UNDERSTANDING THE REGULATION OF CYTOKINESIS IN FISSION YEAST

CHEW TING GANG
(B. Sc. (Hons), NUS)

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Summary

Cytokinesis is the final event of cell cycle during which a membranous physical barrier is established in a mother cell to generate two daughter cells. In most eukaryotes, cytokinesis is accomplished by the constriction of an actomyosin ring and is coordinated spatially and temporally with cellular geometry and nuclear division to ensure genome stability. In recent years, the rod-shaped fission yeast *Schizosaccharomyces pombe* has emerged as an attractive organism for the study of cytokinesis. Like animal cells, *S. pombe* utilizes an actomyosin ring for cell division. Upon entry into mitosis, an anilin-related protein Mid1p shuttles between the nucleus and the cell cortex to guide assembly of an orthogonal actomyosin ring in the middle of the cell. The nucleus provides a spatial cue for division plane specification. The nuclear position is regulated by interphase microtubule array(s) whose organization in turn is determined by cell morphology. Once the division site is specified, the actomyosin ring assembles and constricts to drive membrane assembly and invagination. A GTPase-driven signaling cascade, septation initiation network (SIN), is activated to coordinate actomyosin ring constriction, cell wall and new membrane assembly. SIN signaling is tightly regulated since precocious activation of SIN signaling results in uncontrolled cytokinesis. Mitotic cyclin/CDK1 complex whose activity is high during mitosis has been implicated in negative regulation of SIN signaling to prevent cytokinesis prior to chromosome segregation.

In this thesis, I have used *S. pombe* as a model organism to study the spatio-temporal regulation of cytokinesis.

To study how cytokinesis is regulated temporally, I investigated the role of a TPR-domain containing subunit of anaphase promoting complex (APC/C) Nuc2p. Nuc2p is a core subunit of APC/C and has been suggested to negatively regulate cytokinesis. However, the molecular mechanism behind this regulation remained unknown. In Chapter III of this study, I show that Nuc2p, by antagonizing SIN signaling, functions as a negative regulator of cytokinesis. Cells overexpressing Nuc2p phenocopied *sin* mutants in that the actomyosin rings were not maintained upon completion of mitosis. Examination of SIN proteins in the temperature-sensitive mutant *nuc2-663* showed that SIN signaling was maintained for prolonged period of time. Conversely, overexpression of Nuc2p led to de-localization of SIN component protein kinases Cdc7p and Sid1p from spindle pole body (SPB), and the disruption of binding between the small GTPase Spg1p and Cdc7p. Inactivation of GTPase Activating Protein Cdc16p, interestingly, promoted the localization of Cdc7p to the SPB and allowed septation in cells overexpressing Nuc2p. Genetic evidences further suggested that SIN-inhibitory function of Nuc2p might be independent of the other subunits of APC/C. These experiments established that Nuc2p antagonizes SIN signaling to prevent inappropriate cytokinesis.

To investigate if cell morphology played a role in the spatial regulation of cytokinesis in fission yeast, I have characterized a novel morphogenetic protein Pal1p. In chapter IV, I have shown that a cylindrical-morphology is crucial in positioning of the division plane in fission yeast cells. Pal1p localized to cell growth and division sites and was important for the maintenance of a cylindrical morphology. *pal1*Δ mutants were defective in cell wall integrity and displayed several morphological abnormalities including generation of spherical-shaped cells. Genetic analyses of

*pal1*Δ mutants suggested that Pal1p-mediated mechanism has a primary function in morphogenesis of fission yeast. In the absence of Pal1p, a Kelch-repeat containing protein Tea1p was required to establish a partial-cylindrical morphology. Failure to maintain a cylindrical-axis in *pal1*Δ *tea1*Δ mutants led to mis-positioning of division plane and anueploidy. These experiments established that a cylindrical-morphology provides an optimal spatial regulation of cytokinesis in fission yeast.

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LIST OF ABBREVIATIONS

aa	Amino acid
APC/C	Aanphase promoting complex.cyclosome
CDK	Cyclin-dependent kinase
CHFR	Checkpoint with FHA and ring finger
CKI	CDK inhibitor
DAPI	4', 6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
HU	Hydroxyurea
Kan	Kanamycin
LatA	Latrunculin A
Leu	Leucine
MBC	Methy 1-(butylcarbomyl)-2-benzimidazolecarbamate
mRFP	monomeric red fluorescent protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RING	Really Interesting New Gene
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPB	Spindle pole body
TPR	Tetratricopeptide repeat
UTR	Untranslated region
Ura	Uracil
YFP	Yellow fluorescent protein

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Chapter 1: Introduction

1.1 The cell division cycle

Proper inheritance of genetic material, encoded mainly in the form of deoxyribonucleic acid (DNA), is central to life of all cells. A cell propagates through a series of sequential events, collectively referred to as the cell cycle to transmit its genetic content to its daughter cells. Cell cycle consists of several steps: interphase (comprising G1, S, and G2 phases) and mitosis phase (M phase) which itself is subdivided into metaphase, anaphase, and telophase (see Figure 1; Nurse, 2002).

Interphase is a period that precedes the mitotic phase when cells duplicate their genetic content. During S phase, DNA is replicated and in many cases the spindle pole body (SPB, a functional analog of centrosome in fungus) is duplicated. Concomitantly, cohesion is established between the replicated sister chromatids. After full completion of DNA synthesis and building up a sufficient cell mass in G2 phase, cells enter mitosis, as characterized by chromosome condensation, nuclear membrane disassembly (in "open" mitosis of animal cells), and assembly of a mitotic spindle. The mitotic spindle, composed mainly of microtubules and associated factors, aligns the condensed chromosomes at cell equator during metaphase. Upon loss of cohesion between sister chromatids in anaphase, the mitotic spindle elongates to segregate the replicated chromosomes to opposing cell poles. A final cytoplasmic division termed cytokinesis follows after completion of telophase to divide the mother cell into two daughter cells.

Mitotic entry

Studies in several model organisms such as yeasts, frog, sea urchin, and human cells have led to the discovery of a conserved machinery that triggers mitosis. This machinery consists of a regulatory subunit termed cyclin and a protein kinase subunit termed cyclin-dependent kinase (CDK). The protein complex promotes the entry into mitosis in most, if not all, eukaryotes (Hunt, 2002; Masui, 2001; Nurse, 2002). The CDK activity is regulated by association of the protein kinase subunit with different cyclins to control the G1/S and G2/M transitions (Nurse, 2002). The mitotic cyclin/CDK1 phosphorylates various proteins such as condensins, nuclear lamins, and proteins that organize the mitotic spindle (Enoch *et al*., 1991; Jiang *et al*., 1998; Kimura *et al*., 1998). Mitotic CDK1 activity is positively regulated by the CDC25 family protein phosphatases and negatively regulated by WEE1 protein kinase and CDK inhibitors (CKIs) to control the timing of mitotic entry (Nurse, 2002). WEE1 catalyzes inhibitory phosphorylation of CDK1 at Threonine-14 and Tyrosine-15 residues in higher eukaryotes and Tyrosine-15 residue in yeasts. These residues are de-phosphorylated by CDC25 to promote entry into mitosis. The CDK inhibitors which are composed of INK4 gene family and Cip/Kip family regulate cell cycle by binding to either CDK or cyclin/CDK protein complex in multicellular organisms (Besson *et al*., 2008).

Mitotic exit

The major form of mitotic cyclin/CDK, B-type cyclin/CDK1, is activated during mitosis to promote chromosome condensation, and mitotic spindle assembly. After chromosome segregation, the activity of cyclinB/CDK1 is down-regulated to promote cytokinesis. A

multi-subunit E3 ubiquitin ligase, known as anaphase promoting complex/cyclosome (APC/C), participates in metaphase to anaphase transition and mitotic exit (Sullivan and Morgan, 2007). APC/C ubiquitinates at least two key cell cycle regulators: securin and mitotic cyclin to promote chromosome segregation and mitotic exit (Acquaviva and Pines, 2006). Securin inhibits a protease Separase which cleaves the cohesion complex associated with sister chromatids (Nasmyth *et al*., 2000). Proteolysis of securin following ubiquitination by APC/C activates Separase and triggers chromosome segregation. In addition, APC/C promotes the degradation of mitotic cyclin(s) to inactivate CDK1 (Glotzer *et al*., 1991). The proteolysis of mitotic cyclin allows events such as mitotic spindle disassembly and cytokinesis. In the absence of APC/C function, cells accumulate high B-type cyclin/CDK1 activity and are arrested at metaphase.

The core APC/C is composed of nearly 13 subunits in yeasts and animal cells (Thornton and Toczyski, 2006). A cullin-repeat protein APC2 and a RING finger-containing protein APC11 confer the substrate-dependent catalytic activity to the E3 ubiquitin ligase. APC1/APC4/APC5 provide a structural scaffold for the APC/C holoenzyme. Several Tetratricopeptide repeat (TPR)-containing proteins Cdc23/Cdc16/Cdc27 function primarily in adaptor binding. Two adaptors Cdc20 and Cdh1 activate APC/C and bridge the interaction with its substrates. It appears that there are multiple forms of APC/C that have different sub-cellular localizations and distinct functions (Huang and Raff, 2002; Pal *et al*., 2007). In *Drosophila* syncytial embryos, two TPR-containing APC/C subunits Cdc16 and Cdc27 localize differently, with Cdc27 being enriched at mitotic chromosomes, and Cdc16 excluded from mitotic chromosomes (Huang and Raff, 2002).

The loss of function study of Cdc16 and Cdc27 suggests that these TPR-containing proteins assemble into different forms of APC/C to execute distinct functions in *Drosophila* (Huang and Raff, 2002). Whereas Cdc20 and Cdh1 are present in all organisms, an additional GTPase driven signaling cascade termed Mitotic Exit Network (MEN) is present in the budding yeast *Saccharomyces cerevisiae* to regulate cyclin(s) proteolysis.

Failure to exit from mitosis inhibits replication origin firing, mitotic spindle disassembly, and the actomyosin ring constriction. Thus, the mechanisms inactivating B-type cyclin/CDK1 complex, are as important as the activating pathways. Concerted effects of cyclin/CDK, APC/C, and MEN ensures ordered progression of cell cycle.

1.2 Fission yeast as a model organism

Several organisms have been used to study the process of cell division. Among them, the work in fission yeast has contributed significantly to the identification of machinery and mechanisms involved in mitosis. The Nobel Prize 2001 in Physiology or Medicine awarded to Sir Paul Nurse recognizes his contribution to the understanding of cell division cycle by using fission yeast as a model organism (Nurse, 2002).

The fission yeast *Schizosaccharomyces pombe* is a rod-shaped unicellular organism that undergoes medial fission and polarized growth at the cell tips. A newly divided *S. pombe* cell first undergoes monopolar growth at the old cell end (the pre-existing cell end before

cell division), and then switches to bipolar growth during G2 phase to grow from both cell ends in a process known as new end take off (NETO) (see Figure 1; Mitchison and Nurse, 1985). Similar to animal cells, fission yeast utilizes F-actin and microtubules in cell growth and cell proliferation (Hagan and Hyams, 1988; Marks *et al*., 1986). F-actin in fission yeast is required to target membrane trafficking at the cell ends and division site. In the absence of F-actin, fission yeast is unable to polarize its growth, leading to the loss of polarity and viability. Microtubules are also involved in regulating cell polarity and are essential for chromosome segregation during mitosis. Although fission yeast undergoes a "closed mitosis" where the nuclear envelope remains intact, the processes of chromosome condensation and segregation are very similar to that of higher eukaryotes.

Fission yeast is amenable to genetic manipulations. Mutants defective in various biological processes such as cell cycle, cytokinesis, and morphogenesis have been isolated (Hirano *et al*., 1986; Nurse *et al*., 1976; Snell and Nurse, 1994). Characterization of these mutants has led to the identification of molecular components that participate in these processes. In addition, with its genome sequenced (Wood *et al*., 2002), the functions of a gene can be studied by making targeted knock-out based on homologous recombination. The sub-cellular localization of a protein can also be analyzed by fusing it to a reporter gene such as green fluorescent protein (GFP). The dynamics of the fusion proteins can be observed during cell growth or division by using time-lapse live cell fluorescence imaging. The ability to culture fission yeast in large volume also renders *S. pombe* a good system for proteomics.

Mutants defective in different cell cycle stages have been identified by forward genetic screen in fission yeast. The *cdc* mutants (*c*ell *d*ivision *c*ycle) that fail in various stages of cell cycle such as DNA replication, mitotic entry, and cytokinesis are one of the earliest group of mutants isolated in fission yeast (Nurse *et al*., 1976). Several regulators of mitosis including a cyclin/CDK protein complex Cdc2p-Cdc13p, and its regulators Cdc25p (a protein phosphatase) and Wee1p (a protein kinase) were identified from such genetic screens (Nurse, 2002). Further genetic screens to look for cells that are capable of nuclear division but not cytoplasmic division have led to the isolation of cytokinetic mutants *rng* (*r*i*ng*) and *sin* (*s*eptation *i*nitiation *n*etwork) mutants (Balasubramanian *et al*., 1998). The *rng* genes encode the structural components of the actomyosin-based contractile ring, whereas the *sin* gene products form a signaling cascade that regulates actomyosin ring maintenance and septum assembly (Balasubramanian *et al*., 1998). A group of mutants that is known as *cut* (*c*ell *u*ntimely *t*orn) mutants, is defective in mitosis and undergoes septation in the absence of chromosome segregation (Hirano *et al*., 1986; Samejima *et al*., 1993). Analyses of the *cut* mutants revealed genes that are involved mainly in chromosome condensation, cohesion, segregation, and assembly of anaphase promoting complex (APC/C) (Yanagida, 1998).

The study of cell morphogenesis and cell polarity has also benefited from analyses of *S. pombe* mutants. Mutants defective in establishing and/or maintaining their cell shapes have been isolated and grouped separately into *orb* (orb), *tea* (tip elongation aberrant), and *alp* (altered polarity) (Radcliffe *et al*., 1998; Verde *et al*., 1995). Characterization of *orb* mutants suggests that cell wall biogenesis and F-actin related processes are required

for establishment and maintenance of polarized growth at the cell tips to generate a cylindrical morphology (Verde *et al*., 1995). The *tea* and *alp* gene products are composed mainly of microtubule-associated factors which are responsible for the microtubule-based polarity establishment and the linear growth of fission yeast (Radcliffe *et al*., 1998; Verde *et al*., 1995). In the absence of these genes, fission yeast is bent or forms branches instead of a straight cylindrical morphology. Some of the morphogenetic mutants, in addition to their morphological defects, are also defective in NETO and display growth patterns which are different from wild type (Sawin *et al*., 1999).

Figure 1 The mitotic cell cycle of the fission yeast *Schizosaccharomyces pombe*. Cell cycle of fission yeast consists of G1 phase, S phase, G2 phase, and M phase. Newly-divided *S. pombe* cells first undergo monopolar growth and switch to bipolar growth in G2 phase. Upon entry into mitosis, *S. pombe* cells assemble an actomyosin ring in the middle of the cell and constrict the ring after completion of mitosis.

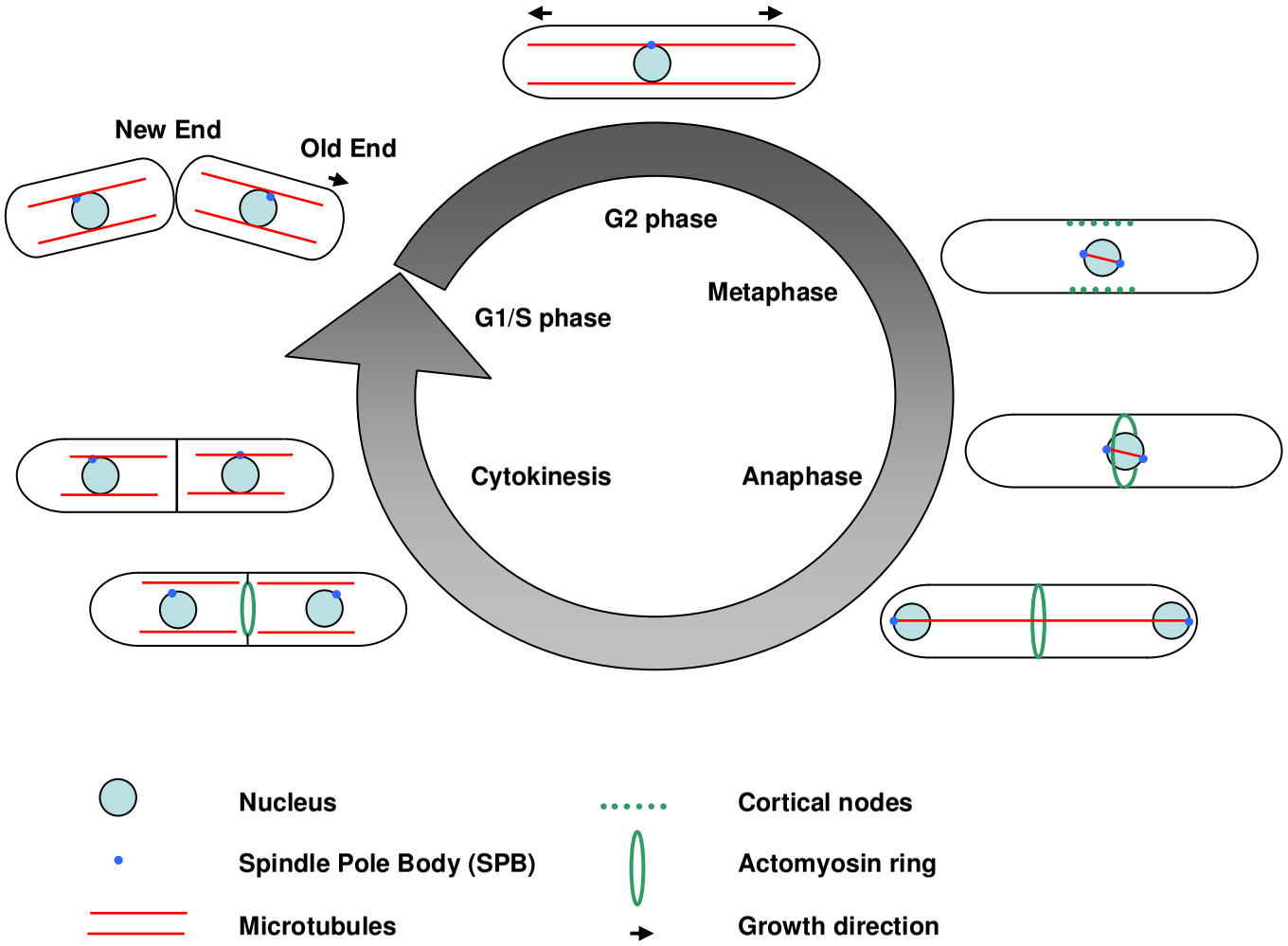


Figure 1 Cell cycle of the fission yeast *Schizosaccharomyces pombe*

1.3 Cytokinesis

The cell division cycle is not considered complete in most cell types until the two daughter cells carrying equal genetic material are separated from each other. The process that contributes to the final division of a mother cell into two daughter cells is known as cytokinesis. In most eukaryotes, particularly metazoans and yeast cells, cytokinesis is accomplished by the assembly and constriction of an actomyosin-based contractile ring. Following chromosome segregation, the actomyosin ring constricts and drives the invagination of plasma membranes in conjunction with assembly of new membrane and cell wall to form a barrier to generate two newly-born cells. Cytokinesis is well-coordinated in time and space with other cell cycle events to ensure genome stability.

Studies from a variety of model organisms ranging from yeast cells to metazoans have revealed the conserved and divergent mechanisms regulating cytokinesis. It appears that the molecular components involved in cytokinesis are evolutionary conserved, however the associated-regulatory processes bear species-specific features. The use of evolutionary conserved machineries for mitosis and cytokinesis in fission yeast has made this unicellular organism an attractive model for cytokinesis studies.

1.3.1 Molecular components of cytokinesis

1.3.1.1 Assembly of the actomyosin ring in metazoans

The cleavage of one cell into two daughter cells has long been observed and described in marine invertebrate embryos by embryologists in early and mid 20th century (Rappaport, 1961). Identification of F-actin and non-muscle myosin, myosin II, at the cleavage furrow led to the concept that an "actomyosin contractile ring" drives cytokinesis in animal cells (Fujiwara and Pollard, 1976; Schroeder, 1968 and 1973). Classic experiments performed by Mabuchi and Okuno (1977) further provided functional evidence that myosin II is the primary motor protein responsible for cytokinesis.

The contractile rings of animal cells are composed of several actin binding proteins such as alpha-actinin, tropomyosin, anillin, filamin, talin, radixin, and cofilin (Field and Alberts, 1995; Fujiwara *et al*., 1978; Gunsalus *et al*., 1995; Mabuchi *et al*., 1985; Nunnally *et al*., 1980; Oegema *et al*., 2000; Sanger *et al*., 1984; Sanger *et al*., 1994). These proteins participate in cross-linking and/or stabilization of F-actin, and anchor the actomyosin ring to plasma membrane. Together, they organize F-actin and myosin II into a dynamic contractile ring at the cleavage furrow. It has recently been shown that myosin II, in addition to its role as a force generator during cytokinesis, is required to regulate the dynamics of F-actin during cytokinesis (Guha *et al*., 2005; Murthy and Wadsworth, 2005).

In addition to F-actin, microtubules are required for assembly of the actomyosin ring in animal cells. The central spindle, which is an antiparallel microtubule is important in cytokinesis and is required to position a small GTPase RhoA at the cleavage furrow. RhoA activity is regulated by RhoGEF ECT2/Pebble/LET-21 and RhoGAP MgcRacGAP/RacGAP50C/CYK-4 (homologues in human, fly, and nematode respectively). Recent studies showed that RacGAP50C at the central spindle interacts with anillin which functions as a scaffold to link RhoA, F-actin, and myosin II at the cell cortex (D'Alvino *et al*., 2008; Gregory *et al*., 2008; Piekny and Glotzer, 2008).

1.3.1.2 Assembly of the actomyosin ring in fission yeast

It was initially thought that cytokinesis in fission yeast was accomplished by the formation of cell plate as in plant cells (Nurse *et al*., 1976). The presence of the F-actin contractile ring in fission yeast was discovered in late 80s and early 90s of 20th century (Balasubramanian *et al*., 1992; Marks *et al*., 1986). Staining of fission yeast cells with fluorescence-labeled phailloidin that recognizes F-actin revealed that the F-actin ring is formed in cell undergoing mitosis (Marks *et al*., 1986). Subsequently, molecular analysis of a tropomyosin mutant defective in the cell division (*cdc*), *cdc8*-110, further suggested that fission yeast, like animal cells, assembles the F-actin contractile ring for cytokinesis (Balasubramanian *et al*., 1992). Thereafter, further analyses of existing mutants and additional isolation of mutants defective in cytokinesis identified most components involved in cytokinesis (Balasubramanian *et al*., 1998; Chang *et al*., 1996).

The actomyosin-based contractile ring of fission yeast consists of a number of proteins: F-actin; profilin Cdc3p; tropomyosin Cdc8p; formin Cdc12p; cofilin Adf1p; FER/CIP homology protein Cdc15p; myosin heavy chain Myo2p; regulatory light chain of myosin Rlc1p; essential light chain of myosin Cdc4p; IQGAP-related protein Rng2p; UCS-domain containing protein Rng3p; alpha-actinin Ain1p; and paxillin Pxl1p (Balasubramanian *et al*., 1992; Balasubramanian *et al*., 1994; Chang *et al*., 1997; Eng *et al*., 1998; Fankhauser *et al*., 1995; Ge and Balasubramanian, 2008; Kitayama *et al*., 1997; Le Goff *et al*., 2000; McCollum *et al*., 1995; Nakano and Mabuchi, 2006; Naqvi *et al*., 2000; Pinar *et al*., 2008; Wong *et al*., 2000; Wu *et al*., 2001). These proteins form an intricate network and assemble into a contractile actomyosin ring during mitosis (Balasubramanian *et al*., 2004).

The cytokinetic proteins assemble sequentially at the division site in late G2 and early M phases (Wu *et al*., 2003). Prior entry into mitosis, an anillin-related protein Mid1p shuttles between nucleus and cell cortex (Wu *et al*., 2003). This leads to recruitment of myosin components: Myo2p, Cdc4p, and Rlc1p; actin-associated factors: Cdc15p, Cdc12p, and Rng2p to the division site (Wu *et al*., 2003). These proteins, similar to Mid1p, form a broad cortical band overlying the nucleus. This broad band of proteins then condenses into a structure containing F-actin and its associated components: Cdc8p and Ain1p (Wu *et al*., 2003). The actomyosin ring is highly dynamic and undergoes dramatic turnover (Pelham and Chang, 2002; Wong *et al*., 2002). The fact that an actin-severing protein Adf1p is required for formation and maintenance of the actomyosin ring

suggests that actin re-modeling might be important for cytokinesis (Nakano and Mabuchi, 2006).

How do multiple proteins assemble into a contractile ring? Two models have been proposed. Firstly, it has been suggested that the actomyosin ring originates from a myosin-progenitor spot that can be detected in interphase cell (Wong *et al*., 2002). Several ring components including Cdc12p, Cdc15p, Myo2p, Rlc1p, and Myp2p assemble into a spot-like structure prior entry into mitosis (Carnahan and Gould, 2003; Chang, 1999; Kitayama *et al*., 1997; Wong *et al*., 2002). It is thought that the spot provides a single nucleation site to assemble actomyosin cables (Mishra and Oliferenko, 2008). Subsequent cross-linking of F-actin network leads to the formation of a tight actomyosin ring (Mishra and Oliferenko, 2008). This model is consistent with the observation that F-actin aster emanates bidirectional from a single nucleation site during early assembly of the actomyosin ring (Arai and Mabuchi, 2002). More importantly, the directionality of individual actin filament has been showed by three-dimensional reconstruction of the F-actin ring at ultra-structural level (Kamasaki *et al*., 2007). Electron microscopic analyses revealed that the F-actin ring consists of two semicircles of parallel actin filaments running in opposite directions during early mitosis (Kamasaki *et al*., 2007). These findings support the hypothesis that the actomyosin ring is nucleated from a single site/spot.

