoan proteins, which are involved in tethering the plasma membrane to the underlying actin cytoskeleton (Snaith and Sawin, 2005). Pom1p is believed to act most down stream of the tip-complex. Pom1p belongs to the DYRK family of kinases (Snaith and Sawin, 2005). This family of proteins are evolutionarily conserved, with members identified from yeast to humans, that participate in a variety of cellular processes. Given that Pom1p is conserved, future studies should also examine whether related molecules in other organisms perform similar functions.

Partially compromised Cdc15p restores tip-occlusion in *mid1* defective as well as in small cells, indicating that cdc15 might be the target of the tip-complex. How does the tip-complex inhibit the function of Cdc15p? Cdc15p belongs to the FCH family (Aspenstrom, 2009; Aspenstrom et al., 2006), which has recently been shown to coordinate membrane and cytoskeletal dynamics (Fankhauser et al., 1995). Since Cdc15p is involved in medial actin ring assembly through recruitment of a formin Cdc12p and the Arp2/3 complex activator Myo1p to the division site, it is possible that Cdc15p might also interact with cell end-localized formin, For3p (Carnahan and Gould, 2003; Feierbach and Chang, 2001). In tip-complex deficient cells, the interaction of Cdc15p and For3p might promote actin ring anchoring at the cells end. Future work screening for *cdc15* alleles that are no longer inhibited by the tip-

complex, and hence allow septum misplacement at the cell tips, even when the tip-complex is intact, would be very helpful to investigate the molecular mechanisms of tip-occlusion.

An alternative mechanism of tip-occlusion is that Cdc15p might be phosphorylated by Pom1p at the cell end during cell division. In *pom1*Δ mutants, Cdc15p might be hypophsphorylated, leading the anchoring of the actomyosin ring at the cell ends. It is known that Pom1p kinase activity is at its lowest during cell division (Bahler and Nurse, 2001) and Cdc15p is known to be hypophospholated when cells form actomyosin rings (Fankhauser et al., 1995). Both pieces of evidence fit this hypothesis. However, careful biochemical analysis of Cdc15p regulation by Pom1p are required to test this hypothesis experimentally.

Another central question in cytokinesis research is how an actomyosin ring is assembled. The components of actomyosin rings in *S. pombe* are highly similar to those in animal cells and include actin, profilin (Cdc3p), formin (Cdc12p), tropomyosin (Cdc8p), IQGAP (Rng2p), type II myosin (Myo2p) and its associated light chains (Rlc1p and Cdc4p), FCH protein (Cdc15p) and anillin-like protein (Mid1p) (Balasubramanian et al., 2004; Balasubramanian et al., 1994; Chang et al., 1997; Chang et al., 1996; Fankhauser et al., 1995; Le Goff et al., 2000; Marks et al., 1986; McCollum et al., 1995; Nakano and Mabuchi, 2006; Sohrmann et al., 1996). For these reasons, *S. pombe* has become a good model for studying the mechanism of actomyosin ring assembly. Recently, there have been conflicting models for

actomyosin ring assembly in *S. pombe*. The 'leading cable model' suggests the actomyosin cables emanate from a single nucleation site. On the other hand, the 'search and capture model' states that actomyosin rings initiate from a series of membrane-bound nodes, and myosin II generates the tension to coalesce the nodes into a focused ring (Mishra and Oliferenko, 2008). However, Rng3p, the myosin II activator, is absent from the nodes in wild type cells and is only localized during ring constriction (Lord and Pollard, 2004; Wong et al., 2000). This is not consistent with the search and capture model.

In chapter IV, I tackled a central prediction of the search and capture model using a *mid1* mutant, in which the nodes do not assemble (Wu et al., 2006). According to the search and capture model, in the absence of nodes, the cells should be unable to assemble actomyosin rings or will assemble abnormal actomyosin rings. However, my studies have shown that normal orthogonal rings assemble in the absence of both Mid1p and cortical nodes, upon SIN activation and septum inhibition. Furthermore, activated SIN is sufficient to promote orthogonal actomyosin ring assembly in *S. pombe*, even in the absence of cortical nodes/Mid1p. Thus, my work challenges the search and capture model of ring assembly and also highlights SIN function in ring assembly.

Consistent with my findings, Viesturs Simanis and his colleges recently found that in SIN mutants although the actomyosin ring are able to assemble, the rings are discontinuous, thereby indicating a role of SIN in actomyosin ring assembly (Hachet

and Simanis, 2008). Furthermore, cells defective in Mid1p and SIN fail to form actomyosin rings. Their work, together with the results presented here, provide evidence that there is a specific role for the SIN in actomyosin ring assembly. Future computer modeling of actomyosin ring assembly should therefore involve also these two elements: Mid1p and SIN. This would generate a more complete understanding of the mechanism of actomyosin ring assembly.

The Mid1p-dependent mechanisms might function in parallel with SIN-dependent mechanisms to trigger actomyosin ring formation. The Mid1p-dependent mechanism might function in early mitosis, whereas a SIN-dependent mechanism would ensure mature homogeneous ring formation later in anaphase. Given that the major actomyosin ring components are conserved, it will be interesting to determine whether similar redundant mechanisms exist in other eukaryotic systems. Anillin, the Mid1p related protein in Drosophila, is known to regulate cytokinesis (Oegema et al., 2000). Anillin mutations cause defects in contractile ring formation. The homologs of SIN in animal cells have been identified (Guertin et al., 2002). However, the role of SIN homologs in ring assembly in animal cells is unclear.

What are the targets of activated SIN for ring assembly? Cdc15p, the FCH protein (Aspenstrom, 2009), is one candidate. In SIN mutants, medial recruitment of Cdc15p is affected (Hachet and Simanis, 2008). Furthermore, SIN activation is sufficient to promote the accumulation of the hypophosphorylated form of Cdc15p, which occurs during actomyosin ring formation in mitosis (Hachet and Simanis, 2008).

Consistently, cells lacking Mid1p and Cdc15p fail to form an actomyosin ring (Wachtler et al, 2006). In SIN mutant cells, actomyosin rings are not homogeneous (Hachet and Simanis, 2008). However, there is no report showing the discontinuous rings in cdc15 mutant cells. It would be very important to carefully examine the morphology of actomyosin ring in cdc15 mutant cells. Activating SIN in interphase cells is sufficient to trigger orthogonal ring formation (Schmidt et al., 1997). However, although overexpression of Cdc15p in interphase cells leads to the rearrangement of the F-actin filaments to the medial region, it is insufficient to trigger proper ring formation (Fankhauser et al., 1995). Hence, Cdc15p might not be the only target of SIN. Identification of SIN targets important for actomyosin ring formation is essential for fully understanding the mechanism of actomyosin ring assembly.

In conclusion, my work has discovered that a novel inhibitory mechanism, 'tip-occlusion', functions at the cell end to position the division plane. This mechanism cooperates with Mid1p-dependent positive regulation, ensuring the fidelity of division plane positioning and cell viability. My work also reveals that activated septation initiation network is sufficient to promote orthogonal ring formation and cortical nodes are not essential for actomyosin ring assembly. Given that the actomyosin-based contractile ring is crucial for cell division in various organisms, my studies presented here might help better understand cytokinesis in other organisms.

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