The second model of actomyosin ring assembly is termed “search-capture-pull-release” mechanism and is mainly based on high-resolution fluorescence microscopy and

computational modeling (Wu *et al*., 2006; Vavylonis *et al*., 2008). Around 60 Mid1p-organized membrane-bound nodes containing Myo2p, Cdc15p, Cdc12p, and Rng2p have been observed at cell equator (Wu *et al*., 2006). In this model, Mid1p recruits Myo2p to the cortical nodes, followed by actin nucleating factor Cdc12p (Wu *et al*., 2006). The actin networks formed by the nucleating activity of Cdc12p are then pulled together by Myo2p (Wu *et al*., 2006). The connections between nodes are unstable and transient; hence the connections are constantly broken and re-established by search and capture mechanism (Vavylonis *et al*., 2008). The continuous activity of "search-capture-pull-release" leads to assembly of the actomyosin ring from various cortical nodes (Vavylonis *et al*., 2008). Thus, this model, in contrast to the first model, suggests that the actomyosin ring is assembled by a broad band of cortical proteins instead of a single nucleation site/spot, and the assembly process is stochastic and dynamic.

1.3.1.3 Signaling pathways involved in cytokinesis

Regulation of RhoA in the cleavage furrow of animal cells

In animal cells, central to regulation of cytokinesis is the function of a small GTPase RhoA and its regulators guanine nucleotide exchange factor (GEF) ECT2/Pebble/LET-21 and GTPase activating protein (GAP) MgcRacGAP/RacGAP50C/CYK-4 (homologues in human, fly, and nematode respectively). RhoA appears to modulate mDia, a formin-related protein, to promote polymerization of unbranched F-actin (Kohno *et al*., 1996; Watanabe *et al*., 1997). In addition, RhoA also regulates myosin II contractility by

controlling proteins that activate the myosin light chain (MLC) function (Matsumura, 2005).

The activity of RhoA depends on the central spindle whose assembly requires microtubules cross-linking protein PRC1 and MAST/Orbit, and centralspindlin (Mishima *et al*., 2002; Mollinari *et al*., 2002). Centralspindlin is an evolutionary conserved tetrameric protein complex that consists of a kinesin-like protein ZEN-4/MKLP1 and a RhoGAP MgcRacGAP/RacGAP50C/CYK-4 (Mishima *et al*., 2002). It has been shown that centralspindlin has microtubule bundling activity and is required to position RhoGEF ECT2/Pebble/LET-21 at the central spindle (Somer and Saint, 2003; Yuce *et al*., 2005). RhoGEF, once localized at the central spindle, positions and activates RhoA at the cleavage furrow (Yuce *et al*., 2005). Recent studies have shown that activation of RhoA requires another RhoGEF, GEF-H1 which is also localized at the central spindle (Birkenfeld *et al*., 2007). It appears that ECT2 is essential for RhoA localization at the cleavage furrow and GEF-H1 is required to load guanine nucleotide triphosphate (GTP) on RhoA (Birkenfeld *et al*., 2007).

Two protein kinases namely, Polo kinase and Aurora B have been implicated in regulating RhoA function. These protein kinases appear to modulate RhoA activity by regulating either RhoGEF or centralspindlin. Interestingly, study of Polo kinase Cdc5 in budding yeast revealed that Cdc5 phosphorylates GEF Tus1 and Rom2 to locally activate Rho1 (RhoA homologue in budding yeast) (Yoshida *et al*., 2006). Pharmacological inhibition of Polo kinase PLK1 in animal cells further demonstrated conservation of this

mechanism in higher eukaryotes (Petroncski *et al*., 2007*)*. Inhibition of PLK1 disrupts the binding between RhoGEF and centralspindlin, and leads to a failure of RhoA activation at the cleavage furrow (Petroncski *et al*., 2007). Aurora B, on the other hand, exerts its activity on RhoA by modulating RhoGAP (Minoshima *et al*., 2003). Aurora B is a component of chromosomal passenger complex which also contains INCENP, Survivin, and Borealin. These proteins re-localize from chromatin to centromere during mitosis, and travel to the central spindle during cytokinesis to specify the cleavage furrow. Aurora B phosphorylates MgcRacGAP and converts it into a RhoGAP of RhoA during cytokinesis (Minoshima *et al*., 2003).

Septation Initiation Network (SIN) in fission yeast

Fission yeast mutants defective in cytokinesis can be classified into two groups based on their phenotypes (Balasubramanian *et al*., 1998). The first group of mutants known as *rng* mutants (***r****i****ng***) does not assemble a proper actomyosin ring and fails to undergo cytokinesis. The second group of mutants is known as *sin* mutants (***s***eptation ***i***nitiation ***n***etwork) are capable of actomyosin ring assembly but are defective in maintaining the cytokinetic apparatus and septum deposition at the division site. Molecular analyses of these mutants revealed that *rng* genes encode for structural components of the actomyosin ring, whereas *sin* genes encode signaling molecules such as small GTPase, protein kinases, and GTPase Activating Protein.

It is now establsihed that SIN is a signal transduction pathway which is driven by a small GTPase to promote septum assembly (Simanis, 2003). It is analogous to Mitotic Exit Network (MEN) of budding yeast that promotes mitotic exit events such as mitotic cyclin proteolysis and mitotic spindle disassembly. The core components of SIN signaling consist of three protein kinases and their associated factors: Cdc7p-Spg1p, Sid1p-Cdc14p, and Sid2p-Mob1p. These molecules localize at the SPBs through the scaffold proteins Cdc11p-Sid4p, which reside at the SPBs throughout cell cycle. Activation of Spg1p is important for the initiation of SIN signaling and its GTPase activity is controlled by a two-component GTPase Activating Protein (GAP) Byr4p-Cdc16p. Activation of SIN signaling leads to delivery of a β-1, 3-glucan synthase Cps1p and septum assembly at the division site (Liu *et al*., 2002). In addition, SIN signaling is required to maintain a *cdc14*-family protein phosphatase Clp1p/Flp1p in the cytoplasm to antagonize cyclin-dependent kinase (CDK)-directed phosphorylation events (Trautmann *et al*., 2001).

The SIN proteins display dynamic localization throughout cell cycle. During interphase, Byr4p-Cdc16p localizes at the SPB to maintain Spg1p in GDP-bound inactive form (Furge *et al*., 1998). Upon entry into mitosis, Cdc16p is lost from the SPBs and Spg1p is converted into the GTP-bound active form. Since GTP-bound Spg1p binds preferentially to Cdc7p, this protein kinase is then recruited to both SPBs during metaphase (Sohrmann *et al*., 1998). In anaphase, Cdc7p is asymmetrically localized at one of the SPBs. After mitotic cyclin proteolysis, Sid1p-Cdc14p is recruited to the SPB where Cdc7p resides and Sid2p-Mob1p re-localizes to the division site from SPBs (Guertin *et al*., 2000; Sparks *et al*., 1999).

Interestingly, studies of NIMA protein kinase Fin1p in fission yeast revealed that the asymmetrically localized SIN proteins: Cdc7p and Sid1p-Cdc14p reside at the new SPB, and Fin1p appears to shut down SIN proteins at the old SPB (Grallert *et al*., 2004). It has been noted that multiple septations occur in cells that lose SIN asymmetry. Upon inactivation of Cdc16p, SIN signaling is hyperactive as characterized by the maintenance of Cdc7p and Sid1p-Cdc14p at both SPBs (Sohrmann *et al*., 1998). This leads to multiple rounds of cytokinesis in the absence of mitosis. Thus, it seems that asymmetrical localization of SIN proteins is crucial to ensure one cytokinetic event per cell cycle.

SIN signaling is regulated by several proteins. One of the regulators is Polo kinase, Plo1p, that functions upstream of SIN signaling to promote septation (Ohkura *et al*., 1995; Tanaka *et al*., 2001). Plo1p participates in various aspects of cell cycle and localizes at the SPBs and the division site during mitosis (Bahler *et al*., 1998). It is currently unclear how Plo1p regulates SIN signaling and cytokinesis. Interestingly, a checkpoint with FHA and ring finger (CHFR)-related protein Dma1p seems to negatively regulate SIN signaling by modulating the activity and/or localization of Plo1p (Guertin *et al*., 2002; Murone and Simanis, 1996). Cell cycle regulation of SIN signaling is crucial for maintaining genome stability since ectopic activation of SIN signaling results in precocious cytokinesis and physical damage to genetic material (The details of regulation of SIN signaling is further discussed in section 1.3.2.2).

Although the identities of SIN components are known, how SIN signaling regulates cytokinesis remains largely unclear. SIN signaling might impinge on cytokinesis by regulating the actomyosin ring components such as Myo2p and its associated factors. It is also possible that SIN signaling regulates membrane trafficking to promote membrane deposition and septum assembly at the division site.

1.3.2 Temporal regulation of cytokinesis

1.3.2.1 Cell cycle regulation of cytokinesis in metazoans

In animal cells, cytokinesis is initiated shortly after anaphase onset when chromosomes have segregated to opposing cell poles. It appears that there is a requirement to down-regulate the mitotic cyclin/CDK1 activity to initiate cytokinesis. Stabilization of mitotic cyclins leads to a prolonged activation of CDK activity and an inhibition of cytokinesis in either fly embryos or mammalian cells (Echard and O'Farrell, 2003; Wheatley et al., 1997).

Mitotic cyclin/CDK1 appears to regulate cytokinesis by inhibiting the activity of regulatory light chain of type II myosin (MRLC) (Satterwhite *et al*., 1992). The inhibition of MRLC by CDK1 ensures timely initiation of cytokinesis after metaphase. Mitotic cyclin/CDK1 also phosphorylates PRC1, which is a microtubule-binding and –bundling protein (Jiang *et al*., 1998). PRC1 is required to localize Polo kinase PLK1 to the central spindle to promote cytokinesis (Barr *et al*., 2004). However, the association of PRC1 and

PLK1 is inhibited during metaphase by mitotic cyclin/CDK1. During exit from mitosis, the inhibitory phosphorylation of PRC1 by CDK1 is alleviated, leading to the targeting of PLK1 to the central spindle (Neef *et al*., 2007; Rape, 2007). Thus, the antagonistic regulation of PRC1 by CDK1 and PLK1 ensures a timely activation of cytokinesis after chromosome segregation.

The multi-subunit E3 ubiquitin ligase APC/C which promotes cyclin proteolysis has been implicated in coordinating mitosis and cytokinesis. A recent study suggested that APC/C has a direct role in promoting cytokinesis (Shuster and Burgess, 2002). By manipulating the distance between the mitotic apparatus and cell cortex in sea urchin embryos, cleavage furrow can be induced even when mitotic cyclin/CDK1 is active (Shuster and Burgess, 2002). Inhibition of APC/C activity by competitive inhibitors or spindle assembly checkpoint (SAC), however, does not allow cytokinesis in the manipulated embryos (Shuster and Burgess, 2002). This study suggests that an inhibitor of cytokinesis, in addition to mitotic cyclin, might be removed by APC/C ubiquitination and proteolysis after anaphase onset to promote cytokinesis (Glotzer and Dechant, 2002).

Onset of cytokinesis is also controlled by phosphorylation and activation of MRLC function. Phosphorylation of MRLC at serine-19/threonin-18 by the protein kinases MRLC kinase and Rho effector kinases: ROCK and citron kinase has been shown to activate type-II myosin during cytokinesis (Matsumura *et al*., 1998). Since these protein kinases are regulated by RhoA which is activated during anaphase, the phosphorylation of MRLC, in principle, is coordinated in time with mitosis (Matsumura, 2005).

Recent studies have shown that F-actin cross-linking protein, alpha-actinin, modulates F-actin dynamics at the division site to ensure timely onset of cytokinesis in mammalian cells (Mukhina *et al*., 2007; Reichl and Robinson, 2007). Excessive alpha-actinin prevents actin turnover and inhibits cytokinesis; in contrast, depletion of alpha-actinin promotes cytokinesis (Mukhina *et al*., 2007). Thus, alpha-actinin and possibly other F-actin associated proteins, might modulate actomyosin-ring dynamics to control the timing of cytokinesis.

1.3.2.2 Cell cycle regulation of cytokinesis in fission yeast

Fission yeast assembles an actomyosin ring upon entry into mitosis, and the ring constricts after chromosome segregation and mitotic exit when the mitotic cyclin/CDK1 activity is low. Early assembly of the actomyosin ring in fission yeast poses great challenge to cells since premature cytokinesis might result in physical damage of genetic material.

Like in animal cells, the onset of cytokinesis in fission yeast is linked to down-regulation of the mitotic cyclin/CDK1 activity. Overexpression of non-degradable mitotic cyclin leads to a constitutively active CDK1 and an inhibition of cytokinesis (Chang *et al*., 2001; Yamano *et al*., 1996). In contrast, inactivation of Cdc2p (CDK1 homolog in fission yeast) using either a temperature-sensitive mutant or a chemical genetic approach leads to premature cytokinesis before chromosome segregation (Dischinger *et al*., 2008; He *et al*.,

1997). These studies collectively suggest a requirement for CDK1 inactivation in promoting cytokinesis. However, a molecular link between the mitotic cyclin/CDK1 and cytokinesis remains largely unclear and awaits future investigation.

Central to temporal regulation of cytokinesis in fission yeast is the small GTPase-driven SIN signaling. Activation of SIN signaling can be divided into three steps. Firstly, the GTP-bound Spg1p recruits Cdc7p to both SPBs in metaphase. Secondly, upon mitotic cyclin proteolysis, Sid1p-Cdc14p complex localizes to one of the SPBs. Lastly, Sid2p-Mob1p complex re-localizes from SPBs to the division site. Since the second step of SIN signaling activation depends on mitotic cyclin proteolysis and inactivation of CDK1 activity, the onset of cytokinesis is coordinated with mitosis (Chang *et al*., 2001; Dishinger *et al*., 2008; Guertin *et al*., 2000).

The spindle assembly checkpoint (SAC) has been suggested to regulate cytokinesis in fission yeast, in addition to its role in ensuring kinetochore-spindle attachment. Inactivation of the SAC in cells defective in chromosome segregation leads to cytokinesis in the absence of karyokinesis (He *et al*., 1997). In contrast, hyperactivation of the SAC by overexpressing a SAC component, Mad2p, leads to an inhibition of metaphase to anaphase transition and cytokinesis. Thus, the SAC might prevent the early-assembled actomyosin ring from constriction before chromosome segregation. It appears that the SAC blocks cytokinesis by regulating SIN signaling. Cells that are defective in chromosome segregation undergo precocious cytokinesis when SIN signaling is ectopically activated (Fankhauser *et al*., 1993). A CHFR-related protein, Dma1p seems to

antagonize Plo1p to inhibit SIN signaling upon the SAC activation (Guertin *et al*., 2002; Murone and Simanis, 1996).

In addition to the SAC, SIN signaling is also negatively regulated by a two-component GTPase-Activating Protein (GAP), Byr4p-Cdc16p (Furge *et al*., 1998). The GAP down-regulates the activity of Spg1p and SIN signaling after completion of cytokinesis. Failure to inactivate Spg1p leads to constitutive cytokinesis and defective cell growth and division (Minet *et al*., 1979; Schmidt *et al*., 1997). Thus, the Byr4-Cdc16p ensures a timely inactivation of SIN and one cytokinetic event in every cell cycle.

1.3.3 Spatial regulation of cytokinesis

1.3.3.1 Positioning of division plane in metazoans

The process of division plane positioning in metazoans has been studied primarily in echinoderms, nematodes, and mammalian cells. It is now clear that the division plane in these animal cells is determined by the position of spindle during anaphase. Depending on the cell type, two parts of spindle: central spindle and astral microtubules function redundantly to specify the cleavage plane. It seems that embryonic cells with bigger cell size use primarily astral microtubules for division plane specification, while smaller somatic cells use preferentially the central spindle to establish the division site.

Classic experiments performed by Ray Rappaport more than 40 years ago in echinoderm embryos have elegantly demonstrated that the mitotic apparatus, more specifically astral microtubules, positions the cleavage furrow (Rappaport, 1961). This mechanism is widely known as astral stimulation model (reviewed in Pollard, 2004; Rappaport, 1961). Manipulation of a fertilized sand dollar egg into a torus-shaped embryo resulted in a pseudo-furrow in between two overlapping astral microtubules. These results strongly suggested that astral microtubules are sufficient to establish cleavage furrow (Rappaport, 1961). Rappaport further proposed that an inductive signal was transported by astral microtubules after mitosis to specify the division plane (Rappaport, 1961).

Recent studies using a nematode, *Caenorhabditis elegans*, that is amenable to genetic manipulation reveal a redundant requirement of astral microtubules and central spindle for cleavage furrow formation (Bringmann and Hyman, 2005; Dechant and Glotzer, 2003). The molecular components of aster-positioned cytokinesis have also been identified in these studies (Bringmann and Hyman, 2005; Bringmann *et al*., 2007; Dechant and Glotzer, 2003). By severing the connection between aster and its associated chromatin mechanically, it was shown that two consecutive signals derived from astral microtubules and central spindle specify the cleavage furrow (Bringmann and Hyman, 2005). A genetic screen to identify proteins that are essential for cytokinesis in the absence of a central spindle revealed an involvement of heterotrimeric G-protein pathway in aster-positioned cytokinesis. This molecular pathway comprises GOA-1/GPA-16 (heterotrimeric G-protein), its regulator GPR-1/2, and a DEP-domain protein LET-99

(Bringmann *et al*., 2007). Further characterization of these proteins might provide mechanistic insights in the aster-positioned cytokinesis.

An opposing model of astral stimulation which is known as astral relaxation or polar relaxation model has been proposed since 1960s. According to the model, astral microtubules at the polar region inhibit the contractility of cell cortex at the cell poles in a density-dependent manner (Glotzer, 2004). The observation that *C. elegans* embryos over-expressing a microtubule-severing protein katanin form short microtubules and ectopic cleavage furrows supports this model (Kurz *et al*., 2002). Mammalian cells that are forced to exit mitosis in the absence of microtubules, similarly, display vigorous unorganized cortical contractility (Canman *et al*., 2000). These studies suggest that microtubules inhibit cortical contractility and possibly cleavage furrow formation. Reduction of local microtubule density in the vicinity of presumptive cleavage furrow by central spindle assembly and centrosome separation appears to promote cytokinesis (Dechant and Glotzer, 2003). In addition, by studying myosin II distribution using fluorescence microscopy with high temporal and spatial resolutions, it was revealed that astral microtubules locally inhibit cortical recruitment of myosin and promote actomyosin ring assembly at the division site (Werner *et al*., 2007).

In certain cell types, the cleavage furrow is positioned normally in the absence of astral microtubules (Bonaccorsi *et al*., 1998; Cao and Wang, 1996). In these cells, it is the central spindle, but not astral microtubules, that plays a pivotal role in the cleavage

furrow specification (Bonaccorsi *et al*., 1998; Cao and Wang, 1996). The central spindle delivers Rho-regulators to convey spatial information to the cell cortex.

The mechanisms to position division plane in animal cells are highly divergent. However, it is clear that astral microtubules and the central spindle act together to specify the cleavage plane in various organisms. The use of multiple pathways that are either stimulatory or inhibitory provides robustness and fidelity in the positioning of the cleavage furrow.

1.3.3.2 Positioning of division plane in fission yeast

Fission yeast divides in the middle to generate two equal-sized daughter cells. How a fission yeast cell determines its division plane precisely in the medial region has been the subject of several studies. In contrast to animal cell cytokinesis, microtubules are not required directly for positioning and assembly of the actomyosin ring in fission yeast. It is possible that the "closed mitosis" in yeast cells prevents an effective interaction between mitotic apparatus and cell cortex.

Genetics analyses of fission yeast mutants revealed the molecular identities of the positional determinants of division plane. One of the factors, Mid1p, plays a pivotal role in the medial positioning of division site. In the absence of Mid1p function, the actomyosin ring and the division septum are not medially-assembled; instead, the

division plane is mis-positioned and mis-oriented, resulting in improper cell division (Balasubramanian *et al*., 1998; Chang *et al*., 1996; Sohrmann *et al*., 1996).

Mid1p, which is related to anillin in animal cells, contains a PH-domain at its C-terminus and displays a dynamic sub-cellular localization during cell cycle. It shuttles between nucleus and cell cortex to form a medial broad band during interphase and early mitosis. It eventually compacts into a tight ring structure prior to ring constriction and leaves the ring during ring constriction (Paoletti and Chang, 2000; Sohrmann *et al*., 1996). Shuttling of Mid1p between nucleus and cortical medial broad band is partly dependent on Polo kinase, Plo1p (Bahler *et al*., 1998; Paoletti and Chang, 2000). Once Mid1p is localized at the medial cell cortex, it interacts with the Myosin heavy chain, Myo2p, to promote assembly of the actomyosin ring (Motegi *et al*., 2004). The fact that Mid1p shuttles between nucleus and cell cortex suggests that Mid1p links the position of nucleus to that of the division site (Paoletti and Chang, 2000). The nucleus is maintained in the middle of cell through the pushing forces exerted by interphase microtubule arrays (Daga and Chang, 2005; Tran *et al*., 2001). A cell-end localized DYRK-family protein kinase Pom1p appears to restrict the cortical localization of Mid1p to the medial cortical region by inhibiting Mid1p at one of the cell ends (Celton-Morizur *et al*., 2006; Padte *et al*., 2006). However it is not known what blocks Mid1p at the old end. This negative regulation of Mid1p by Pom1p might explain the mis-placed division plane phenotype displayed in *pom1* deficient cells (Bahler and Pringle, 1998).

Interestingly, careful characterization of cells defective in Mid1p function suggests an additional mechanism participates in the spatial regulation of cytokinesis. It was frequently noted that the mis-positioned and mis-oriented septa in *mid1* defective cell are "occluded" from the cell ends. Compromising the functions of cell-end-localized polarity factors (tip complex): Tea1p (Kelch-repeat protein), Tea4p/Wsh3p (SH3-domain protein), and Pom1p in *mid1* defective cell leads to septum assembly at the cell ends. These findings suggest that the tip complex negatively regulates the division plane positioning (Huang *et al*., 2007). The tip-complex appears to delay septum assembly at the cell tips by inhibiting the FER/CIP homology protein Cdc15p (Huang et al., 2007).

Collectively, positioning of the division plane in fission yeast is regulated by a dual mechanism in which Mid1p positively promotes actomyosin ring assembly in the medial region, while the tip complex prevents septum assembly at the cell tips. These mechanisms collaboratively ensure assembly of the actomyosin ring and the division septum at cell equator.

1.3.3.3 Morphogenesis and spatial regulation of cytokinesis

Positioning of the division plane in metazoans and fission yeast is directly or indirectly determined by microtubules. In animal cells, spindle orientation specifies the axis of division. The fission yeast, on the other hand, uses interphase microtubule arrays to position nucleus, and the nucleus in turn, determines the division axis. Thus, factors that

influence spindle orientation in animal cells, or organization of interphase microtubule arrays in fission yeast might contribute to the spatial regulation of cytokinesis.

In certain animal cells, the spindle orientation and division plane are influenced by cell shape. It appears that, in the absence of cell polarity cues, the longitudinal cell axis acts as a default determinant to position the mitotic spindle and the division machinery. The mitotic spindle tends to align with cell's long axis to specify the cytokinetic apparatus. This geometrical rule is applicable to cultured cell lines such as rat epithelial cell, and the early dividing cells in *Caenorhadbitis elegans* embryos and mouse embryos (Gray *et al*., 2004; O'Connell and Wang, 2000). It is thought that the tension in astral microtubules is stronger when microtubules are longer and parallel to the surface. Thus, it is more feasible for a cell to divide its chromosomes along the long cell axis (O'Connell and Wang, 2000; Tsou *et al*., 2003). Interestingly, a similar requirement of cell geometry has been suggested in fission yeast cytokinesis that is not dependent on astral microtubules (Sipiczki *et al*., 2000).

Cells divide perpendicular to the long cell axis to avoid physical damage to genetic material. The cell geometry itself allows for ample space for mitotic spindle to elongate. However, often cells do not follow the geometrical rule for their division. In some cases, particularly in polarized cell types, positioning of the division plane largely depends on cell polarity. This is more evident in embryos where the embryonic axes determine the cell division orientation. Studies of *Drosophila* neuroblast division show that embryonic

axes determine distribution of polarity factors to guide spindle orientation and cleavage plane positioning (Wodarz, 2005).

In fission yeast, the polarized cell growth is regulated by several polarity factors that localize at the cell ends. In the absence of these polarity factors, the cells are unable to maintain their linear growth pattern, and assume a bent or branched morphology. In some cases, these cells misplace their division plane (Arellano *et al*., 2002). The cell-end localized polarity factors consist of a Kelch-repeat protein Tea1p, a Tea1p-related protein Tea3p, and a SH3 domain protein Tea4p. These proteins form a complex and are delivered by microtubules from vicinity of the nucleus to the cell ends (Martin *et al*., 2005; Snaith *et al*., 2005). A kinesin motor Tea2p, together with the CLIP170 homologue Tip1p and the EB1 protein Mal3p, are responsible for the microtubule-based transport of these cell-end polarity factors (Browning *et al*., 2003). These polarity factors affect microtubule dynamics and organization to promote polarized growth (Behrens and Nurse, 2002). These polarity factors establish an interphase microtubule array that runs parallel to cell's long axis. Such an organization of microtubules ensures the medial-positioning of the nucleus and division plane (Tran *et al*., 2001). Thus, cells that are unable to localize the polarity factors at cell ends display an off-centre division septum, partly because of the perturbed organization of interphase microtubule arrays and partly due to Mid1p at cell ends.

1.4 Aims of the study

Constriction of the actomyosin ring cleaves one cell into two daughter cells. Proper spatio-temporal coordination of cytokinesis is crucial to maintain genome stability. Mis-regulation of cytokinesis might lead to aneuploidy which is a hallmark of tumor cells or malignant cancer cells.

This study aimed to elucidate the temporal and spatial regulation of cytokinesis in fission yeast. First, I investigated how cytokinesis is coordinated in time with mitosis. An evolutionary conserved TPR-containing subunit of APC/C, Nuc2p, has been suggested to negatively regulate cytokinesis. The molecular mechanism and the physiological roles of Nuc2p in cytokinesis were further examined in this study.

Secondly, this study also aimed to investigate if cell morphogenesis played any role in spatial regulation of cytokinesis. Fission yeast establishes and maintains a cylindrical morphology during cell growth. I investigated the importance of cell geometry on positioning of the division plane.

Chapter 2: Materials and methods

2.1 *S. pombe* strains, media, and reagents

S. pombe strains used in the studies are listed in Table 1A and 1B. YES medium (5g/l Difco yeast extract, 30g/l Glucose, 82.5mg/l histidine, 82.5mg/l leucine, 112.5mg/l adenine, 82.5mg/l uracil) or Minimal medium (3g/l potassium hydrogen phthalate, 2.2g/l disodium hydrogen phosphate, 5g/l ammonium chloride, 20g/l glucose, salt solution, minerals solution, vitamins stock) with appropriate supplements were used to culture fission yeast cells. Strains were constructed by either random spore germination method or by tetrad dissection. For crossing with *nuc2*-663 mutant, *nuc2*-663 cells complemented with plasmids encoding full length *nuc2* (pNUC2, a generous gift from Dr. Mitsuhiro Yanagida) were used in mating with other strains. After obtaining the strains, the plasmid was selected away by growing the cells on YES medium.

To generate *nmt1-nuc2* strain, a DNA fragment was first generated by Polymerase Chain Reaction (PCR) using the primers MOH2187 and MOH2188, and plasmid pCDL956 as template. The amplified DNA fragment contained kan^R cassette and *nmt1* promoter, and was flanked by 80bp of the 5'UTR of *nuc2* at the 5'end and 80bp of DNA sequences downstream of the start codon of *nuc2* at the 3' end. The amplified DNA fragment was introduced into wild type *S. pombe* MBY192. The positive clone was selected with YES medium containing 100μg/ml Geneticin (Sigma) and confirmed by PCR. To overexpress Nuc2p in the *nmt1-nuc2* strain, cells were first grown in minimal medium containing

15μM thiamine. The culture was then washed three times and re-inoculated into fresh medium without thiamine to induce Nuc2p expression. Similar approaches were used to overexpress Slp1p from wild type cells carrying *pREP1-slp1*.

To arrest cells in S phase, cells were treated with 12 mM hydroxyurea (HU; Sigma) for 6 hours prior to any experimental manipulation. To synchronize *nuc2*-663 mutant at metaphase, *nda3*-KM311 (control) and *nuc2*-663 *nda3*-KM311 mutants were first grown at 27.5°C in YES medium supplemented with 1.2 M sorbitol. Cells were washed three times with YES before shifting to 19°C in YES medium to achieve mid-mitosis arrest. The cells were stained by Alexa Fluor-488 (A12379, Molecular Probes) phalloidin after 6 hours of temperature shift to score for the presence of an actin ring (indication of mitotic cells). The cultures were released to permissive temperature of *nda3*-KM311 after 50% of cells were scored to have an actin ring.

To disrupt F-actin, cells were treated with 15μM of latrunculin A (T-119, BioMol International). To test if *pal1*Δ mutant was unable to grow in the presence of low dosage of microtubules depolymerizing drug, the cells were grown on YES agar plate containing 8μg/ml methyl 1-(butylcarbomyl)-2-benzimidazolecarbamate (MBC; Aldrich Chemical, Milwaukee, WI).

Table 1A: Yeast strains used in study of Chapter III

Name	Genotypes	Source
MBY192	*ura4*-D18 *leu1*-32 h^-	Laboratory collection
MBY286	*cdc16*-116 *leu1*-32 *ura4*-D18 *ade6*-M210 h^+	Paul Nurse
MBY533	*nuc2*-663 *leu1*-32 *ura4*-D18 h^-	Mitsuhiro Yanagida
MBY604	*GFP-sid1::ura4*$^+$ *leu1*-32 h^+	Dan McCollum
MBY644	*sid2*-GFP*::ura4*$^+$ *leu1*-32 h^+	Dan McCollum
MBY645	*sid2*-13MYC*::kan*^{R} *leu1*-32 h^-	Dan McCollum
MBY1323	*dma1::ura4*$^+$ *ura4*-D18 *leu1*-32 h^-	Dan McCollum
MBY1434	*cut9*-665 *leu1*-32 *ura4*-D18 h^-	Mitsuhiro Yanagida
MBY1588	*sid4*-GFP*::kan*^{R} h^-	Kathy Gould
MBY1590	*spg1*-GFP*::kan*^{R} h^+	Kathy Gould
MBY1722	*nda3*-KM311 *ura4*-D18 *leu1*-32 h^+	Mitsuhiro Yanagida
MBY2331	*cdc15*-GFP*::kan*^{R} *ura4*-D18 *leu1*-32 h^-	Kathy Gould
MBY2899	*nmt1-nuc2::kan*^{R} *rlc1*-GFP*::leu*$^+$ *uch2*-GFP*:ura4*$^+$	This study
MBY2942	*nmt1-nuc2::kan*^{R} *sid4*-GFP*::kan*^{R} h^+	This study
MBY2944	*nmt1-nuc2::kan*^{R} *cdc15*-GFP*::kan*^{R}	This study
MBY3057	*nmt41-nuc2*-GFP*::ura4*$^+$ h^-	This study
MBY3355	*nmt1-nuc2::kan*^{R} *cdc7*-GFP*::ura4*$^+$ *rlc1*-GFP*::leu1*$^+$ h^-	This study
MBY3356	*nmt1-nuc2::kan*^{R} GFP-*sid1::ura4*$^+$ *rlc1*-GFP*::leu1*$^+$ h^+	This study
MBY3397	*nmt1-nuc2::kan*^{R} *cdc7*-3XHA*::ura4*$^+$ h^+	This study
MBY3405	*nuc2*-663 *cdc7*-GFP*::ura4*$^+$	This study
MBY3458	*nuc2*-663 GFP*::sid1::ura4*$^+$	This study
MBY3560	*nmt1-nuc2::kan*^{R} *sid2*-GFP*::ura4*$^+$ *rlc1*-mRFP::ura4$^+$ *sid4*-mRFP*::ura4*$^+$	This study
MBY3572	*nmt1-nuc2::kan*^{R} *dma1::ura4*$^+$	This study
MBY3719	*nuc2*-663 *sid2*-GFP*::ura4*$^+$	This study
MBY4472	*nmt1-nuc2::kan*^{R} *sid2*-13MYC*::kan*^{R} *ura4*$^+$	This study
MBY4494	*nmt1-nuc2::kan*^{R} *cdc16*-116 *ura4*$^+$	This study
MBY4536	*nuc2*-663 *dma1::ura4*$^+$	This study
MBY4539	*nmt1-nuc2::kan*^{R} *spg1*-GFP*::kan*^{R} *cdc7*-3XHA*::ura4*$^+$	This study
MBY4623	*nda3*-KM311 *nuc2*-663	This study
MBY4736	*nmt1-nuc2::kan*^{R} *cdc16*-116 *cdc7*-GFP*::ura4*$^+$	This study
MBY4900	*lid1*-6 *ura4*-D18 *leu1*-32 h^-	Kathy Gould
MBY4927	*nda3*-KM311 *cut9*-665	This study
MBY4929	*nda3*-KM311 *lid1*-6	This study
MBY5043	*pREP1-slp1*(pCDL1270) in MBY192	This study

Table 1B: Yeast strains used in study of Chapter IV

Name	Genotypes	Source
MBY102	*ade6*-210 *ura4*-D18 *leu1*-32 *h+*	Laboratory collection
MBY103	*ade6*-216 *ura4*-D18 *leu1*-32 *h-*	Laboratory collection
MBY169	*wee1*-50, *leu1*-32, *h-*	Paul Nurse
MBY192	*ura4*-D18 *leu1*-32 *h-*	Laboratory collection
MBY411	*orb2*-34 *leu1*-32 h^+	Paul Nurse
MBY412	*orb3*-167 *leu1*-32 h^-	Paul Nurse
MBY415	*orb6*-25 *leu1*-32 h^-	Paul Nurse
MBY1186	*sph2*-3 ade*3*-58 *h90*	Matthias Sipiczki
MBY1247	*tea1::ura4*$^+$ *rlc1-GFP::leu1*$^+$ *leu1*-32 *ura4*-D18 *ade6*-210 *h+*	Laboratory collection
MBY1422	*cdc13*-YFP	Paul Nurse
MBY1442	*tea1*-YFP*::kan*^{R} *ura4*-D18 *leu1*-32 h^-	Paul Nurse
MBY2204	*pal1*-GFP*::ura4*$^+$ *ura4*-D18 *leu1*-32 *h-*	This study
MBY2211	*pal1::ura4*$^+$ *ura4*-D18 *leu1*-32 *h-*	This study
MBY2219	*pal1*-GFP*::ura4*$^+$ *cdc25*-22	This study
MBY2220	*pal1*-GFP*::ura4*$^+$ *mid1*Δ*::ura4*$^+$	This study
MBY2233	*pal1*::*ura4*$^+$, *wee1*-50, *ura4*-D18	This study
MBY2237	*pal1::ura4*$^+$ *ade6*-210, *h+*	This study
MBY2238	*pal1::ura4*$^+$ *ade6*-216, *h-*	This study
MBY2239	*pal1::ura4*$^+$ *ade6*-216, *h+*	This study
MBY2240	*pal1*-GFP*::ura4*$^+$ *h+*	This study
MBY2249	*orb2*-34 *pal1*-GFP*::ura4*$^+$	This study
MBY2250	*orb3*-167 *pal1*-GFP*::ura4*$^+$	This study
MBY2253	*tea1::ura4*$^+$ *pal1*-GFP*::ura4*$^+$	This study
MBY2260	*pal1::ura4*$^+$ *tea1::ura4*$^+$	This study
MBY2262	*pal1::ura4*$^+$ *expressing pREP1GFP-*α*-tubulin*	This study; *pREP1GFP-*α*-tubulin is a gift from* Yasushi Hiraoka
MBY2264	*pal1::ura4*$^+$ *tea1*-YFP*::kan*^{r}	This study
MBY2335	*orb6*-25, *wee1*-50	This study
MBY2336	*nda3*-KM311 *pal1*-GFP*::ura4*$^+$	This study
MBY2338	*pal1*-GFP*::ura4*$^+$ *ade6*-210 *leu1*-32 *ura4*-D18 *h90*	This study
MBY2655	*cdc13*-YFP, *pal1*::*ura4*$^+$	This study

2.2 Molecular methods and yeast methods

2.2.1 Standard recombinant DNA technique

Digestions of DNA with restrictive enzymes were carried out at conditions stated by the manufacturers. Ligation of DNAs was carried out at room temperature or 19°C from 2 hours to overnight. Purification of DNA was performed using kits from QIAGEN. The BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) was used to determine the DNA sequences.

To PCR amplify DNA fragments for molecular cloning, a reaction mixture containing 1x Expand High Fidelity buffer containing 1.5mM $MgCl_2$, 200µM of each dNTP, 0.3µM of primers, 0.75µl/50µl reaction volume of Expand HiFi enzyme (11732641001, Roche), and DNA template at pico-gram (pg) to nano-gram (ng) range was setup and subject to thermal-cycle according to the manufacturer's protocol. In most cases, purified genomic DNA or plasmid DNA was used as template. In cases of yeast colonic PCR, the yeast colonies were picked with yellow tips and resuspended in 15µl of TE buffer. The cells were boiled at 100°C for 2 minutes and were spun down. Ten microliters of the supernatant were used as the template in a PCR mixture. Additional $MgCl_2$ was added to reaction mixture to achieve final concentration of 2.5mM.

2.2.2 Chemical transformation of *S. pombe* by LiAc approach

Yeast transformation was performed by the lithium acetate (LiAc) method (Okazaki *et al*., 1990). Briefly, 20ml to 30ml of *S. pombe* cells was cultured to $O.D_{595}$ of 0.5. Cells were spun down at 3400rpm for 1~2 minutes and cell pellet was washed once with equal volume of sterile water. After resuspended in 1ml of sterile water, cells were transferred to an Eppendorf tube. The cells were spun down again and washed with 1ml of LiAc/TE buffer (1M LiAc, pH7.5; 0.1M Tris-HCl; 0.01M EDTA, pH7.5) and resuspended in 100µl of LiAC/TE. Two microliters of 10mg/ml sonicated sperm DNA and 10µl of DNA fragments were added to the cells. After 10 to 20 minutes of incubation at room temperature, 240µl of PEG/LiAc/TE was added and mixed gently with the cells. After an hour at 30°C (or room temperature for temperature sensitive mutants), 43µl of DMSO was added and the mixture was heated at 42°C for 5 minutes. Cells were spun down and washed once with 1ml of sterile water and resuspended in 100µl of sterile water for plating. Transformants were selected by growth on YES medium containing Geneticin, or supplemented minimal medium lacking uracil or leucine (in the presence or absence of 15µM thiamine). In all cases, correct integration was confirmed by PCR.

2.2.3 Extraction of *S. pombe* genomic DNA

To purify genomic DNA from *S. pombe*, cells were first spun down from 5ml of liquid culture of 0.5 $O.D_{595.}$ The cell pellet was resuspended in 500µl of TE and transferred to an Eppendorf tube. After pelleting down the cells, 500µl of Buffer 1 (1.2M sorbitol;

0.1M EDTA; 1% β-mercaptoethanol; 2mg/ml Zymolase) was added and incubated at 37°C for at least 30 minutes. The progress of cell wall digestion was monitored by mixing an aliquot of cells with 10% SDS and examining under microscope. The protoplasts were then spun down and resuspended in 500µl of Buffer 2 (100mM NaCl; 50mM EDTA; 50mM Tris, pH9). To the mixture, 50µl of 10% SDS and 10µl of 20mg/ml ProteinaseK were added and kept at 65°C for 30 minutes. Then, the mixture was cooled down on ice for 10 minutes, and 200µl of 5M potassium acetate was added and kept on ice for 10 minutes. Cell debris and proteins were removed by centrifuging at 14,000rpm at 4°C for 20 minutes. The supernatant was recovered and mixed with 800µl of isopropanol. The DNA was precipitated down after centrifuging at 14,000rpm at room temperature for 10 minutes, and was subsequently washed by 70% ethanol. After washing, ethanol was removed and the DNA pellet was dried and resuspended in 50µl of sterile distilled water.

2.3 Gene deletion and epitope tagging

To create the *pal1*::ura4^{+} disruption cassette, a 0.7-kb KpnI/XhoI fragment representing the 5' UTR of the *pal1* open reading frame (ORF) was obtained by PCR from *S. pombe* genomic DNA using the primers MOH1532 and MOH1533, and cloned into KpnI/XhoI sites of the pCDL126 vector (contains *ura4* in *pBSK*+ plasmid). A 0.7-kb XbaI/SacI fragment representing the 3' UTR of *pal1* was also obtained by PCR using the primers MOH1534 and MOH1535 and cloned into the modified plasmid containing the 5' UTR and the marker gene *ura4*, to generate pCDL863. To generate a *pal1* gene deletion strain,

a 3.2-kb fragment from pCDL863 was generated by digestion with KpnI and SacI and was introduced into the wild type haploid strain MBY192.

For green fluorescent protein (GFP) tagging of *pal1*, a 1-kb KpnI/SmaI fragment of *pal1*, encoding the C-termini, was obtained by PCR using the primers MOH1466 and MOH1467. The fragments were cloned into the KpnI/SmaI sites of pJK210-GFP. The resulted plasmid was linearized with SalI and transformed into the wild type strain MBY192.

To tag *rlc1* with monomeric red fluorescent protein (mRFP), a 555-bp KpnI/SmaI DNA fragment containing full length *rlc1* was first amplified by PCR using primers MOH461 and MOH462, and then cloned into the KpnI/SmaI sites of pJK210-mRFP. The product was partially digested using restriction enzymes EcoRI and integrated into the *rlc1* locus of wild type *S. pombe* strain MBY192. For monomeric red fluorescent protein (mRFP) tagging of *sid4*, a 1-kb KpnI/SmaI DNA fragments containing C-terminal sequences of *sid4* was amplified by PCR with primers MOH2460 and MOH2461, and cloned into the KpnI/SmaI sites of pJK210-mRFP. The product was linearized using restriction enzymes and integrated into the *sid4* locus of wild type strain MBY192.

To express *slp1* from thiamine regulatable promoter (*nmt1*), the *slp1* was first amplified by PCR from *S. pombe* genomic DNA using primers MOH3105 and MOH3106. The DNA fragment was digested by NdeI and BamHI and cloned into the NdeI/BamHI sites of *pREP1* vector. The plasmid *pREP1-slp1* was transformed into MBY192 and grown in supplemented minimal medium without leucine but containing 15μM thiamine.

Table 2: Primer used in the studies

Name	Primer sequences
MOH461	GAGAGCTGGTACCTGAATGTTCTCTTCGAAGGAA
MOH462	GAGAGTGCCCGGGATTGCTATCTTTTGACCC
MOH1466	CGGGGTACCTAATCTGCGGAGGCATTAGCCGAACG
MOH1467	TCCCCCGGGCGACTTTTTGTGGAATAATCGTC
MOH1532	CGGGGTACCTTATTGCAGCTCTTTATATC
MOH1533	CCGCTCGAGCCCGTATTGCACCCAAACGATAC
MOH1534	GCTCTAGAGCTTTTCAGGCTTGAAACTTTATTTAC
MOH1535	CGAGCTCCCAATTTGGCACCGACTCTAAAAAAC
MOH2187	CAATAACAACCACCTGTTTGTACCCACATGTTTTTGTTGACATTA ACTCCCATCGTTTCCAAAACTTTAATAGATTTGTCGAATTCGAGC TCGTTTAAAC
MOH2188	CGTTCTGAATAAAAAATTGAATTATCATAATTCTGATTATCAAT GCAATACCATATTAAACATTTCAATCGATCTGTCATCATGATTTA ACAAAGCGACTATA
MOH2460	CGGGGTACCTAAGGAGATGAATGCCACAATACAATC
MOH2461	TCCCCCGGGCAAACTACGTTTTTTAAGCTCCC
MOH3105	TTGGATACGTTTCTGTCCTTTGATATTCGCTTCTTGCATTATTTTT CTTTTACATATATATTTTGAGCGAGTTAATCAAAGAATTCGAGCT CGTTTAAAC
MOH3106	CTGTTAGGGAAGACTAAGTTCCTTTTCTTTGTGGGTGTTGAAAAT GTAGGCGATATGGTTGAAGAATTACCTGCTATCTCCATGATTTA ACAAAGCGACTATA

2.4 Immunoprecipitation and Western blotting

The total cell extracts of fission yeast were prepared by glass bead disruption and solubilized in buffer containing 1% Triton X-100, 150mM NaCl, 2mM EDTA, 6mM Na2HPO4, 4mM NaH2PO4, and complete protease inhibitors (Roche). Cell extracts were then clarified by centrifugation at 14,000rpm for 10minutes at 4°C. To immunoprecipitate protein complexes, 500µl of soluble protein was incubated with 5 µl of rabbit polyclonal GFP antibodies (a generous gift from Dr. Ramanujam Srinivasan) for 1–2 h at 4°C. Protein A-Sepharose beads CL-4B (100 µl, Amersham Biosciences) were then added to the antigen-antibody immunecomplex and incubated for 45 min at 4°C. After six washes with buffer containing 1% Triton X-100, the beads were resuspended in SDS-PAGE loading buffer and heated at 95 °C for 5 minutes. The Protein A-Sepharose beads were spun down at 14,000 rpm for 5 min and the supernatants were subject to SDS-PAGE.

To detect GFP, Myc, or HA-tagged proteins, antibodies recognizing GFP, Myc, or HA were used to probe PVDF membranes containing separated proteins. The membrane was blocked with 5% nonfat milk in phosphate-buffered saline-Tween 20 (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.05% Tween 20, pH 7.4) for 1 h at room temperature or overnight at 4°C. Primary rabbit polyclonal α-GFP (ab290, Abcam), mouse monoclonal α-Myc 9E10 (M5546, Sigma) and mouse monoclonal α-HA 12CA5 (1666606, Roche) antibodies were used at 1:2000 dilutions. Peroxidase-conjugated α-rabbit (711-035-152, Jackson Immuno Research) and α-mouse IgG (715-035-151,

Jackson Immuno Research) were used at 1:4000 dilutions, and the chemiluminescent signal was detected using a 1:1 mixture of ECL1 (2.5 mM 3-aminophytaldrazide dissolved in dimethyl sulfoxide (DMSO), 0.4 mM p-coumaric acid, 100 mM Tris-HCl, pH 8.5), and ECL2 (0.02% H2O2, 100 mM Tris-HCl, pH 8.5).

2.5 Microscopy

2.5.1 Staining of nucleus, F-actin, and cell wall/septum

To visualize F-actin cytoskeleton, cells were fixed with 7% formaldehyde and permeabilized with PBS containing 1% Triton X-100 and stained with Alexa Fluor-488 phalloidin (A12379, Molecular Probes). Septum/cell wall and DNA were stained with 0.5µg/ml aniline blue (Sigma) and 4′, 6-diamidino-2-phenylindole (DAPI) (1µg/ml DAPI, 1mg/ml p-phenylenediamine, 50% glycerol), respectively.

2.5.2 Indirect immunofluorescence

For immunofluorescence studies, cells were fixed either with formaldehyde or with methanol kept at -20°C. For formaldehyde fixation, one fifth volume of 37% formaldehyde was added to liquid culture containing *S. pombe* cells and incubated for 10-15 minutes. For methanol fixation, cell pellet was collected from 20ml-30ml of liquid culture and was mixed with cold methanol. The mixture was incubated at -20°C for 8 minutes and spun down. In both fixations, the cell pellet was washed three times with

PBS. For immunofluorescence, cells were processed as described (Balasubramanian *et al*., 1997). Briefly, the fixed cells were protoplasted by incubating the cells with 3mg/ml Zymolase and 5mg/ml lysing enzyme for at least 5-10 minutes in the presence of PBS containing 1.2M sorbitol. After 5-10 minutes at room temperature, an aliquout of cells was mixed with 10% SDS to monitor the progress of cell wall digestion by microscope. After 50% of cells were protoplasted, 1ml of PBS containing 1% Triton X-100 was added to the suspension. The protoplast was spun down at speed of no more than 3000 rpm and washed three times with PBS. The protoplasts were further blocked at room temperature for minimum 30 minutes by resuspending in 1 ml of PBAL (PBS, 1% BSA, 100mM Lysine hydrochloride, 50μg/ml Carbenicillin). Then, respective primary antibodies were added to 200μl of protoplast and incubated at room temperature for overnight. After washing three times with PBAL, 1μl of Alexa Fluor conjugated secondary antibody (Molecular Probes) was incubated with the mixture for an hour at room temperature. The cells were then mounted on the glass slide in the presence of DAPI after washing three times with PBAL. Primary rabbit polyclonal antibody to Cdc4p (laboratory collection) and mouse monoclonal antibody to α-tubulin (a generous gift from Dr. Keith Gull) were used as the primary antibodies to stain the actomyosin ring and microtubules, respectively.

2.5.3 Time-lapse live cell imaging

For fluorescence time-lapse microscopy, cells expressing GFP-fusion proteins were cultured to $O.D_{595}$ of 0.2~0.5 and 1ml of cells was concentrated to less than 10μl. One

microliters of the concentrated cells was spotted on a thin agarose pad with appropriate medium and imaged using a Leica DMIRE2 microscope (100x/1.4NA Plan Apo objective lens) or a Zeiss LSM 510 confocal microscope (63×/1.4NA Plan Apo objective lens). The cover slip was sealed tightly with VALAP (Vaseline/lanolin/paraffin 1:1:1) to prevent evaporation.

To monitor the growth patterns of *S. pombe*, cells were concentrated from early log phase culture and spotted on a glass slide containing an agar pad with appropriate medium. The time-lapse analysis was conducted at room temperature (22–24°C) using a Leica DMLB microscope (100x/1.3NA Plan Fluotar objective lens). Assembly of images was done using ImageJ 1.32 (National Institutes of Health, Bethesda, MD).

2.5.4 Image acquisition

For widefield fluorescence imaging, cells were examined with either an Olympus IX71 microscope (100x/1.45NA TIRFM Plan Apo objective len) or a Leica DMLB microscope (100x/1.3NA Plan Fluotar objective lens) or a Leica DMIRE2 microscope (100x/1.4NA Plan Apo objective lens). The images were captured using a Photometrics CoolSNAP ES camera. A Piezo Z-axis controller PZM-2000 (Applied Scientific Instrumentation) was attached to an Olympus IX71 microscope (100x/1.45NA TIRFM Plan Apo objective lens) to acquire Z-sections of a cell. All images were processed with MetaMorph 6.1 and were assembled in Photoshop 7.0 (Adobe Systems). For confocal imaging, images were

acquired with the Zeiss LSM 510 confocal microscope equipped with a 63×/1.4NA PlanApo objective lens. The images were anaylsed by LSM image browser.

2.5.5 Electron microscopy

Electron microscopy was performed on permanganate-fixed *S. pombe* as described (Armstrong *et al*., 1993). Fission yeast cells were grown at appropriate temperatures, washed three times in sterile water, and fixed for 1 h in 2% potassium permanganate at room temperature. The fixed cells were harvested by centrifugation and washed three times in sterile water, resuspended in 70% ethanol, and incubated overnight at 4°C. The samples were dehydrated and treated with propylene oxide before infiltration with Spurr's medium, followed by another change of medium and incubation at 65°C for 1 h. Finally, they were embedded in Spurr's resin, and the resin was allowed to polymerize at 60°C overnight. Ultrathin sections were cut on a Jung Reichert microtome (Leica Mikroskopie and Systeme GmbH, Wetzler, Germany) and examined using a JEM1010 transmission electron microscope (Jeol, Tokyo, Japan) at 100 kV.

Chapter 3: Temporal regulation of cytokinesis

3.1 Introduction

The eukaryotic cell cycle is composed of an invariant sequence of events, in which DNA replication precedes mitosis and mitosis in turn precedes cytokinesis (Hartwell and Weinert, 1989). Cells also possess mechanisms to ensure that DNA replication, chromosome segregation and cytokinesis occur only once per cell cycle (Minet et al., 1979; Romanowski and Madine, 1996). While much progress has been achieved in understanding the temporal regulation of DNA synthesis and chromosome segregation, the mechanisms by which cytokinesis is restricted to once per cell cycle has not been fully explored. Septation initiation network (SIN) coordinates mitosis and cytokinesis in fission yeast and is central to the regulation of cytokinesis. How the SIN pathway is inactivated following cytokinesis and how its precocious activation in interphase is prevented, while the CDK activity is low, remain poorly understood.

This chapter describes the roles of a Tetratricopeptide repeat (TPR)-domain containing subunit of APC/C, Nuc2p in regulating SIN signaling and cytokinesis. A previous study has showed that overproduction of Nuc2p inhibits cytokinesis. My study further demonstrates that Nuc2p exerts its effects on cytokinesis by modulating the nucleotide state of the Spg1p-GTPase and thereby down regulating the SIN. This study provides a mechanism to inactivate SIN pathway after cytokinesis and to prevent inappropriate cytokinetic events following cell division.

3.2 Results

3.2.1 Cells overexpressing Nuc2p phenocopy *sin* mutants phenotype

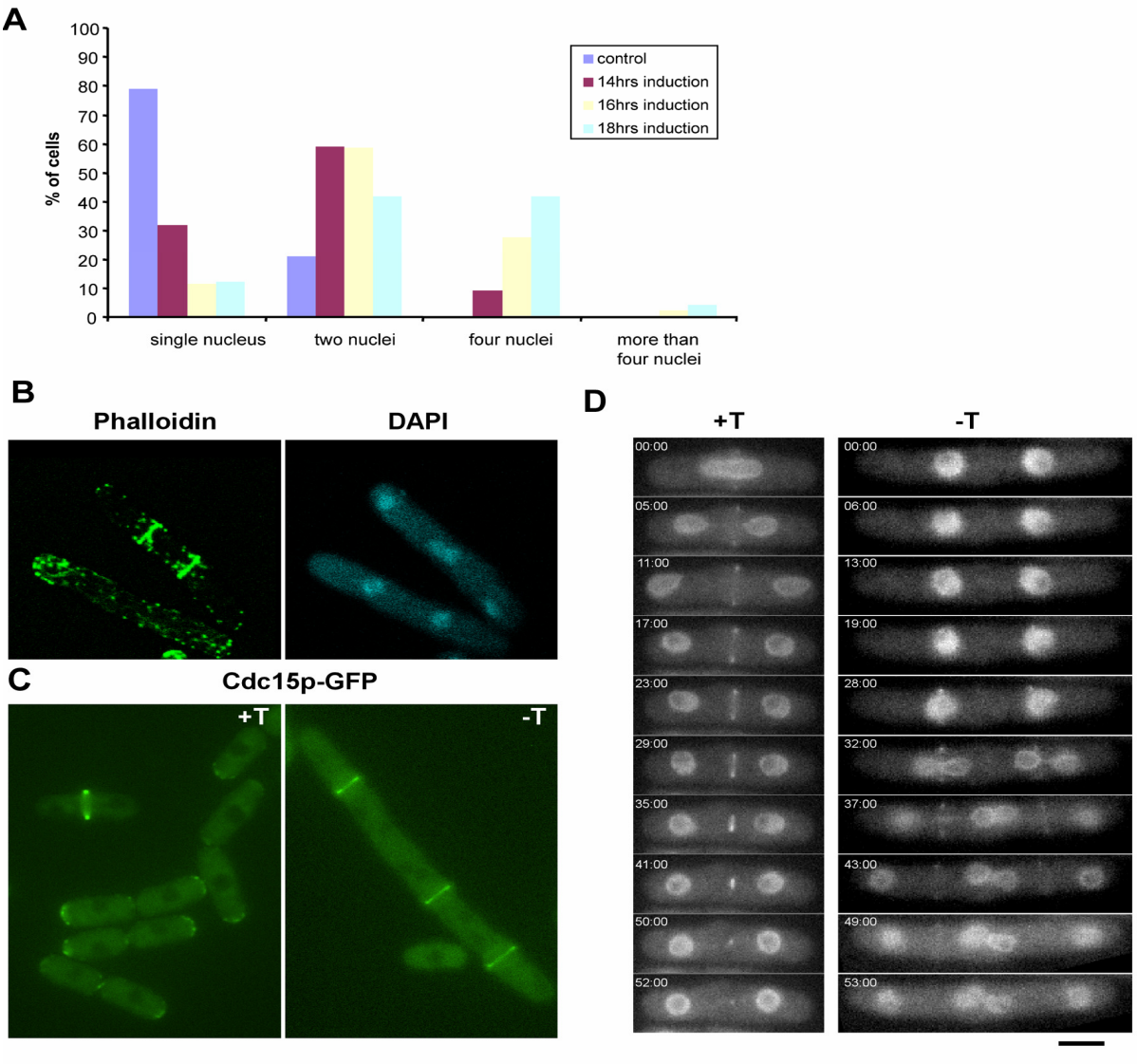
Previous study has showed that high level of Nuc2p blocks septation, and results in a failure of cytokinesis in fission yeast (Kumada *et al*., 1995). However, the mechanism underneath the inhibition of cytokinesis by Nuc2p remained unclear. To investigate the inhibitory roles of Nuc2p in cytokinesis, a yeast strain carrying thiamine-repressible promoter, *nmt1*, integrated at *nuc2* gene locus (hereafter referred to as *nmt1-nuc2*) was generated and analyzed. The integrant resembled wild type cells upon growth on medium supplemented with thiamine. In order to analyze the overexpression phenotype more thoroughly, I generated *nmt1-nuc2* cells expressing a nuclear marker Uch2p-GFP (Ubiquitin C-terminal hydrolase fused to GFP) to visualize nuclear division versus cell division. When this strain was grown in medium containing thiamine, cells were found to contain either a single nucleus or two nuclei, depending on the cell cycle stage (Figure 3.1A). Upon removal of thiamine from the medium (leading to overexpression of Nuc2p), a high proportion of cells accumulated two or more nuclei (Figure 3.1A). In particular, after 18 h of derepression of the *nmt1* promoter, more than 40% of the cells contained 4 or more nuclei, while another 40% of the cells contained two nuclei, most of which were of a post-mitotic configuration (Figure 3.1A).

Since the cytokinesis of fission yeast requires the assembly and constriction of an actomyosin ring, it is plausible that the failure of cytokinesis in cells overexpressing

Figure 3.1 Actomyosin rings are assembled, but not maintained, at the division site in cells overexpressing Nuc2p

(A) Quantification of the number(s) of nuclei/cell and the frequency of their appearance upon overexpression of Nuc2p. Cells expressing Uch2p-GFP as a nuclear marker were used in the experiment. (B) Visualization of F-actin. Cells overexpressing Nuc2p were fixed and stained with phalloidin and DAPI to visualize the F-actin cytoskeleton and nuclei, respectively. (C) Visualization of Cdc15p. Cells carrying Cdc15p-GFP were induced to overexpress Nuc2p and visualized by fluorescence microscopy. +T/−T indicates medium supplemented with or without thiamine. (D) Time-lapse fluorescence microscopy to image the dynamics of actomyosin ring assembly and constriction. Cells were grown in medium with or without thiamine and imaged by time-lapse microscopy. The nucleus and actomyosin ring were marked by Uch2p-GFP and Rlc1p-GFP, respectively. Scale bar, 5 μm.

Figure 3.1


A
100
90
80
70
60
50
40
30
20
10
0
% of cells
control
14hrs induction
16hrs induction
18hrs induction
single nucleus
two nuclei
four nuclei
more than four nuclei
B
Phalloidin
DAPI
C
Cdc15p-GFP
+T
-T
D
+T
-T
00:00
05:00
11:00
17:00
23:00
29:00
35:00
41:00
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52:00
00:00
06:00
13:00
19:00
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32:00
37:00
43:00
49:00
53:00

Nuc2p was a result of the defective formation of the contractile apparatus. To examine this possibility, I grew the *nmt1-nuc2* cells on medium lacking thiamine to overexpress Nuc2p and stained the cells with phalloidin to reveal the actin cytoskeleton. In cells overexpressing Nuc2p, F-actin assembled into ring structures in mitotic cells, as in control cells, suggesting that the recruitment and assembly of F-actin into the actomyosin ring was not affected (Figure 3.1B). In addition, I tested the effect of Nuc2p overexpression on the FCH domain protein, Cdc15p, which is essential for actomyosin ring maintenance and septum assembly. Similar to the case of F-actin, cells were able to assemble Cdc15p-GFP rings upon Nuc2p overexpression (Figure 3.1C).

Next, I examined the effect of Nuc2p overexpression on the dynamics of actomyosin ring assembly and constriction. To this end, I performed time-lapse microscopy on *nmt1-nuc2* cells expressing Rlc1p-GFP (regulatory light chain of myosin fused to green fluorescent protein) and Uch2p-GFP as the markers for actomyosin ring and nucleus, respectively. In control cell, which Nuc2p is not overexpressed, the Rlc1p assembled into ring structure at the division site, and the ring constricts after nuclear division (Figure 3.1D, +T panel). In contrast, upon the growth on medium lacking thiamine to induce Nuc2p expression, the cells assembled Rlc1p rings overlying the position of the interphase nuclei, however, these rings were not stable and collapsed in late anaphase and failed to constrict, leading to failure of septation (Figure 3.1D, -T panel). Collectively, these studies established that the presence of excess Nuc2p led to defects in maintenance of actomyosin rings in late anaphase, leading to failure of division septum assembly. This phenotype of Nuc2p

overexpression is similar to *sin* mutants which are defective in signaling pathway, septation initiation network (SIN).

3.2.2 Prolonged maintenance of SIN signaling in *nuc2*-663 mutant

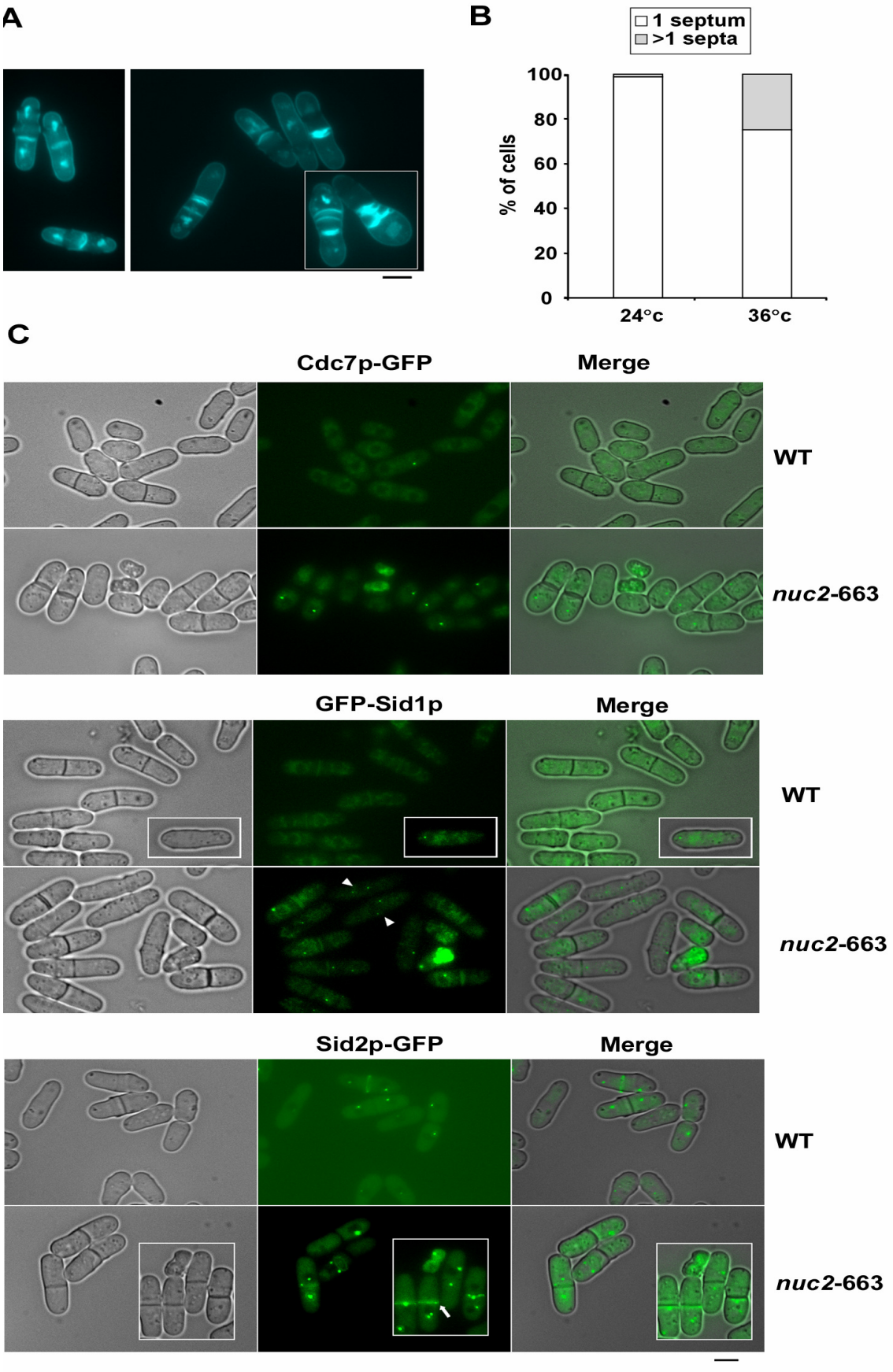
Since the cells overproducing Nuc2p phenocopy the loss of function mutants of *sin* (septation initiation network), I considered the possibility that the SIN signaling is up-regulated in temperature-sensitive allele of *nuc2*, *nuc2*-663. To test this possibility, I analyzed the phenotype of *nuc2*-663 mutant grown at restrictive temperature with focus on the organization and function of the cytokinetic machinery. Interestingly, I observed that approximately 28% of *nuc2*-663 cells (n = 112/404) displayed multiple septa, in addition to *cut* phenotype in which cells undergo septation in the absence of chromosome segregation (Figure 3.2A and B). This suggests that the cytokinetic machinery might be constitutively active in these cells, which is indicative of SIN activation.

Next, I examined if the SIN proteins were affected by the inactivation of Nuc2p function. Previous studies have shown that Cdc7p and Sid1p localize to spindle pole body (SPB) during mitosis and disappear from SPB upon completion of septation. Interestingly, inactivation of Nuc2p function resulted in the persistence localization of Cdc7p and Sid1p at the SPB even after completion of septation (Figure 3.2C, top and middle panel). In addition, Sid1p was detected at two SPBs in *nuc2*-663 cells at restrictive temperature instead of one SPB as in wild type cell (Figure 3.2C, indicated by arrowhead). The protein kinase Sid2p appears to be the most downstream kinase of the SIN. In wild type

Figure 3.2 Hyperactivation of SIN signaling in *nuc2*-663 mutant

(A) *nuc2*-663 cells were stained with DAPI and aniline blue to visualize nuclei and septum material, respectively. The left panel shows *nuc2*-663 cells at permissive temperature. The right panel shows *nuc2*-663 cells at restrictive temperature. (B) Quantification of multiseptated cells in the *nuc2*-663 mutant. (C) Wild type and *nuc2*-663 cells expressing GFP tagged versions of Cdc7p, Sid1p, and Sid2p were grown at 36 °C for 4 h and were visualized using fluorescence microscopy. Arrowheads indicate mitotic cells with Sid1p at both SPBs. Arrows indicate cells in which Sid2p-GFP signal persisted at the division site after completion of cytokinesis. Scale bar, 5 μm.

Figure 3.2


A
B
1 septum
>1 septa
% of cells
100
80
60
40
20
0
24°c
36°c
C
Cdc7p-GFP
Merge
WT
nuc2-663
GFP-Sid1p
Merge
WT
nuc2-663
Sid2p-GFP
Merge
WT
nuc2-663

cells, Sid2p localizes to the SPBs throughout the cell cycle and is also detected at the cell division site during cytokinesis. Interestingly, in the *nuc2*-663 mutant, Sid2p was detected at the division site in fully septated cells (Figure 3.2C, indicated by arrow). Furthermore, additional ring like structures containing Sid2p were also detected in these cells (Figure 3.2C). Similar localization of Sid2p was not seen in wild type cells, in which the medial localization of Sid2p was lost upon completion of septation.

Collectively, these data suggest that SIN signaling is up-regulated when *nuc2* is defective.

3.2.3 De-localization of Cdc7p and Sid1p from SPBs in cells overexpressing Nuc2p

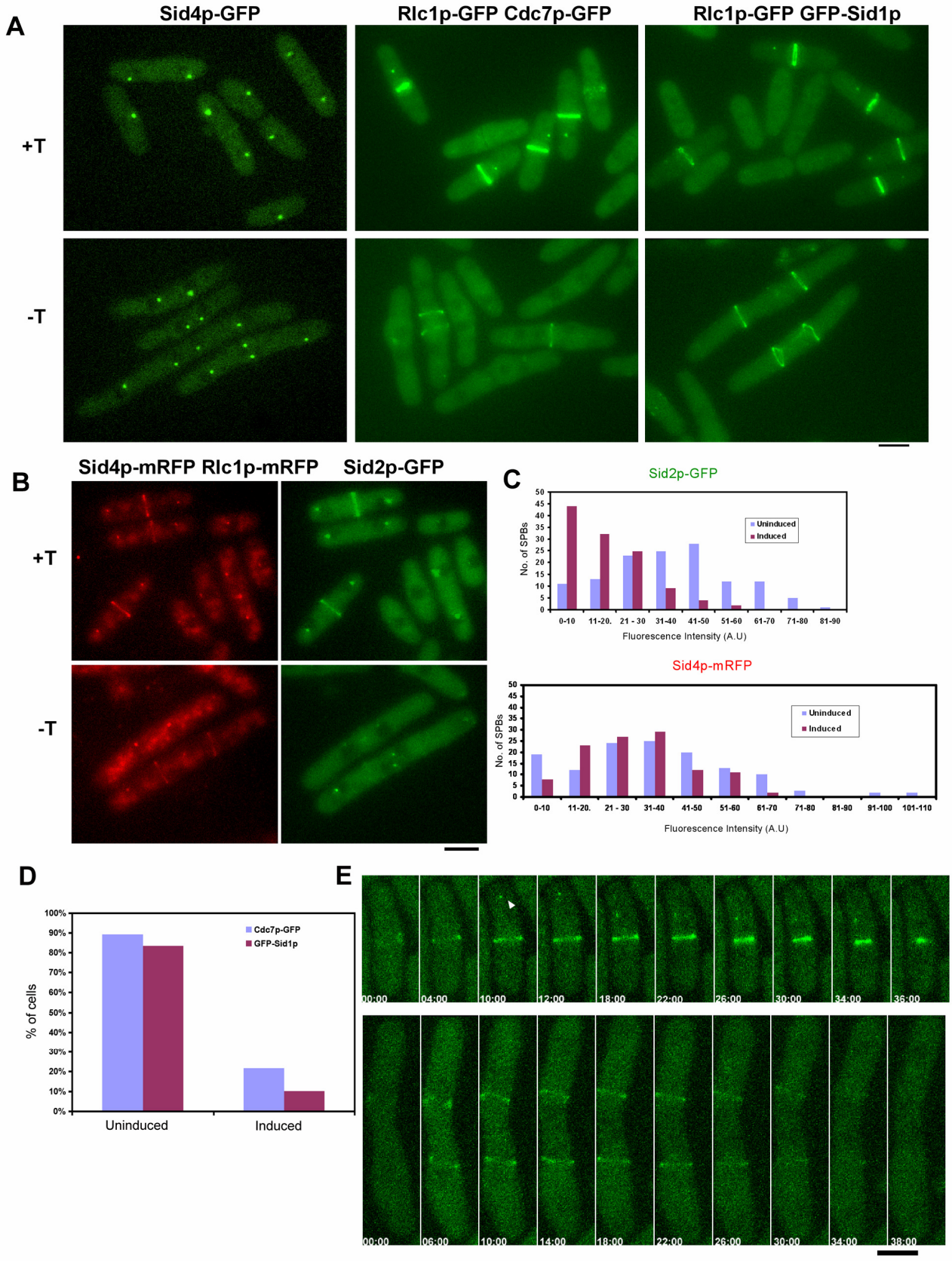
Having established that SIN signaling is activated for prolonged period of time in *nuc2*-663 mutant and cells overexpressing Nuc2p resembles *sin* mutant, I tested if overexpression of Nuc2p affected the functions of SIN proteins. To this end, I examined the effect of Nuc2p overexpression on the SIN scaffold proteins: Cdc11p, Sid4p; the small GTPase: Spg1p; and the downstream kinases: Cdc7p, Sid1p, and Sid2p. Of these proteins, Sid4p, Cdc11p, Spg1p, and Sid2p are known to localize to the SPBs throughout the cell cycle, while Cdc7p and Sid1p localize during cytokinesis to one of the two SPBs (which has been shown to contain GTP-bound Spg1p).

The overexpression of Nuc2p did not affect the SPB-localization of Cdc11p, Sid4p, and Spg1p (Figure 3.3A and data not shown). Interestingly, the localization of Cdc7p and

Figure 3.3 Overexpression of Nuc2p inhibits SIN signaling

(A) *nmt1-nuc2* cells expressing Sid4p-GFP, Cdc7p-GFP, and GFP-Sid1p were visualized by fluorescence microscopy in the presence (+T, top panel) or absence (−T, bottom panel) of thiamine. The actomyosin ring (visualized with Rlc1p-GFP) was used as the marker for cells undergoing cytokinesis. (B) Fluorescence signal of Sid2p is reduced in cells overproducing Nuc2p. nmt1-nuc2 cells expressing Sid2p-GFP were visualized using fluorescence microscopy upon growth on medium supplemented with or without thiamine. SPBs and actomyosin ring were marked by Sid4p-mRFP and Rlc1p-mRFP, respectively. (C) Quantification of the relative fluorescence intensities of Sid2p-GFP and Sid4p-mRFP in Nuc2p-uninduced and -induced cells. (D) Quantification of actomyosin ring containing cells with Cdc7p-GFP or GFP-Sid1p at the SPBs. At least 80 cells were counted for each category. (E) Cdc7p is not recruited to the SPBs in cells overproducing Nuc2p. Top panel, control cell grown on minimal medium supplemented with thiamine. Arrowhead indicates localization of Cdc7p-GFP at the SPB. Bottom panel, cell grown in medium without thiamine to overexpress Nuc2p. Rlc1p-GFP was used in these cells to label actomyosin ring. Cells were grown on agarose pad containing growth medium and were imaged by laser scanning microscopy. Scale bar, 5 μm.

Figure 3.3


A
Sid4p-GFP
Rlc1p-GFP Cdc7p-GFP
Rlc1p-GFP GFP-Sid1p
+T
-T
B
Sid4p-mRFP Rlc1p-mRFP
Sid2p-GFP
+T
-T
C
Sid2p-GFP
No. of SPBs
Uninduced
Induced
0-10
11-20.
21 - 30
31-40
41-50
51-60
61-70
71-80
81-90
Fluorescence Intensity (A.U)
Sid4p-mRFP
No. of SPBs
Uninduced
Induced
0-10
11-20.
21 - 30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
101-110
Fluorescence Intensity (A.U)
D
% of cells
Cdc7p-GFP
GFP-Sid1p
Uninduced
Induced
E
00:00
04:00
10:00
12:00
18:00
22:00
26:00
30:00
34:00
36:00
00:00
06:00
10:00
14:00
18:00
22:00
26:00
30:00
34:00
38:00

Sid1p was severely affected in mitotic cells overexpressing Nuc2p (Figure 3.3A). Quantification shows a significant reduction in the number of cells with Cdc7p and Sid1p at the SPBs upon Nuc2p overexpression. Cdc7p was detected at the SPBs in 12/61 cells with actomyosin rings, while Sid1p was detected in 6/71 cells with rings, compared to uninduced cells in which at least 80% of cells with actomyosin rings contained Cdc7p-GFP and GFP-Sid1p at the SPBs (Figure 3.3D). In cells that displayed Cdc7p and Sid1p at the SPBs, the fluorescence intensity is significantly lower than that in uninduced control cells. Furthermore, the Sid2p kinase that localizes constitutively to SPBs during cell cycle and to division site during cytokinesis, is also affected by Nuc2p overexpression. The fluorescence signal is not detectable at the division site upon Nuc2 overexpression, and its intensity at the SPBs has reduced extensively compared to uninduced control cells (Figure 3.3B). Quantification of the fluorescence intensity of Sid2p at the SPBs showed that the cells that overexpress Nuc2p have more SPBs with lower fluorescence intensity than uninduced control cells (Figure 3.3C). Thus, overexpression of Nuc2p has strong effect on the localization of the three protein kinases in SIN signaling: Cdc7p, Sid1p, and Sid2p, and there is no drastic effect on the scaffold proteins: Sid4p, Cdc11p and the small GTPase Spg1p.

Next, I determined if overexpression of Nuc2p affected the recruitment or the maintenance of SIN components at the SPBs. To this end, I performed time-lapse microscopy to observe the localization of Cdc7p during cytokinesis. We found that Cdc7p was recruited to one of the SPBs during actomyosin ring constriction in cells grown in the presence of thiamine (Figure 3.3E, top panel). In contrast, in cells

overexpressing Nuc2p, Cdc7p was not detected at the SPBs throughout the cell division cycle and the actomyosin ring disassembled at the end of mitosis (Figure 3.3E, bottom panel). Taken together, these studies suggest that overexpression of Nuc2p affects the recruitment of the SIN components, Cdc7p and Sid1p to SPB.

3.2.4 Overexpression of Nuc2p does not affect the steady-state level of SIN kinases

Since Nuc2p is a component of anaphase promoting complex (APC/C) and is involved in protein degradation (Yamada *et al*., 1997), I considered the possibility that overexpression of Nuc2p enhances the activity of anaphase promoting complex and results in the proteolysis of SIN proteins. The steady state levels of Cdc7p, Sid1p, and Sid2p were determined by Western Blotting in cells overexpressing Nuc2p. I found that the steady state levels of Cdc7p, Sid1p, and Sid2p were not significantly altered upon overexpression of Nuc2p (Figure 3.4). Thus, Nuc2p appears to affect the SPB localization of the SIN kinases but not their stability.

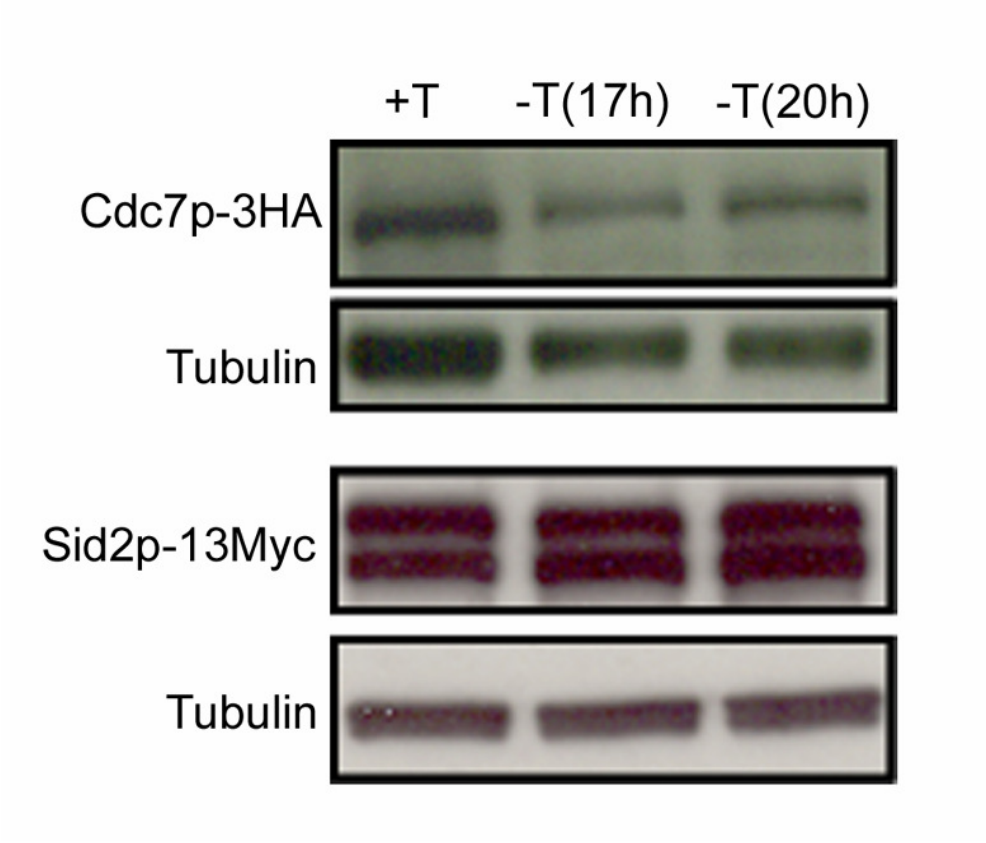
3.2.5 The binding of Spg1p and Cdc7p is affected upon overexpression of Nuc2p

Previous studies have shown that the small GTPase, Spg1p, upon its activation, binds and recruits Cdc7p to the SPB during mitosis (Sohrmann *et al*., 1998). Cdc7p binds preferentially to GTP-bound Spg1p, which is thought to be the activated form of this GTPase (Sohrmann *et al*., 1998). Since I observed a defective recruitment of Cdc7p to the SPBs in cell overexpressing Nuc2p, it is possible that the binding between Spg1p and

Figure 3.4 Overexpression of Nuc2p does not affect the steady-state levels of Cdc7p-3HA and Sid2p-13Myc

Protein levels of Cdc7p-3HA and Sid2p-13Myc in *nmt1-nuc2* cells grown in medium with or without thiamine. Tubulin serves as the loading control.

Figure 3.4



Cdc7p is affected under this condition. To test if this was the case, I overexpressed Nuc2p and examined the physical binding of Spg1p and Cdc7p using co-Immunoprecipitation assay. Immunoprecipitating the Spg1p-GFP using antibody recognizing GFP epitope pull-down Cdc7p-HA when cells were grown in medium containing thiamine. Interestingly, immunecomplexes generated with GFP-antibodies from cells overproducing Nuc2p contained very little or no Cdc7p-HA (Figure 3.5). The specificity of the immnuoprecipitation procedure was confirmed by the absence of Cdc7p-HA in immunecomplexes generated from cells expressing Cdc7p-HA but not Spg1p-GFP (Figure 3.5). Comparable protein level of Cdc7p-3HA was detected in both strains used as well as under both conditions (+ or - thiamine) used indicating that similar amount of protein was used as input for co-immunoprecipitation experiment (Figure 3.5). These experiments established the binding between Cdc7p-3HA and Spg1p-GFP was interrupted in cells overproducing Nuc2p.

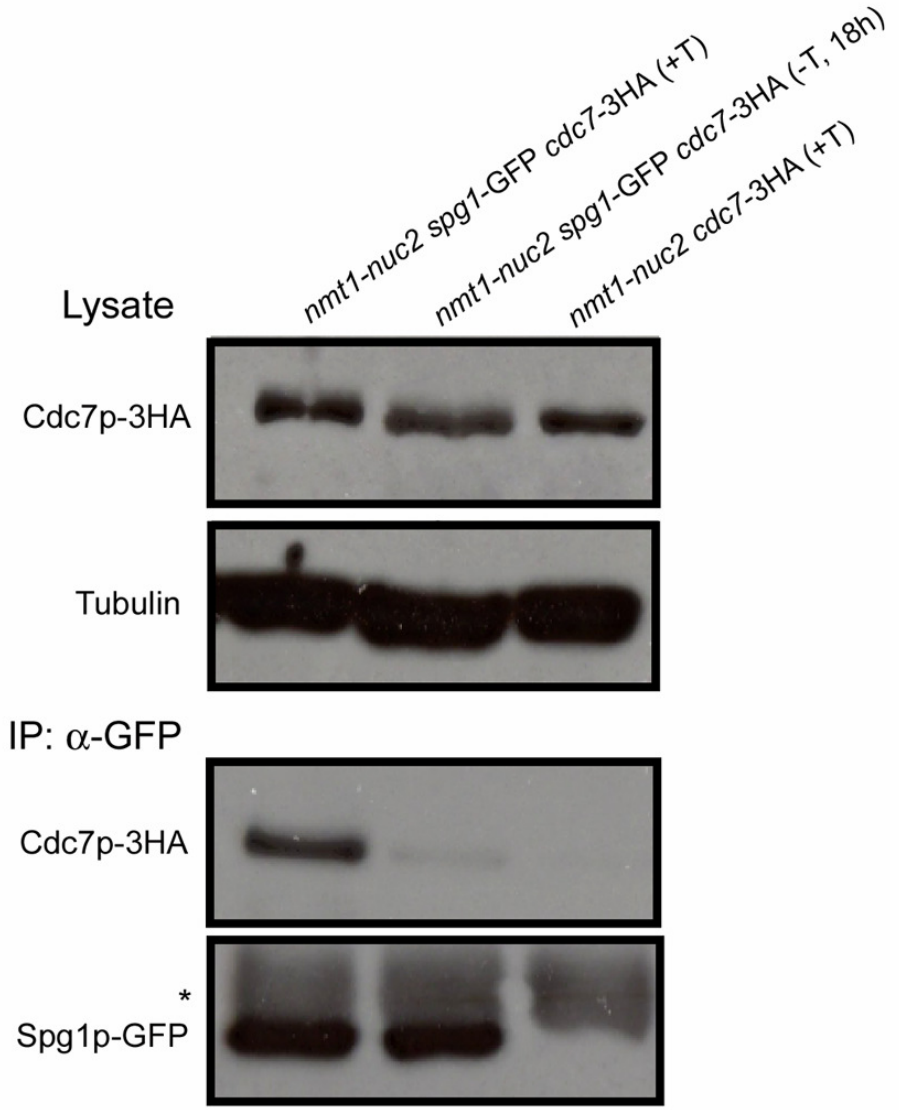
3.2.6 Inactivation of the GAP subunit, Cdc16p promotes the localization of Cdc7p to SPBs and allows septation in cells overexpressing Nuc2p

I have shown that overexpression of Nuc2p affects the binding of Spg1p and Cdc7p. Since it has been shown that the physical interaction between Spg1p and Cdc7p is determined by the nucleotide state of Spg1p, it could be hypothesized that the Spg1p is kept at GDP-bound form upon overexpression of Nuc2p. It is possible that high level of Nuc2p either down-regulates a guanine nucleotide exchange factor (GEF) that activates Spg1p, or promotes the activity of the two-component GTPase activating protein (GAP).

Figure 3.5 Overexpression of Nuc2p disrupts the binding of Spg1p-GFP and Cdc7p-3HA

Lysates from cells expressing Cdc7p-3HA and Spg1p-GFP in the presence or absence of Nuc2p overexpression were immunoprecipitated with GFP antibodies and immunoblotted with antibodies against the HA epitope. Lysates from a strain without Spg1p-GFP was included as the control. Tubulin serves as the loading control. Asterisk indicates heavy chain of IgG.

Figure 3.5


nmt1-nuc2 spg1-GFP cdc7-3HA (+T)
nmt1-nuc2 spg1-GFP cdc7-3HA (-T, 18h)
nmt1-nuc2 cdc7-3HA (+T)
Lysate
Cdc7p-3HA
Tubulin
IP: α-GFP
Cdc7p-3HA
*
Spg1p-GFP

Since protein that functions as GEF for SIN signaling has not been identified, I considered the possibility that overexpression of Nuc2p might result in the activation of Byr4p-Cdc16p, which is the two-component GAP of SIN signaling. I examined this possibility by overexpressing Nuc2p in *cdc16*-16 mutant and assayed the ability of these cells to localize Cdc7p to SPB and to assemble division septa (Figure 3.6A). In contrast to the cell with functional Cdc16p and high level of Nuc2p, inactivation of Cdc16p function in cells overexpressing Nuc2p resulted in the recruitment / maintenance of Cdc7p on SPB and septation (Figure 3.6B and C). When multinucleated cells were quantitated, more than 90% of the tetranucleate cells were able to undergo cytokinesis and septation (n = 190/203) (Figure 3.6D). In contrast, less than 25% of the tetranucleate cells in which Cdc16p was functional underwent cytokinesis and septation (n = 44/204) (Figure 3.6D). These experiments suggest that overproduction of Nuc2p might lead to activation of Byr4p-Cdc16p, thereby to the inability to maintain SIN function and septation.

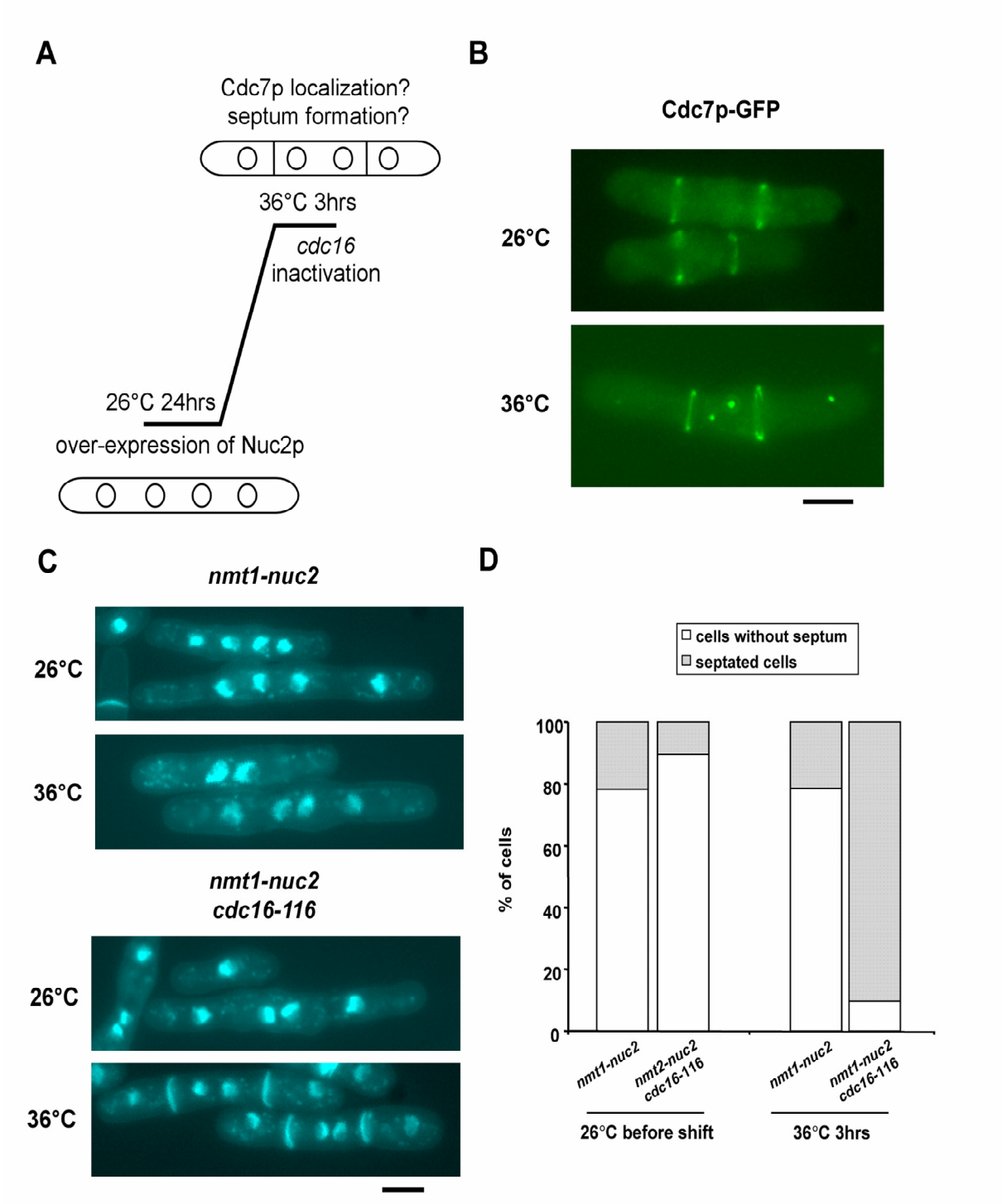
3.2.7 Nuc2p prevents inappropriate cytokinesis after septum assembly

Having established that Nuc2p functions upstream of SIN signaling to inhibit cytokinesis, I seek for the physiological significance of this regulatory mechanism. To this end, I inactivated Nuc2p function after passage through anaphase to study its function during cytokinesis (Figure 3.7A). A yeast strain *nuc2*-663 *nda3*-KM311 was generated so as to allow for the inactivation of Nuc2p function following anaphase. The *nda3*+ gene encodes the β-subunit of the tubulin heterodimer and the *nda3*-KM311 mutant results in

Figure 3.6 Inactivation of Cdc16p function promotes localization of Cdc7p to SPBs and allows septation in cells overexpressing Nuc2p

(A) Diagram schematically illustrates the experimental design. Nuc2p was induced for 24 h at 26 °C, and the culture was shifted to 36 °C to inactivate Cdc16p and examined after 3 h of incubation. (B) The localization of Cdc7p-GFP in *cdc16*-116 cells overexpressing Nuc2p was visualized by fluorescence microscopy at the permissive or restrictive temperatures. (C) Septum assembly is restored upon inactivation of Cdc16p in cells overexpressing Nuc2p. DAPI and aniline blue staining of formaldehyde fixed cells is shown. (D) Quantification of septated cells versus non-septated cells in *nmt1-nuc2* and *nmt1-nuc2 cdc16*-116. Note that only cells with four nuclei were counted. Scale bar, 5 μm.

Figure 3.6


A
Cdc7p localization?
septum formation?
36°C 3hrs
cdc16
inactivation
26°C 24hrs
over-expression of Nuc2p
B
Cdc7p-GFP
26°C
36°C
C
nmt1-nuc2
26°C
36°C
nmt1-nuc2
cdc16-116
26°C
36°C
D
cells without septum
septated cells
% of cells
100
80
60
40
20
0
nmt1-nuc2
nmt2-nuc2
cdc16-116
nmt1-nuc2
nmt1-nuc2
cdc16-116
26°C before shift
36°C 3hrs

the defective assembly of microtubules, cold-sensitivity and lethality (Hiraoka *et al*., 1985). The *nda3*-KM311 allows the synchronization of cells at mid-mitosis due to the activation of the spindle checkpoint, caused by the loss of β-tubulin function and hence formation of mitotic spindles. The product of the cold-sensitive allele *nda3*-KM311 resumes its ability to polymerize into microtubules within 6 minutes of return to the permissive temperature and allows progression through chromosome segregation and mitotic exit (Hiraoka *et al*., 1985).

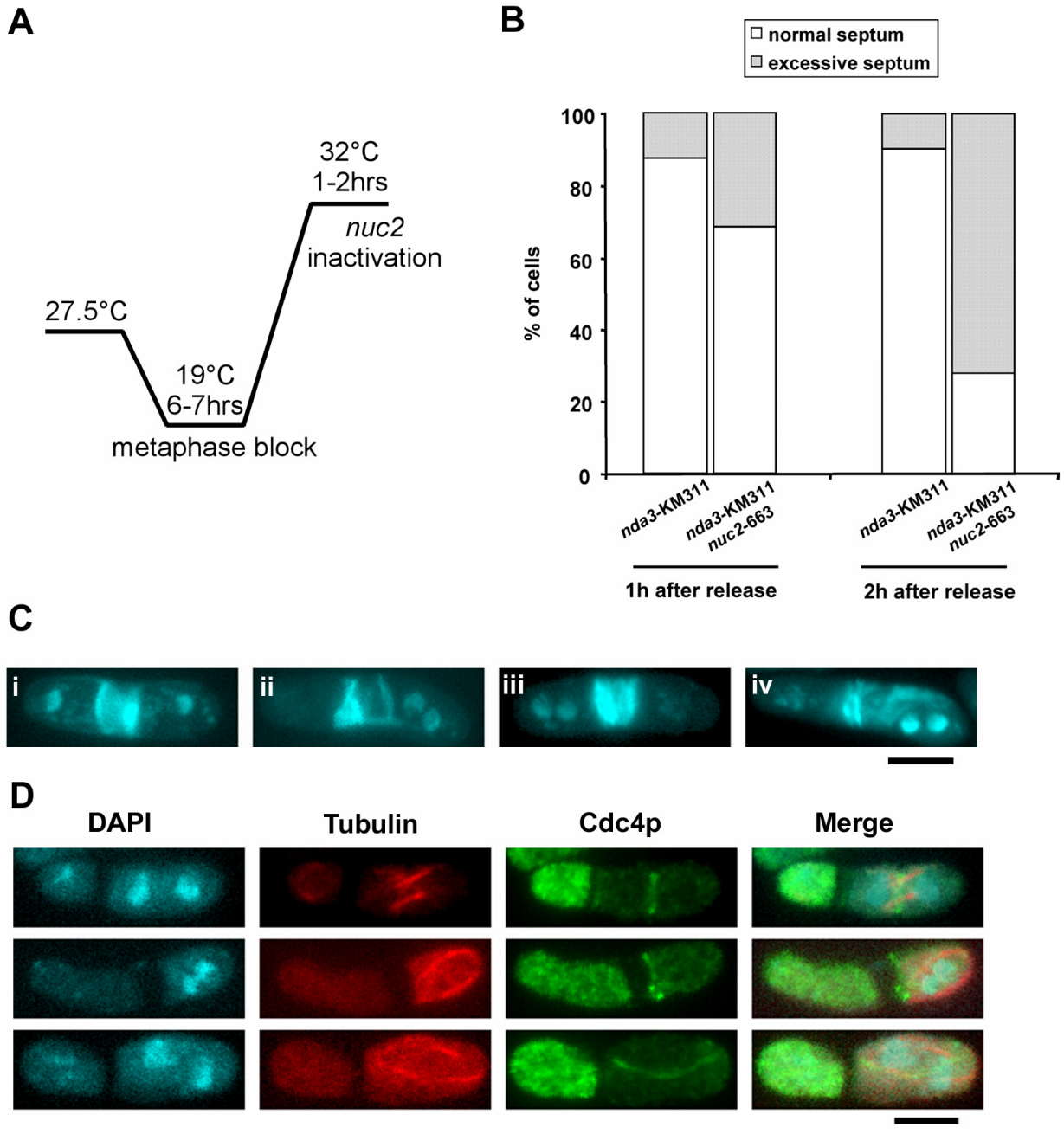
The *nuc2*-663 *nda3*-KM311 and *nda3*-KM311 (as a control) cells were first cultured at 19 °C to inactivate Nda3p function. Under these conditions, at least 50% of cells arrested at metaphase due to the activation of the spindle assembly checkpoint. Subsequently, these cells were shifted to 32 °C to inactivate Nuc2p function and to reactivate Nda3p function. Since reactivation of Nda3p function (based on the immediate assembly of the mitotic spindle and 40% of total cells formed an elongated anaphase spindle after 45 min at 32 °C) occurred more rapidly than the inactivation of Nuc2p, *nuc2*-663 *nda3*-KM311 (and the control *nda3*-KM311) underwent anaphase after the release from metaphase block and completed one round of septation.

Interestingly, maintenance of *nuc2*-663 *nda3*-KM311 at 32 °C led to the accumulation of cells that either formed multiple septa, or deposited excessive septum material in the vicinity of the first septum (Figure 3.7C, cells i-iii). Cells with mis-oriented ectopic septa were also frequently observed (Figure 3.7C, cell iv). These effects were observed less frequently in the control *nda3*-KM311 cells. Quantification results showed that more than

Figure 3.7 Ectopic actomyosin ring and septum formation in the *nuc2*-663 mutant after septation

(A) The diagram schematically illustrates the experimental design. (B) Quantification of cells with normal or excessive septa after 1–2 h release from metaphase block. At least 200 cells were counted in each category. (C) Examples of septum patterns in *nuc2*-663 *nda3*-KM311 cells. Four examples of cells with excessive septum material are shown. i and ii, multiseptated cells; iii, excessive deposition of septum material at division site; iv, cell with ectopic misoriented septum. (D) Visualization of actomyosin rings in *nuc2*-663 *nda3*-KM311 cells by immunofluorescence microscopy. Top, middle, and bottom panels show cells with actomyosin rings that were formed straight, close to, or misoriented with respect to the previous division site, respectively. Microtubules were stained with TAT-1 antibody, and the actomyosin ring was stained with anti-Cdc4p antibody.

Figure 3.7


A
32°C
1-2hrs
nuc2
inactivation
27.5°C
19°C
6-7hrs
metaphase block
B
normal septum
excessive septum
100
80
60
40
20
0
% of cells
nda3-KM311
nda3-KM311
nuc2-663
nda3-KM311
nda3-KM311
nuc2-663
1h after release
2h after release
C
i
ii
iii
iv
D
DAPI
Tubulin
Cdc4p
Merge

70% of *nuc2*-663 cells displayed excessive septation phenotype compared to *nuc2*+ cells (Figure 3.7B). In addition, immunostaining of cells with antibody that recognizes the myosin light chain Cdc4p to visualize actomyosin ring showed that additional actomyosin rings were also detected in *nuc2*-663 *nda3*-KM311 cells (Figure 3.7D). These rings were either orthogonally placed or mis-oriented (Figure 3.7D, Cdc4p panel). The staining of microtubule cytoskeletons revealed that these cells contained interphase microtubule arrays running along the long axis, suggesting that the assembly of additional actomyosin rings was not linked to re-entry into mitosis (Figure 3.7D, tubulin panel). Collectively, these experiments showed that Nuc2p might be required to inhibit SIN signaling to ensure one round of cytokinesis per round of mitosis.

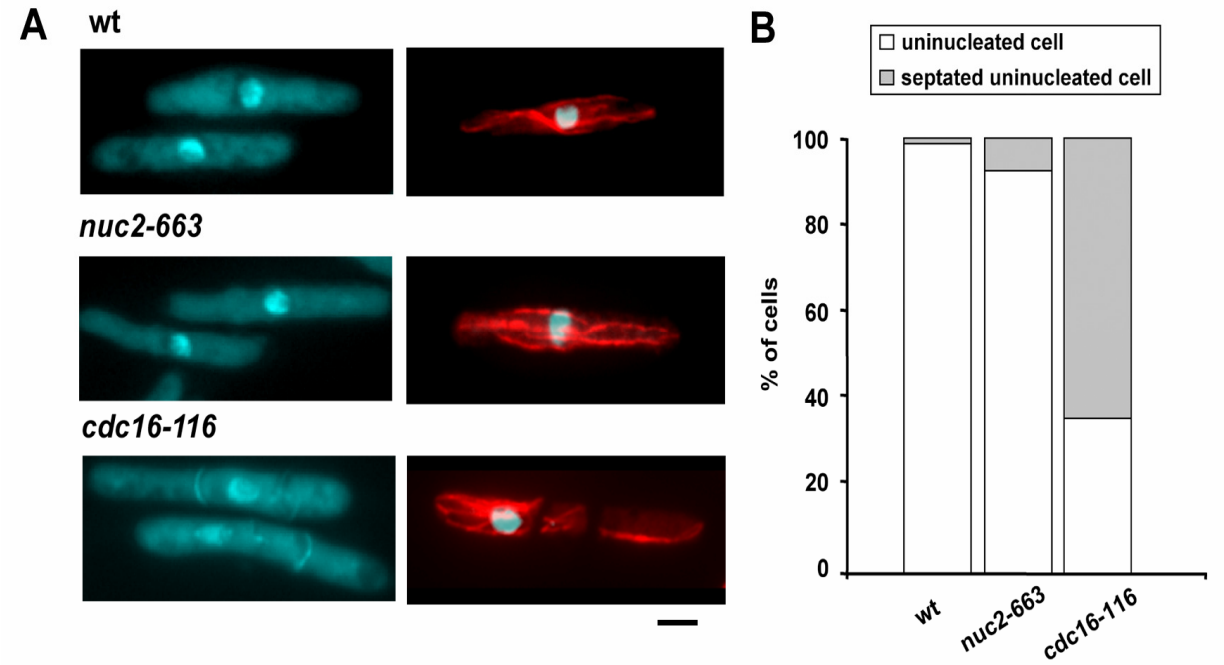
3.2.8 Inactivation of Nuc2p function does not trigger septation in S-phase arrested cells

The fact that *nuc2*-663 mutant assembled excessive septum after one round of cytokinesis raised the possibility that the high SIN signaling in *nuc2*-663 mutant might be able to trigger ectopic cytokinesis in interphase cells as in *cdc16*-116 mutant (Cerutti and Simanis, 1999). To test if this is the case, *nuc2*-663 mutant and *cdc16*-116 mutant were treated with hydroxyurea to block cells at G1/S boundary, and were shifted to restrictive temperature to heat inactivate *nuc2* and *cdc16*. As previously reported, heat inactivation of *cdc16* led to formation of septated cells during interphase as indicated by the interphase microtubule organization and septum in the cell (Figure 3.8A and B, *cdc16*-116 panel). The *nuc2*-663 mutant at restrictive temperature, however, similar to wild type

Figure 3.8 S-phase arrested *nuc2*-663 cells do not septate

(A) Septum assembly in S-phase–arrested cells. Wild type, *nuc2*-663, and *cdc16*-116 cells arrested in S phase by hydroxyurea treatment were either formaldehyde fixed for DAPI and aniline blue staining or methanol fixed for immunostaining with TAT-1. (B) Quantification of septated cells versus non-septated cells upon hydroxyurea treatment. At least 300 cells were counted for each category. Scale bar, 5 μm.

Figure 3.8


A
wt
nuc2-663
cdc16-116
B
uninucleated cell
septated uninucleated cell
% of cells
100
80
60
40
20
0
wt
nuc2-663
cdc16-116

cells did not form septum after hydroxyurea treatment (Figure 3.8A and B, *wt* and *nuc2*-663 panels). Thus, *nuc2*-663 mutant only loses the ability to restrain cytokinesis but does not induce ectopic cytokinesis in interphase arrested cells.

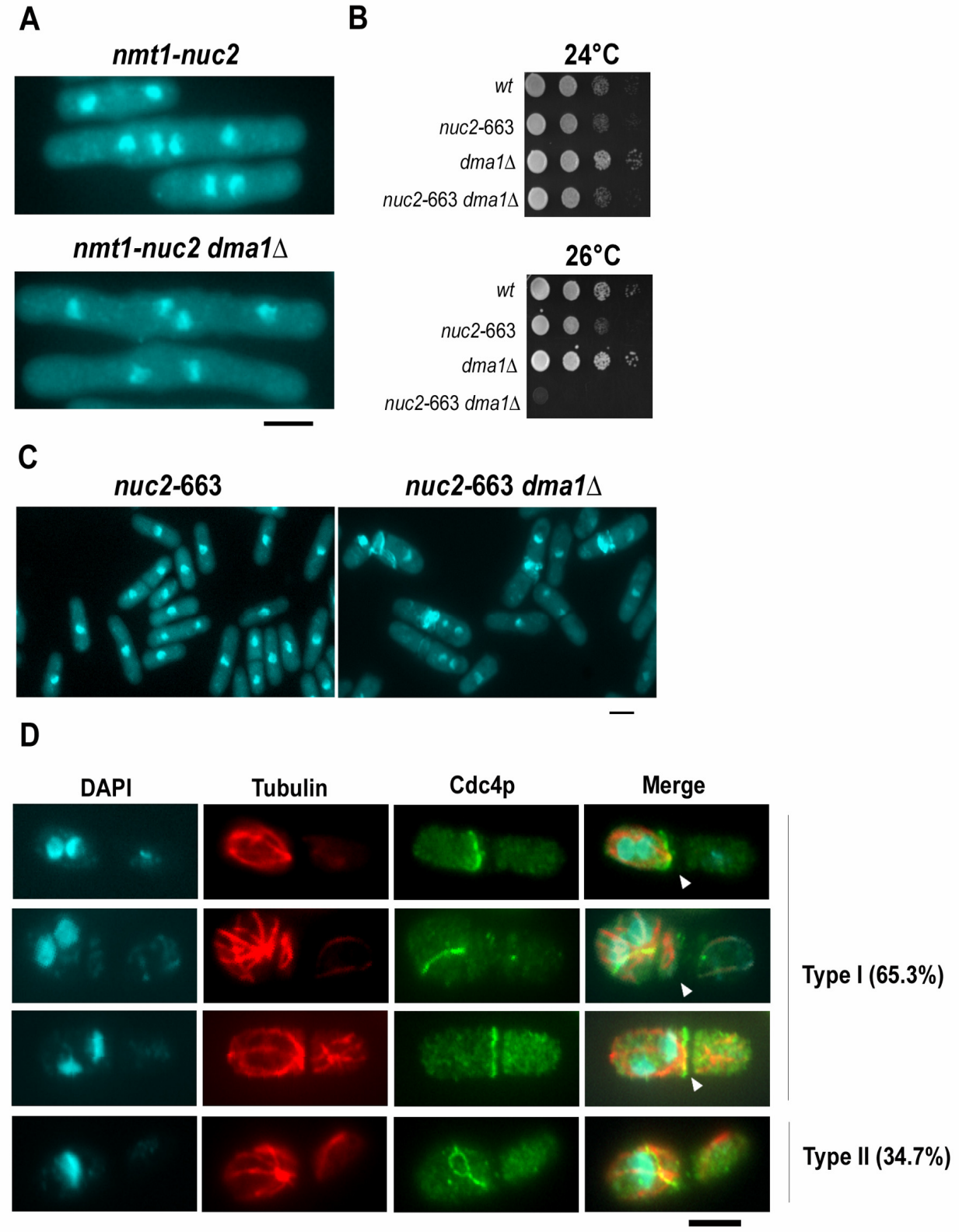
3.2.9 Nuc2p acts independently of Dma1p to inhibit SIN

Previous studies have shown that overproduction of Dma1p, a fission yeast protein related to human CHFR, leads to inhibition of SIN function and defective cytokinesis (Guertin *et al*., 2002; Murone and Simanis, 1999). Since overproduction of Nuc2p also led to defects in SIN signaling, I tested if the cytokinesis-inhibitory effect upon Nuc2p overproduction depended on Dma1p. To this end, Nuc2p was overproduced in cells lacking Dma1p. Cytokinesis defects were observed in control (*dma1*+) cells as well as *dma1*Δ cells overproducing Nuc2p (Figure 3.9A). This experiment suggested that the SIN-inhibitory effect caused by overproduction of Nuc2p was independent of Dma1p. To firmly establish if this was the case, double mutants defective in *nuc2* and *dma1* were constructed. Interestingly, whereas *nuc2*-663 and *dma1*Δ cells were able to form colonies at 26 °C, the double mutants were unable to do so (Figure 3.9B). Staining of DNA and septum material revealed that the *nuc2*-663 *dma1*Δ, but not *nuc2*-663, cells displayed an aberrantly septated phenotype, similar to that observed in *nuc2*-663 cells at higher temperatures (Figure 3.9C). Immunostaining of *nuc2*-663 *dma1*Δ cells with antibodies against Cdc4p and tubulin revealed that 65.3% of septated cells contained segregated DNA, interphase microtubules and actomyosin cables/rings (Figure 3.9D, Type I panel). Furthermore, 34.7% of the septated cells contained unsegregated DNA, interphase

Figure 3.9 Nuc2p acts independently of Dma1p to regulate cytokinesis

(A) Overexpression of Nuc2p in wild type or *dma1*Δ mutant. (B) Wild type, *nuc2*-663, *dma1*Δ, and *nuc2*-663 *dma* Δ strains were diluted serially and spotted on YES agar and incubated at 24 °C or 26 °C. (C) Septum patterns in *nuc2*-663 and *nuc2*-663 *dma1*Δ mutants grown at 28 °C. Cells were fixed and stained with DAPI and aniline blue. (D) Visualization of actomyosin rings and microtubules in *nuc2*-663 *dma1*Δ cells by immunofluorescence microscopy. Microtubules were stained with TAT-1 antibody and the actomyosin ring was stained with antibodies against Cdc4p. About 150 cells were counted in each category. Arrowhead indicates septum assembled in previous cell division.

Figure 3.9


A
nmt1-nuc2
nmt1-nuc2 dma1Δ
B
24°C
wt
nuc2-663
dma1Δ
nuc2-663 dma1Δ
26°C
wt
nuc2-663
dma1Δ
nuc2-663 dma1Δ
C
nuc2-663
nuc2-663 dma1Δ
D
DAPI
Tubulin
Cdc4p
Merge
Type I (65.3%)
Type II (34.7%)

microtubules, and actomyosin rings/cables (Figure 3.9D, Type II panel). Since the multi-septate phenotype is largely detected in cells in which chromosome segregation is not affected, we conclude that the effect of septation in the *nuc2*-663 *dma1*Δ double mutants is not purely due to synthetic effects on APC/C function. Collectively, these studies established that Nuc2p and Dma1p inhibited the SIN by different mechanisms.

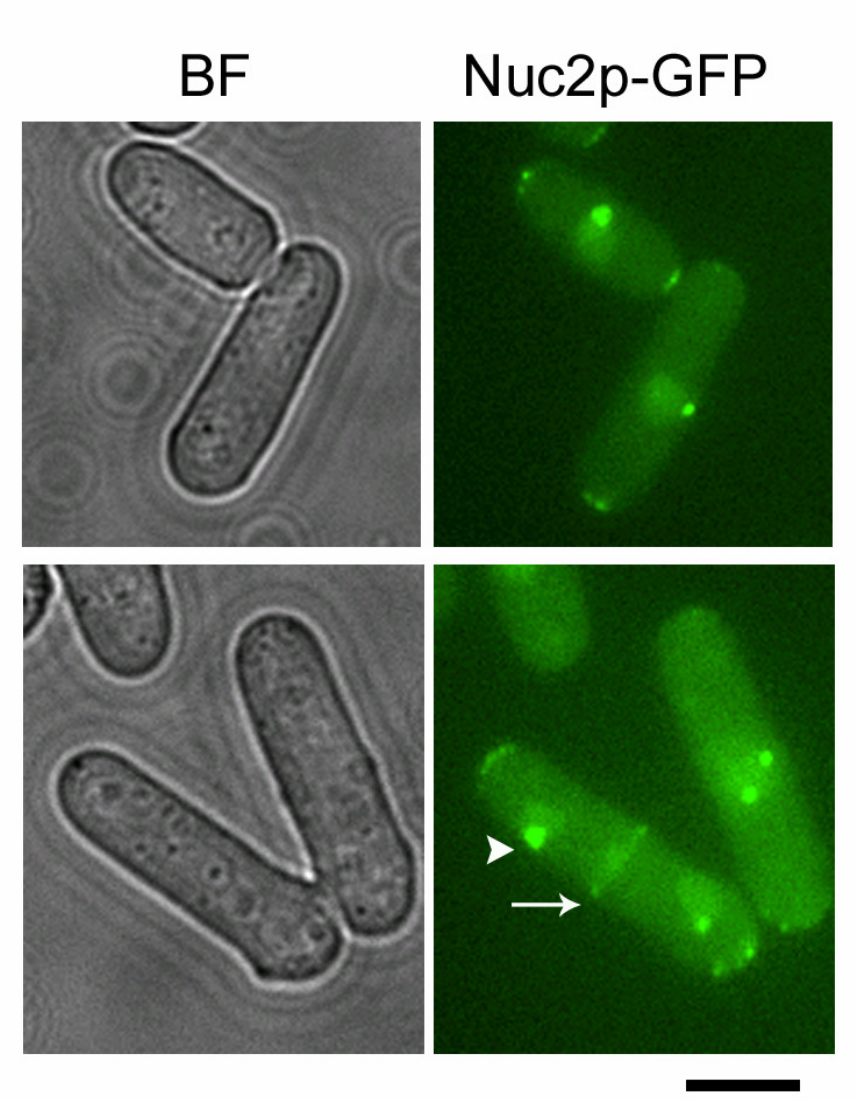
3.2.10 Analysis of the sub-cellular localizations of Nuc2p

To further understand the functions of Nuc2p, I examined the sub-cellular localizations of Nuc2p by fusing the gene with green fluorescent protein epitope (GFP). The expression of *nuc2*-GFP driven by its native promoter was not detectable under fluorescence microscopy. To enhance the fluorescence intensity, I replaced the endogenous *nuc2* promoter with medium strength thiamine-repressible promoter, *nmt41*. Upon the growth on medium lacking thiamine to induce Nuc2p-GFP expression, strong GFP signals could be detected at the structures resemble SPBs (Figure 3.10). In addition, GFP signals were also observed at the division site, cell tips, and nucleus (Figure 3.10). Since proteins involved in the regulation of cytokinesis normally localize at the SPBs and division sites, the detection of Nuc2p-GFP at these subcellular localizations strengthens its roles in cytokinesis. The localization of Nuc2p-GFP at cell tips and nucleus also suggests a possible role of Nuc2p at these subcellular localizations.

Figure 3.10 Localization of Nuc2p-GFP in *S. pombe*

Cells expressing Nuc2p-GFP under medium strength *nmt41* promoter grown on minimal medium without thiamine were visualized by fluorescence microscopy. Arrow indicates Nuc2p-GFP at the division site and arrowhead shows Nuc2p-GFP at the SPB. Scale bar: 5μm.

Figure 3.10



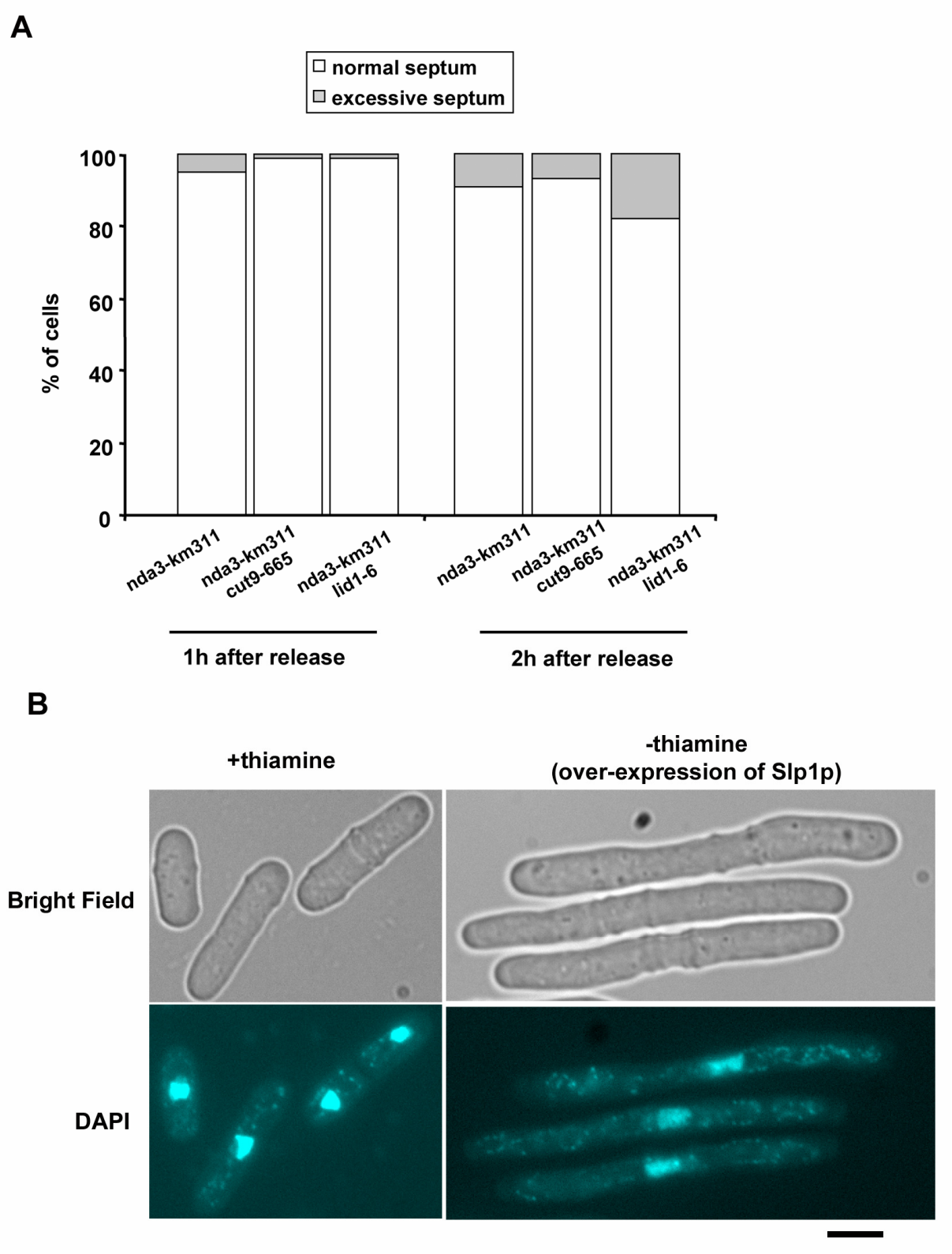
3.2.11 Nuc2p might function independently of APC/C to regulate cytokinesis

Since Nuc2p is a subunit of the APC/C, it was possible that the entire APC/C might function to inhibit inappropriate cytokinesis. Alternatively, it was possible that Nuc2p regulated cytokinesis in a manner independent of other subunits of the APC/C. To distinguish between these possibilities, I assayed the ability of *cut9*-665 and *lid1*-6 (two essential components of the APC/C) mutants to accumulate multiple septa. As before, I tested for the presence of multiple and excessive septa upon shift of synchronous *nda3*-KM311 *cut9*-665 and *nda3*-KM311 *lid1*-6 to conditions that inactivated the APC/C components. In these experiments, *nda3*-KM311 *cut9*-665 and *nda3*-KM311 *lid1*-6 behaved similar to *nda3*-KM311 cells and did not accumulate multiple and excessive septa (Figure 3.11A). Furthermore, activation of APC/C by overexpression of Slp1p or Ste9p did not lead to septation defects, indicating that APC/C activation per se does not lead to septation defects (Figure 3.11B). Collectively, these experiments suggested that the inhibition of inappropriate cytokinesis by Nuc2p might not require the other subunits of the APC/C.

Figure 3.11 Analysis of septation in other APC/C mutants, *cut9*-665 and *lid1*-6, and overexpression of Slp1p

(A) Cells were first synchronized at metaphase using cold sensitive allele of β-tubulin, nda3-*KM311*. The cultures were shifted to 36 °C to release cells from the metaphase block and to inactivate Cut9p and Lid1p functions. Cells were then collected after 1 to 2 h release from the metaphase block and scored for septation phenotype (normal versus excessive septum) by staining the cells with DAPI and aniline blue. (B) The APC/C activator Slp1p was overexpressed from *nmt1* promoter in wild type cells. Shown are DAPI stained images of cells grown in the presence (repressing) and absence (inducing) of thiamine. Scale bar, 5 μm.

Figure 3.11


A
normal septum
excessive septum
% of cells
100
80
60
40
20
0
nda3-km311
nda3-km311 cut9-665
nda3-km311 lid1-6
nda3-km311
nda3-km311 cut9-665
nda3-km311 lid1-6
1h after release
2h after release
B
+thiamine
-thiamine (over-expression of Slp1p)
Bright Field
DAPI

3.3 Discussion

Previous studies have characterized the role of Nuc2p, a TPR-containing subunit of APC/C, in the regulation of progression through events of mitosis (Samejima and Yanagida, 1994; Yamada *et al*., 1997; Yamashita *et al*., 1999). Previous studies have also proposed that Nuc2p might function as an inhibitor of septation, although the mechanism of this inhibition was not investigated (Kumada *et al*., 1995). In the current study we show that Nuc2p prevents septum assembly by inhibiting the septation initiation network (SIN).

Cells defective for *nuc2* display a "cut" phenotype, characterized by septum assembly in the absence of proper segregation of chromosomes (Hirano *et al*., 1988). Interestingly, we have found that prolonged incubation of *nuc2*-663 mutants at the restrictive temperature leads to the accumulation of cells with multiple septa. The specific effects of Nuc2p on septation are particularly clear in *nuc2*-663 mutants shifted to the restrictive temperature after execution of Nuc2p function in mitosis. The multiseptate phenotype of *nuc2*-663 is reminiscent of that displayed by cells in which the SIN is constitutively activated, such as in cells defective in Cdc16p, which together with Byr4p functions as a GTPase activating protein for the Spg1p-GTPase (Furge *et al*., 1998; 1999). As in the case of *cdc16*-116 cells, the SIN components Cdc7p and Sid1p are retained at the SPBs even after septum assembly in *nuc2*-663 cells. In addition, Sid2p, considered to be the most downstream element of the SIN, is retained at the cell cortex after septum assembly in *cdc16*-116 and *nuc2*-663 mutants. Interestingly, overproduction of Nuc2p has been shown to cause

defects in cytokinesis (Kumada *et al*., 1995 and this study). Furthermore, overproduction of Nuc2p leads to defects in actomyosin ring maintenance and localization of Cdc7p, Sid1p, and Sid2p to the SPB, suggestive of defective SIN signaling. Thus, while *nuc2* mutants phenocopy *cdc16* mutants (in which SIN is constitutively active), overproduction of Nuc2p leads to a phenotype indistinguishable from that displayed by SIN-defective mutants. These results imply that Nuc2p might be a bona fide inhibitor of the SIN. Interestingly, whereas activation of SIN, by loss of Cdc16p function, promotes events of cytokinesis (actomyosin ring and division septum assembly) in premitotic interphase arrested cells, the loss of Nuc2p function in premitotic interphase does not lead to actomyosin ring and septum assembly. Thus, although Nuc2p appears to be an inhibitor of the SIN, Nuc2p might inhibit SIN specifically after cell division.

How does Nuc2p inhibit the SIN? Cells overexpressing Nuc2p phenocopy mutants defective in SIN function, which is a Spg1p GTPase driven signaling cascade. Interestingly, the localization of the upstream components of SIN, Cdc11p, Sid4p and Spg1p are not altered upon overproduction of Nuc2p. The increased level of Nuc2p, however, specifically affects the localization of downstream kinases: Cdc7p, Sid1p and Sid2p. Since Spg1p, but not its effectors Cdc7p and Sid1p, remains at the SPB, after Nuc2p overexpression, it can be speculated that the Spg1p in cells overexpressing Nuc2p is GDP-bound. Consistent with this, we have found that the physical interaction between Cdc7p and Spg1p is dramatically reduced in cells overproducing Nuc2p. Two possibilities can be envisaged for the mechanisms that maintain Spg1p in GDP-bound form. First, it is possible that overexpression of Nuc2p inhibits a putative guanine nucleotide exchange factor (GEF) for Spg1p, thereby preventing the loading of GTP onto

Spg1p and the activation of SIN signaling. The second possibility is that Nuc2p promotes the activation of the two-component GTPase activating protein (GAP), Cdc16p-Byr4p, leading to the conversion of GTP-Spg1p into GDP-Spg1p. We do not favour the first possibility since no guanine nucleotide exchange factor for Spg1p has been identified to date. Our genetic analysis (restoration of Cdc7p localization and septum assembly in *cdc16*-116 cells overproducing Nuc2p) points to the second possibility that Nuc2p might function as an activator of Cdc16p-Byr4p. However, it also remains possible that Nuc2p might prevent the conversion of GDP-Spg1p to GTP-Spg1p by acting as a GDP dissociation inhibitor (GDI). Future studies should test these possibilities.

What is the physiological function of the inhibition of SIN by Nuc2p? Both cell division and cell growth utilize actin cytoskeleton and its modulators. Persistent SIN signaling might sequester actin cytoskeleton at the division site and thereby block actin remodeling at the growth sites. Thus, prevention of cytokinesis after cell division might ensure proper growth polarity establishment. Several mechanisms have been uncovered in fission yeast that prevent cytokinesis by inhibiting SIN (McCollum and Gould, 2001). During metaphase, the human CHFR protein homolog in fission yeast, Dma1p, inhibits SIN signaling to prevent precocious cytokinesis (Guertin *et al*., 2002; Murone and Simanis, 1996). Prior to mitotic exit (in metaphase and anaphase A), the inhibition of SIN function by high CDK activity ensures the coordination of mitosis and cytokinesis (Guertin *et al*., 2000). Our studies show that Nuc2p inhibits SIN function after completion of cytokinesis. Genetic analysis suggests that Nuc2p and Dma1p, both inhibitors of SIN, might function independently of each other. Our study also points to an additional function for Dma1p after cell division in the prevention of SIN activation. Thus, it

appears that high CDK activity and Dma1p inhibit SIN during metaphase, while Nuc2p and Dma1p are required to prevent SIN activation and inappropriate cytokinesis after completion of cell division

The current study shows that Nuc2p inhibits SIN signaling. However, it remains unclear whether Nuc2p acts independently or requires a specific set of APC/C components in order to inhibit septation. We have not been able to detect a multiseptate phenotype upon inactivation of function of two additional subunits of the APC/C, namely Cut9p and Lid1p, after passage through anaphase. These observations might suggest that Nuc2p, rather than the entire APC/C, is required for the inhibition of SIN following cell division. In addition, hyperactivation of APC/C by overexpression of Slp1p (this study) and Ste9p (Kitamura *et al*., 1998; Yamaguchi *et al*., 1997) does not lead to septation defects, suggesting that activation of APC/C does not lead directly to abnormal septation. In contrast, we have found that overproduction of Cut23p gives weak cytokinesis defects (Matsuyama *et al*., 2006), suggesting a role for the entire APC/C in prevention of inappropriate cytokinesis. Collectively, our present studies lean toward a specific role for Nuc2p (rather than the entire APC/C) in the inhibition of cytokinesis. However, additional studies with stronger alleles of other APC/C components, which inactivate faster than the currently available alleles, might be required to firmly conclude if Nuc2p inhibition of the SIN requires the entire APC/C.

Chapter 4: Morphogenesis and spatial regulation of cytokinesis*

4.1 Introduction

Cell morphology contributes to the positioning of division plane in animal cells. The mitotic spindle segregates chromosomes along the longitudinal axis, and specifies the division plane perpendicular to the long axis of a cell. The long cell axis provides a default determinant to position the division plane. It is more feasible to divide along the long cell axis as the tension in astral microtubules appears to be stronger when the microtubules are aligned in parallel with the cellular axis. Interestingly, a longitudinal axis has been proposed to play an important role in the regulation of fission yeast cytokinesis that is independent of astral microtubules.

In this study, we further characterize the importance of a cylindrical axis in spatial regulation of cytokinesis in fission yeast. Through the identification and characterization of a novel protein Pal1p that participates in morphogenesis, we provide insights on the relationship between cellular morphology and cytokinesis in fission yeast. We found that Pal1p regulates morphogenesis by modulating the cell wall synthesis, and it is required to maintain a cylindrical-morphology of fission yeast. The *pal1*Δ mutant that lacks *pal1* open reading frame (ORF) re-polarizes to establish a cylindrical axis in a Tea1p (a Kelch-repeat containing protein)-dependent manner. In the absence of Pal1p and Tea1p, the fission yeast is unable to establish/maintain a cylindrical-shaped and undergoes mitosis in

* This is a collabrative work with Mr. Wanzhong Ge. The work has been published in ***Molecular Biology of the Cell. 2005 Sep;16(9):4124-38***, where WG and TGC are joint first author.

a spherical morphology. Thus, the double mutant provides a useful tool to investigate cellular geometry and cytokinesis. Analysis of the double mutant revealed that spherical morphology is inefficient in the proper positioning of the division plane, suggesting that a long cell axis is crucial for spatial regulation of cytokinesis in fission yeast.

4.2 Results

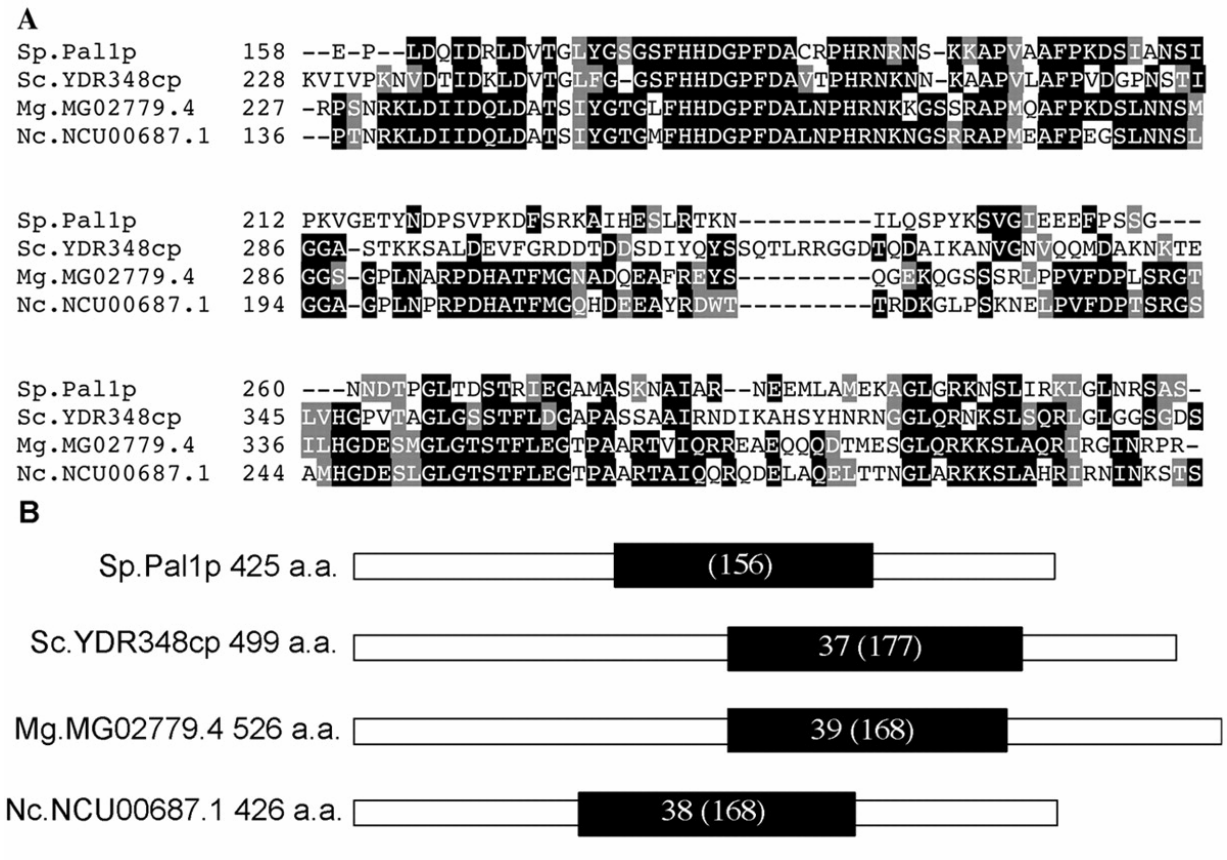
4.2.1 Identification of a novel protein Pal1p that associates with active growth zones and cell division site

We identified a gene, pal1 (pears and lemons; encoded by SPCP1E11.04c), based on its homology to a novel uncharacterized ORF from budding yeast (YDR348c). This gene was chosen because its protein product localizes to the cell division site, as determined from a large-scale effort aimed at cataloging the intracellular localization of all budding yeast proteins (Huh *et al*., 2003). Proteins related to Pal1p were detected in several ascomycetes. Figure 4.1A shows the alignment of the conserved part of the amino acid sequence of Pal1p with related members from *S. cerevisiae*, *Magnaporthe grisea*, and *Neurospora crassa*. Proteins related to Pal1p are variable in length and share an ~160 amino acid region of high similarity, which ends roughly 100 amino acids from the C-termini of these proteins (Figure 4.1B). Proteins related to Pal1p were not readily identified in plants and metazoans.

Figure 4.1 Identification of Pal1p in fission yeast.

(A) *Schizosaccharomyces pombe* Pal1p is aligned with *S. cerevisiae* YDR348cp, *Magnaporthe grisea* MG02779.4, *Neurospora crassa* NCU006871. Identical amino acids are shaded in black and similar amino acids are shaded in gray. (B) Schematic representation of the homology between Sp.Pal1p and other related proteins. Identities (%) and the number of amino acids in parentheses are indicated in the conserved region.

Figure 4.1


A
Sp.Pal1p 158 --E-P--LDQIDRLDVTGLYGSGSFHHDGPFDACRPHRNRNS-KKAPVAAFPKDSIANSI
Sc.YDR348cp 228 KVIVPKNVDTIDKLDVTGLFG-GSFHHDGPFDAVTPHRNKNN-KAAPVLAFPVDGPNSTI
Mg.MG02779.4 227 -RPSNRKLDIIDQLDATSIYGTGLFHHDGPFDALNPHRNKKGSSRAPMQAFPKDSLNNSM
Nc.NCU00687.1 136 --PTNRKLDIIDQLDATSIYGTGMFHHDGPFDALNPHRNKNGSRRAPMEAFPEGSLNNSL
Sp.Pal1p 212 PKVGETYNDPSVPKDFSRKAIHESLRTKN---------ILQSPYKSVGIEEEFPSSG---
Sc.YDR348cp 286 GGA-STKKSALDEVFGRDDTDDSDIYQYSSQTLRRGGDTQDAIKANVGNVQQMDAKNKTE
Mg.MG02779.4 286 GGS-GPLNARPDHATFMGNADQEAFREYS---------QGEKQGSSSRLPPVFDPLSRGT
Nc.NCU00687.1 194 GGA-GPLNPRPDHATFMGQHDEEAYRDWT---------TRDKGLPSKNELPVFDPTSRGS
Sp.Pal1p 260 ---NNDTPGLTDSTRIEGAMASKNAIAR--NEEMLAMEKAGLGRKNSLIRKLGLNRSAS-
Sc.YDR348cp 345 LVHGPVTAGLGSSTFLDGAPASSAAIRNDIKAHSYHNRNGGLQRNKSLSQRLGLGGSGDS
Mg.MG02779.4 336 ILHGDESMGLGTSTFLEGTPAARTVIQRREAEQQQDTMESGLQRKKSLAQRIRGINRPR-
Nc.NCU00687.1 244 AMHGDESLGLGTSTFLEGTPAARTAIQQRQDELAQELTTNGLARKKSLAHRIRNINKSTS
B
Sp.Pal1p 425 a.a.
(156)
Sc.YDR348cp 499 a.a.
37 (177)
Mg.MG02779.4 526 a.a.
39 (168)
Nc.NCU00687.1 426 a.a.
38 (168)

To determine the intracellular distribution of Pal1p, we fused the ORF to DNA sequences encoding the GFP. The sole copy of the gene encoding the Pal1p-GFP fusion was expressed under the control of the native promoter sequence. Pal1p showed a cell cycle-dependent intracellular distribution (Figure 4.2). Pal1p was detected at one end of the cell in a small proportion of uninucleate cells (Figure 4.2; cell 1) and was visualized at both ends of the vast majority of uninucleate cells (Figure 4.2; cell 2). In cells undergoing cytokinesis, Pal1p-GFP was present at the medial cell division site (Figure 4.2; cells 3–6). Thus Pal1p localizes to the cell tips in interphase and to the division site during mitosis and cytokinesis.

4.2.2 Pal1p localizes to growth zones independent of F-actin and microtubules

The fact that Pal1p was detected at one or both ends suggested that Pal1p localizes to the actively growing end(s). In the *orb2*-34 mutant grown at the permissive temperature (known to grow only at the old end; Verde *et al*., 1995), Pal1p-GFP was detected at the growing end as determined by costaining with the cell wall stain aniline blue (Figure 4.3, A and B). Pal1p-GFP was detected at both ends of heat-arrested *cdc25*-22 cells that are known to grow in a bipolar manner (Figure 4.3B). Pal1p-GFP was found to be localized to a larger region of the cell cortex in spherical and isotropically growing mutants *orb3*-167 at the restrictive temperature (Figure 4.3C) and was detected at the additional tips generated in a *tea1*Δ mutant (marked with arrows in Figure 4.3D). Furthermore, Pal1p-GFP localized to the mispositioned septa in a *mid1*Δ mutant (Figure 4.3E). Taken

Figure 4.2 Localization of Pal1p in wild type cells.

Cells expressing functional GFP-tagged Pal1p grown on YES plates were visualized by fluorescence microscopy. Pal1p localizes to the growing end(s) during monopolar and bipolar growth (cells 1 and 2) and to the medial cell division site during cytokinesis (cells 3–6). Scale bar, 5 µm.

Figure 4.2

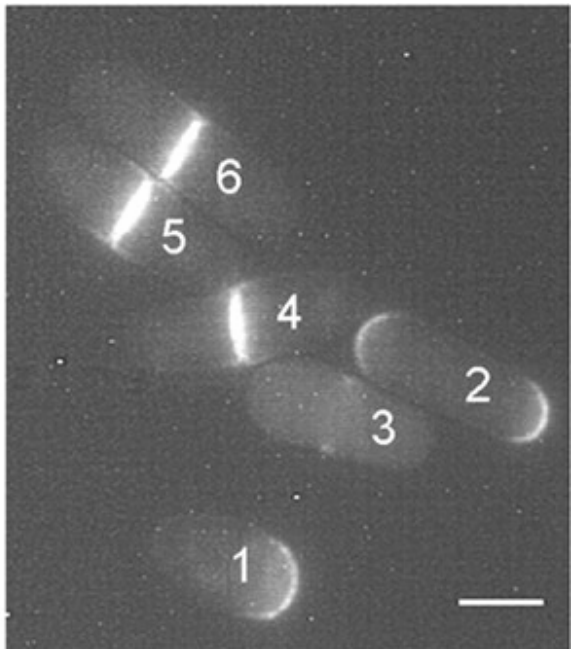
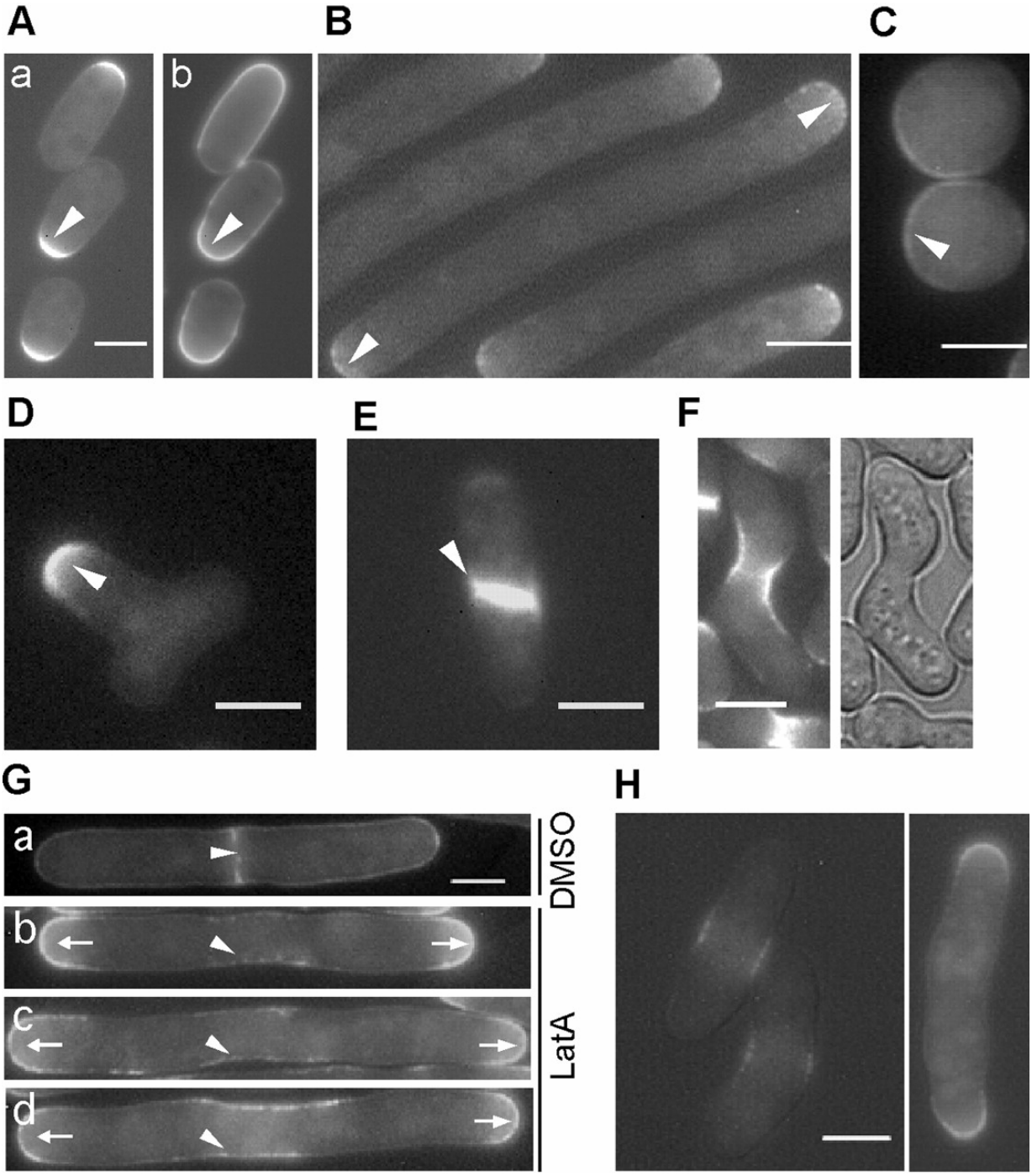


Figure 4.3 Pal1p localizes to the sites of cell growth and division in an F-actin- and microtubule-independent manner.

Different strains with functional GFP-tagged Pal1p were grown on YES agar plate at 24°C and then shifted to 36°C for 6 h. (A) *orb2*-34 at 24°C; a shows Pal1p-GFP; b shows aniline blue staining; (B) *cdc25*-22 at 36°C; (C) *orb3*-167 at 36°C; (D) *tea1*Δ; (E) *mid1*Δ; (F) Pal1p localizes to the zone of cell fusion in the *h90* strain. (G) Role of F-actin in Pal1p localization. *cdc25*-22 *pal1-GFP* cells were blocked for 4 h at 36°C in YES medium and then released to 24°C into medium containing 15 µM Lat A or DMSO. Cell samples were taken at different time points (a, 60 min; b, 60 min; c, 120 min; d, 180 min) after release and visualized by fluorescence microscopy. (H) Role of microtubules in Pal1p localization. *nda3*-KM311 *pal1-GFP* cells were grown on a YES agar plate at 32°C for 1 d and shifted to 19°C for 6 h. Scale bar, 5 µm.

Figure 4.3



together, these data suggested that Pal1p localizes to sites of polarized growth and secretion.

Fission yeast cells are known to reinitiate polarized growth toward a mating partner of opposing mating type. This growth occurs in random directions, determined by the position of the mating partner (Nielsen and Davey, 1995). During mating, Pal1p-GFP was detected at the zone of cell fusion (Figure 4.3F). Thus, Pal1p localizes to sites of polarized growth and secretion in vegetative and mating cells.

Given that both F-actin and microtubule cytoskeletons are important for proper polarized growth and cell division, we studied the role of F-actin and microtubule cytoskeletons in the localization of Pal1p-GFP to the cell tips and the cell division site. To test the role of F-actin in assembly and maintenance of Pal1p at the cell tips and the division site, a *cdc25*-22 strain was arrested at the G2/M boundary by shift to the restrictive temperature. Cells were then treated with Lat A to cause eventual loss of all F-actin structures and returned to the permissive temperature to allow resumption of the mitotic cycle. Cells were treated with the solvent DMSO as a control. In DMSO-treated cells, Pal1p was detected at the division site in well-formed septa (Figure 4.3G; cell a). Interestingly, in Lat A-treated cells, Pal1p continued to be present at the cell tips, suggesting that maintenance of Pal1p at the cell tips is F-actin independent (Figure 4.3G: cells b–d, marked with arrows). In addition, Pal1p-GFP also accumulated at the division site in Lat A-treated cells (Figure 4.3G: cells b–d, marked with arrowheads), although Pal1p-GFP

was not organized into a proper medial ring structure in the absence of an actomyosin ring.

To study the role of the microtubule cytoskeleton in Pal1p-GFP localization, we arrested the cold-sensitive β-tubulin mutant *nda3*-KM311 at the restrictive temperature and assayed the localization of Pal1p-GFP. Interestingly, Pal1p-GFP was clearly detected at the presumptive cell division site (Figure 4.3H) as well as at the cell tips (Figure 4.3H). Thus, Pal1p accumulation at the cell division site and its maintenance at the cell tips and the division site are independent of F-actin and microtubule function.

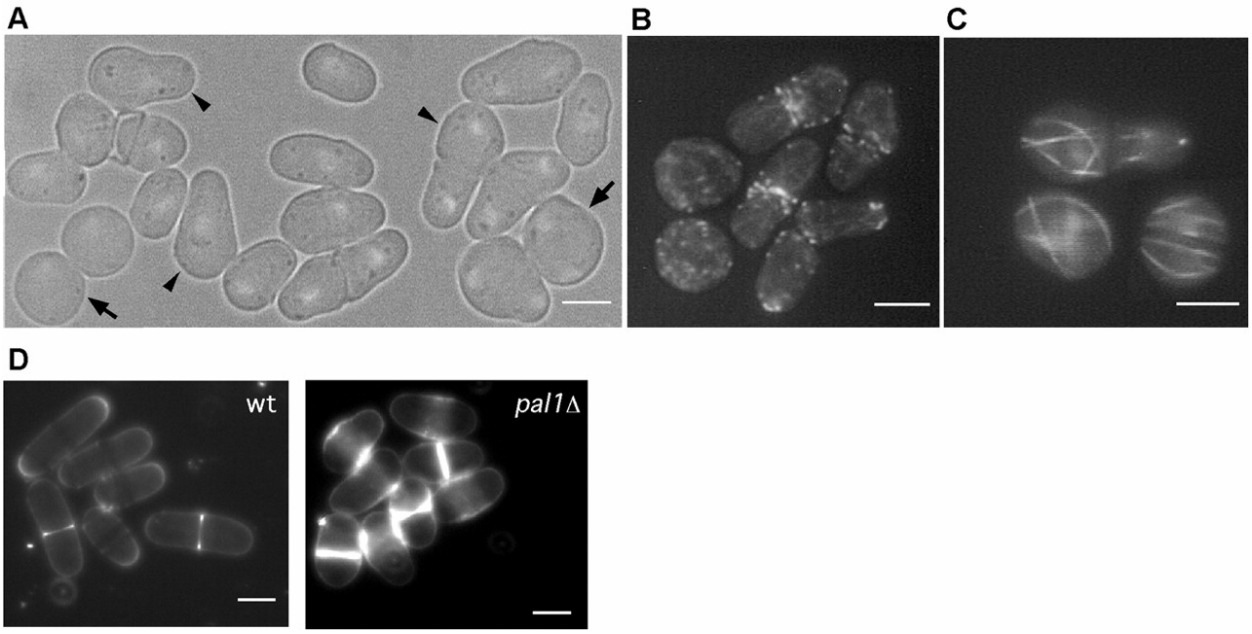
4.2.3 Pal1p is important for maintenance of a cylindrical shape

To study the phenotypes resulting from the loss of Pal1p function, a strain in which the entire coding region of *pal1* was deleted was constructed. The resultant strain, *pal1::ura4* (referred to as *pal1*Δ) was viable, although upon microscopic observation several morphological abnormalities were detected. The morphological phenotypes in *pal1*Δ cells were classified into three groups; Spherical (short to long axis ratio of 0.80 or higher; Figure 4.4A; cells marked with arrows), Abnormal (pear-like appearance; Figure 4.4A; cells marked with arrowheads), and Normal (cylindrical). In spherical cells F-actin and microtubules were disorganized (Figure 4.4, B and C). F-actin was detected in patches over the entire cortex, as opposed to the pattern of localization of F-actin patches at the cell tips observed in wild type cells. In spherical cells microtubules were observed to crisscross the cell, as opposed to the presence of properly organized bundles of

Figure 4.4 *pal1*Δ cell has defects in cell morphology and cell wall.

(A) Microscopic analysis of *pal1* Δ cells. *pal1*Δ cells were grown in minimal medium at 30°C and stained with DAPI to visualize DNA. Arrows, spherical cell; arrowheads, abnormal shaped cell. (B) *pal1*Δ cells were grown in minimal medium, fixed, and stained with Alexa Fluor-488-conjugated phalloidin to show the F-actin localization. (C) *pal1*Δ cells expressing GFP-α-tubulin were used to visualize interphase microtubules. (D) Wild type and *pal1*Δ cells were grown in YES liquid medium and stained with Calcofluor. Scale bar, 5 μm

Figure 4.4



microtubules observed in wild type cells (Figure 4.4C). Thus, the *pal1*Δ mutant is defective in maintenance of a cylindrical cellular morphology and displays abnormally organized F-actin and microtubules. Staining with calcofluor revealed cell wall abnormalities in *pal1*Δ cells (Figure 4.4D). In wild type cells, intense staining is only detected at hemispherical cell ends during growth and is observed at the division site during cytokinesis. In contrast, in growing interphase *pal1*Δ cells, the intensity of calcofluor staining was more in the cylindrical part than that in the hemispherical part of the cell, suggesting that cell wall assembly was occurring at improper sites.

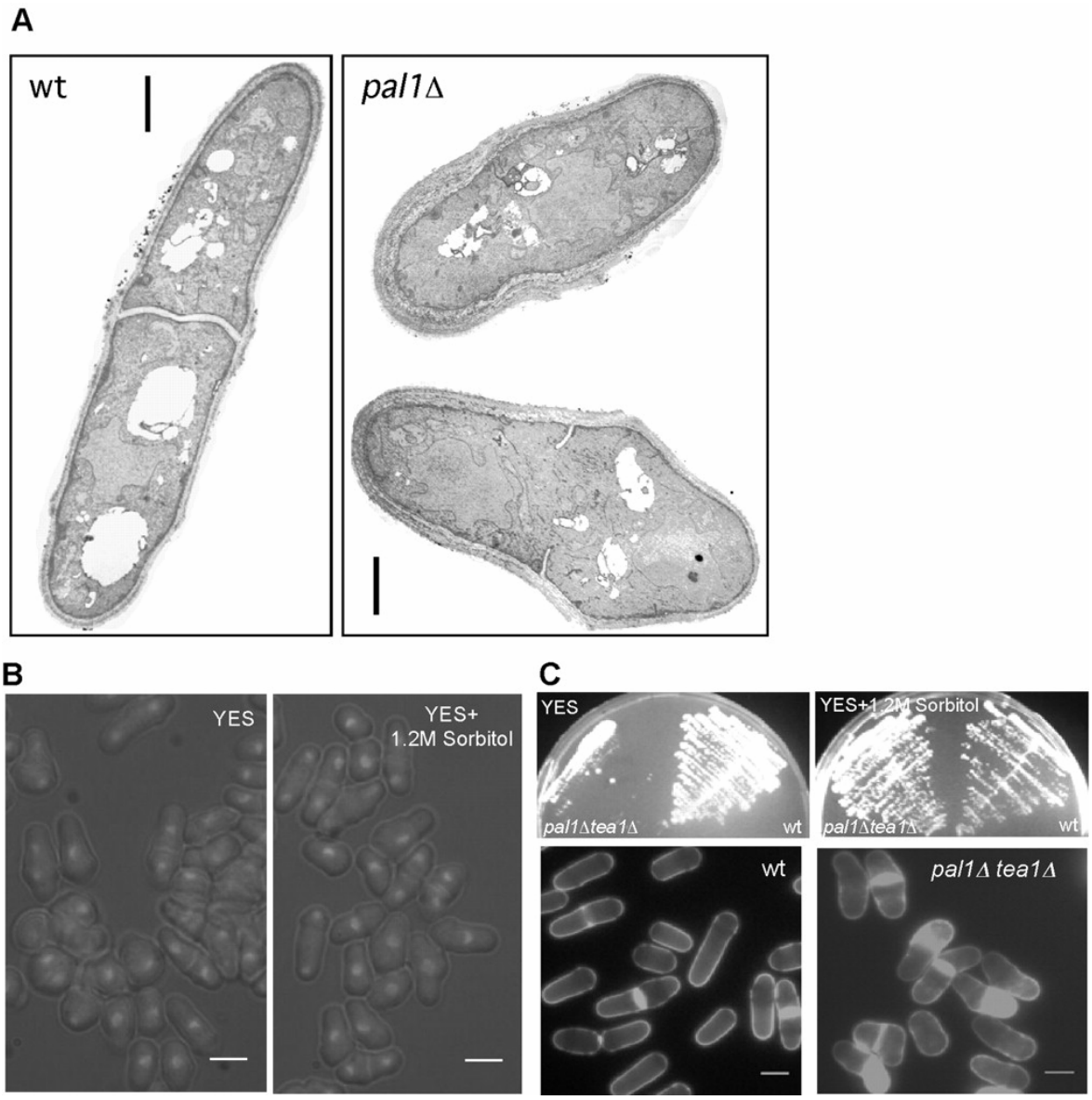
4.2.4 Pal1p is important for cell wall integrity

In experiments involving calcofluor staining, we found that the cell wall of *pal1*Δ cells was intensely fluorescent, suggesting that the cell wall in *pal1*Δ cells might be altered. To test this, we studied thin sections of wild type and *pal1*Δ cells by electron microscopy. Interestingly, whereas the cell wall of wild type cells was of uniform thickness, the cell wall in *pal1*Δ cells was significantly thicker (Figure 4.5A). Thus, *pal1*Δ cells assemble abnormal cell walls. It remained possible that the spherical morphology might have resulted from improper cell wall integrity. If this were the case, addition of an osmolyte such as sorbitol would be expected to restore cylindrical morphology of *pal1*Δ cells. This was found to be the case (Figure 4.5B). An increased number of spherical and abnormal cells was detected when *pal1*Δ cells were grown in YES compared with those grown in YES medium containing sorbitol.

Figure 4.5 *pal1*Δ cell has abnormally thick cell wall and is rescued by growth on sorbitol.

(A) Exponentially growing wild type, *pal1*Δ cells were processed for thin section electron microscopy. Electron micrographs of wild type, *pal1*Δ cells, are shown in the left, middle, and right panels, respectively. Scale bar, 1 μm. (B) Morphology of *pal1*Δ mutants is suppressed by sorbitol. *Pal1*Δ cells were grown in YES and YES plus 1.2 M sorbitol medium. Cells were stained with DAPI and visualized by fluorescence microscopy. (C) *pal1*Δ phenotype can be suppressed by sorbitol in *pal1*Δ *tea1*Δ mutant. *pal1*Δ *tea1*Δ and wild type cells were grown on YES plate with or without 1.2 M sorbitol for 2 d at 36°C (top panel). Cells from the plates were stained with aniline blue to visualize the cell shape (bottom panel).

Figure 4.5


A
wt
pal1Δ
B
YES
YES+
1.2M Sorbitol
C
YES
YES+1.2M Sorbitol
pal1Δtea1Δ
wt
pal1Δtea1Δ
wt
pal1Δ tea1Δ

We have found that Pal1p is essential in cells lacking the kelch-repeat protein Tea1p (discussed in a later section). To more rigorously test the conclusion that cells lacking Pal1p exhibited abnormal cell wall integrity, we assessed the ability of exogenously added sorbitol to restore viability of *pal1*Δ *tea1*Δ double mutant. Although *pal1*Δ *tea1*Δ double mutants were incapable of colony formation at 36°C on YES plates (Figure 4.5C; top panel), the double mutants formed healthy colonies on YES plates containing 1.2 M sorbitol (Figure 4.5C; top panel). Microscopic examination of double mutants grown on sorbitol-containing plates revealed the establishment of a cylindrical morphology in these cells (Figure 4.5C; bottom right panel). These double mutant cells, however, displayed a *tea1*Δ phenotype, because the osmolyte only suppressed cell wall-associated defects. Collectively, these studies established that the cell shape defects in *pal1*Δ cells might result from improper cell wall architecture.

4.2.5 Spherical *pal1*Δ cells polarize in G2 to establish pear-shaped morphology

Interestingly, we found that spherical binucleate cells were rarely (<1%) seen, whereas ~ 6% of the uninucleate cells were spherical in appearance (Figure 4.6A). The vast majority of uninucleate and binucleate cells were abnormal (pear shaped) in appearance (Figure 4.6A; >60% in both cases). The fact that spherical cells were rarely detected with two nuclei suggested that a mechanism might exist to polarize spherically born *pal1*Δ cells before mitosis. To test if this was the case, spherical *pal1*Δ cells were imaged by time-lapse microscopy (Figure 4.6B). Twelve of 15 spherical cells imaged underwent polarized growth, leading to the attainment of a pear-shaped morphology. Cells shown in

Figure 4.6 Establishment of cylindrical morphology in spherical cells of *pal1*Δ.

(A) Correlation of cell shape and nuclear number in *pal1*Δ cells. Cells were grown in minimal medium at 30°C to log phase, fixed, and stained with DAPI to visualize DNA. At least 600 cells each were counted for uninucleate and binucleate categories. (B) Time-lapse analysis of the growth of *pal1*Δ mutant. Cells were imaged by time-lapse light microscopy on minimal medium agar pads at room temperature. Time in minutes is indicated on the top right of each panel at the commencement of observation (t = 0). Cells are numbered for tracking, a and b are used to indicate the products of cell division. (C) Spherical shaped *pal1*Δ cells repolarize while the Cdc13-YFP signal is present in the nucleus. *pal1*Δ cells expressing Cdc13-YFP were imaged by time-lapse laser scanning confocal microscopy on YES medium agar pads at room temperature. (D) Wee1p is required for the viability and the repolarization of *pal1*Δ mutant. Wild type, *pal1*Δ, *wee1*-50, and *pal1*Δ *wee1*-50 were streaked on YES agar plate and incubated for 2 d at 36°C. (E) Microscopic analysis of *wee1*-50 and *pal1*Δ *wee1*-50 mutants. Cells were stained with DAPI to show DNA. (F) Quantification of spherical cells with one or two nuclei in *pal1*Δ, *wee1*-50, and *pal1*Δ *wee1*-50 mutants. Cultures were grown in YES medium at 24°C to log phase and shifted to 36°C for 4 h. Cell samples were stained with DAPI and at least 300 cells were counted for each strain. Scale bar, 5 μm.

Figure 4.6

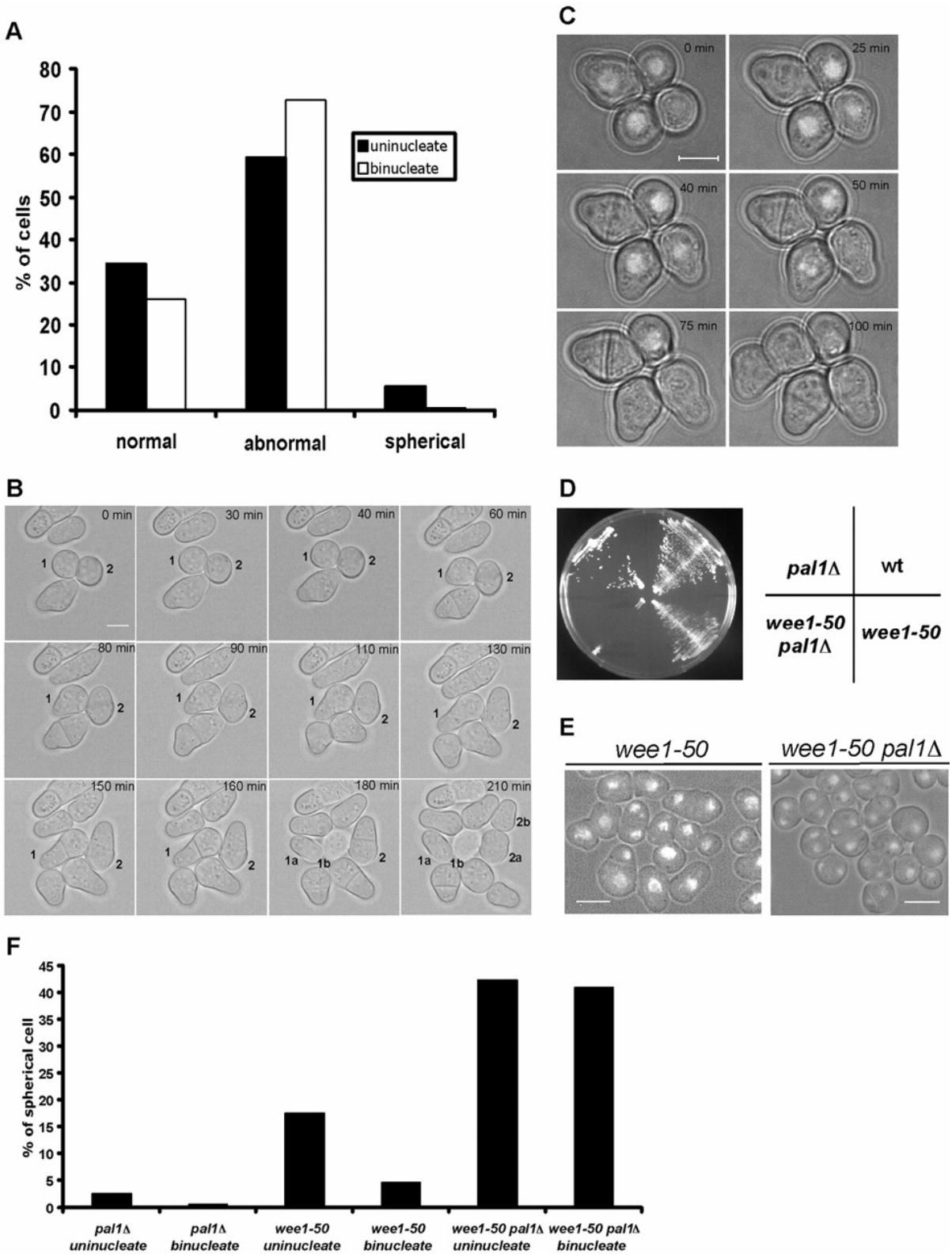

A
80
70
60
50
40
30
20
10
0
% of cells
uninucleate
binucleate
normal
abnormal
spherical
C
0 min
25 min
40 min
50 min
75 min
100 min
B
0 min
30 min
40 min
60 min
80 min
90 min
110 min
130 min
150 min
160 min
180 min
210 min
1
2
1a
1b
2a
2b
D
pal1Δ
wt
wee1-50 pal1Δ
wee1-50
E
wee1-50
wee1-50 pal1Δ
F
% of spherical cell
45
40
35
30
25
20
15
10
5
0
pal1Δ uninucleate
pal1Δ binucleate
wee1-50 uninucleate
wee1-50 binucleate
wee1-50 pal1Δ uninucleate
wee1-50 pal1Δ binucleate

Figure 4.6B were found to establish a partially cylindrical morphology and assemble division septa, indicative of successful completion of mitosis. To determine if the polarization of spherical cells took place in interphase (as opposed to during mitosis), we followed the repolarization in *pal1*Δ cells expressing Cdc13p-YFP. Cdc13p, the predominant B-type cyclin in fission yeast accumulates in the nucleus in interphase and is transferred to the mitotic spindle and the spindle pole body in metaphase before eventual degradation at anaphase A (Decottignies *et al*., 2001). We found that the polarization in spherical cells always occurred in cells in which Cdc13p-YFP was detected in the nucleus, suggesting that the polarization event occurred in interphase, and possibly in G2, because this phase represents the majority of interphase in fission yeast (Figure 4.6C). We then addressed if shortening of the G2 phase abrogated polarization of spherical *pal1*Δ cells. To this end, double mutants of the genotype *pal1*Δ *wee1*-50 were constructed. Wee1p regulates timing of entry into mitosis by inhibitory phosphorylation of Cdc2p and wee1 mutants exhibit a shortened G2 phase. Interestingly, we found that *pal1*Δ cells displayed synthetic lethality in combination with *wee1*-50 at 36°C (Figure 4.6D) and nearly 40% of binucleate cells were spherical in shape (Figure 4.6, E and F), whereas <6% of binucleate *wee1*-50 single mutants were spherical in morphology (Figure 4.6F). We therefore conclude that spherical *pal1*Δ cells polarize and achieve a partially cylindrical morphology before entry into mitosis in a Wee1p-dependent manner. It is interesting to note that previous studies have suggested the existence of a morphogenesis checkpoint which is involved in coordinating cell morphogenesis and mitotic entry (Hirata *et al*., 2002; Lew, 2003; Verde *et al*., 1998). Similar to *pal1*Δ cells, these mutants have morphological defects and are delayed in G2 phase in a Wee1p-dependent manner.

4.2.6 Kelch-repeat protein Tea1p is required for polarization of spherical *pal1*Δ cells

We have shown that spherically shaped *pal1*Δ cells polarize in G2 and become abnormally shaped with a clearly defined long and short axis before septation. We noticed that microtubules converged into the newly forming cylindrical projections that assemble on the spherical cell bodies (Figure 4.7A). The microtubule cytoskeleton is important for ensuring proper antipodal growth, although it is not required for establishment of cylindrical morphology (Beinhauer *et al*., 1997; Mata and Nurse, 1997; Sawin and Nurse, 1998; Browning *et al*., 2000). It was possible that microtubules were important for the establishment of cylindrical morphology in spherically born *pal1*Δ cells. Previous studies have established a strong (but not absolute) requirement for the microtubule cytoskeleton in localizing the polarity factor Tea1p (Mata and Nurse, 1997; Sawin and Nurse, 1998; Behrens and Nurse, 2002). Tea1p was localized in several spots over the cortex in spherical *pal1*Δ cells and was detected at the ends of abnormal as well as normal *pal1*Δ cells (Figure 4.7B, marked with arrows). It was therefore possible that Tea1p was also important in the polarization process in spherical *pal1*Δ cells. To address this question, *pal1*Δ *tea1*Δ double mutants were constructed. Although *tea1*Δ and *pal1*Δ cells were capable of growth and colony formation at 36°C, the double mutant was unable to form colonies at 36°C and grew poorly at 30°C (Figure 4.7C). Furthermore, the percentage of spherical uninucleate and binucleate cells increased dramatically in the double mutants. Although <1% of binucleate *pal1*Δ cells were spherical, ~25% of binucleate *pal1*Δ *tea1*Δ cells were spherical at 36°C (Figure 4.7D). Time-lapse studies

performed with the *pal1*Δ *tea1*Δ double mutants further confirmed the inability of a majority of spherical double mutants to polarize and these cells septated while still spherical (Figure 4.7E). These experiments led to two conclusions. First, Tea1p is important for polarization in spherical *pal1*Δ cells. Second, the loss of both Pal1p and Tea1p has a deleterious combinatorial effect, suggesting that the formation of cylindrical morphology is important for maximal cell viability.

4.2.7 Coordination between mitosis and cytokinesis is altered in spherical cells

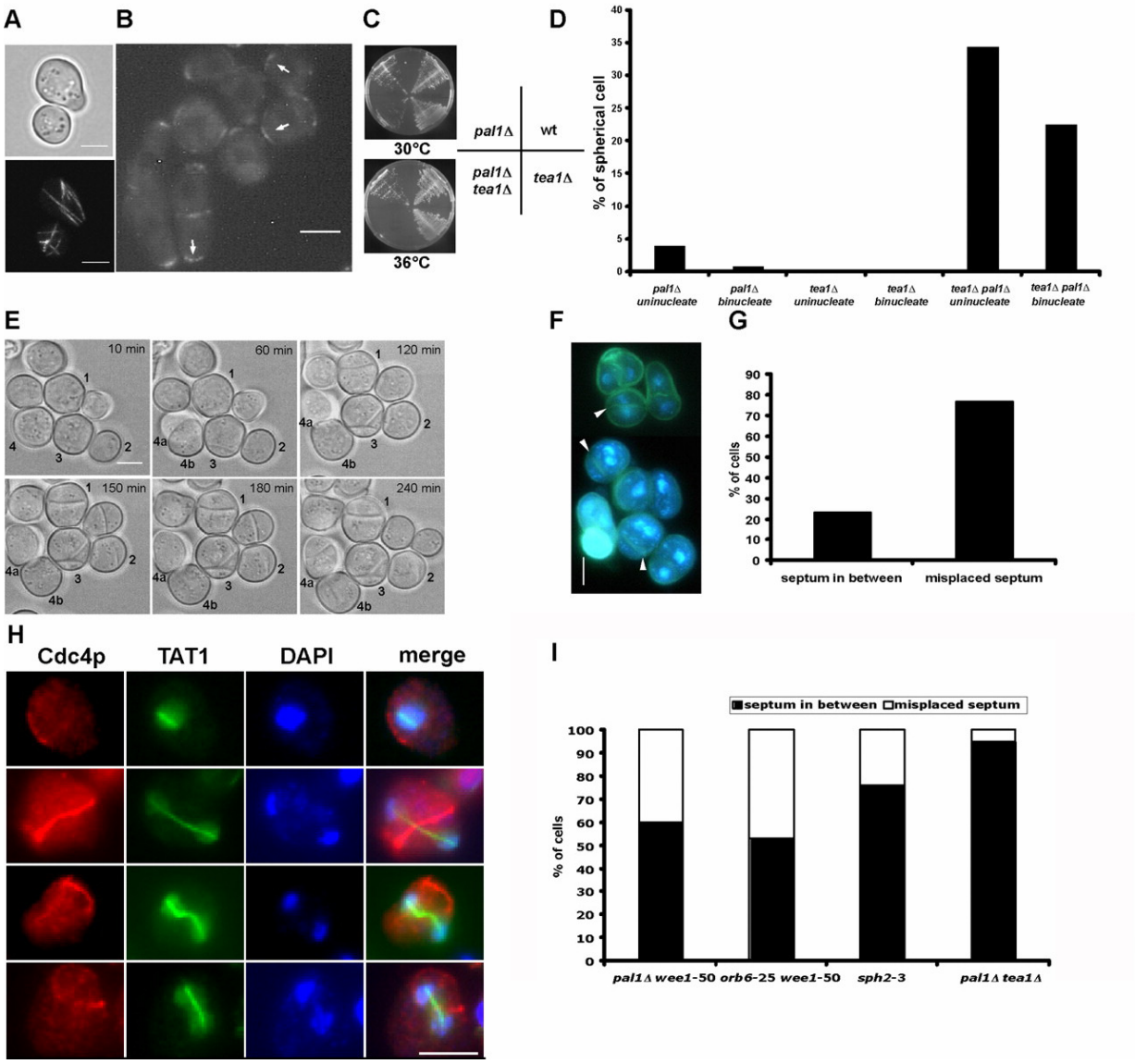
We have shown a deleterious effect upon combining *pal1*Δ with *tea1*Δ. To understand the basis of this lethality, we shifted *pal1*Δ *tea1*Δ cells to 36°C, fixed, and stained with DAPI and aniline blue to visualize nuclei and septa, respectively. As controls, *pal1*Δ and *tea1*Δ cells were used. We noticed that a high proportion of double mutants, but not either of the single mutants, contained both DNA masses on one side of the division septum, leading to the production of an anucleate and a binucleate compartment. Such abnormally septated cells did not separate to produce anucleate and binucleate daughters (Figure 4.7, F and G). *pal1*Δ, *tea1*Δ, and *pal1*Δ *tea1*Δ cells were also fixed and stained with antibodies against Cdc4p and α-tubulin to visualize the actomyosin ring and microtubules. Normally the actomyosin ring and the anaphase B spindle are aligned at right angles to each other. Interestingly, in spherical cells of the double mutant this alignment was severely altered and strikingly some spindles did not intersect the plane of the actomyosin ring at all, whereas in other cells the spindle was not placed perpendicular

Figure 4.7 Tea1p is essential for the viability and repolarization of *pal1*Δ cells.

(A) Microtubules converge into the cylindrical projection during the repolarization of *pal1*Δ cells. *pal1*Δ cells expressing *pREP1GFP-α-tubulin* (driven under *nmt1* promoter) were grown on minimal medium agar pad in the presence of 15μM thiamine at room temperature and imaged by confocal microscopy. (B) Tea1p-YFP is spread over the cortex in spherical *pal1*Δ cells and localizes at tips generated in spherical *pal1*Δ cells. *pal1*Δ cells expressing Tea1p-YFP were used to visualize the localization of Tea1p-YFP. (C) Wild type, *pal1*Δ, *tea1*Δ, and *pal1*Δ *tea1*Δ were streaked on YES agar plate and incubated for 2 d at 30 or 36°C. (D) Quantification of the percentages of spherical cells with one or two nuclei in *pal1*Δ, *tea1*Δ, and *pal1*Δ *tea1*Δ mutants. Cultures were grown in YES medium at 24°C to log phase and shifted to 36°C for 6 h. Cell samples were stained with DAPI and at least 300 cells were counted for each strain.(E) The growth of *pal1*Δ *tea1*Δ was imaged by time-lapse light microscopy on YES agar pads at room temperature. Time is indicated in minutes from the beginning of observation (t = 0). Cells are numbered for tracking, a and b are used to indicate the daughter cells produced after cell division.(F) Microscopic analysis of *pal1*Δ *tea1*Δ cells. Cells were stained with DAPI and aniline blue to visualize DNA and septum. Arrowheads indicate the septum. (G) Quantification of the percentages of spherical cells with properly positioned septum in between two nuclei or misplaced septum. (H) Localization of Cdc4p in *pal1*Δ *tea1*Δ double mutant. Exponentially growing cells were fixed with formaldehyde, processed for immunofluorescence, and stained with DAPI to visualize DNA, α-Cdc4p to visualize actomyosin ring, and α-TAT1 to visualize microtubules. Merge shows that the mitotic

spindle (green) is not aligned properly with respect to the Cdc4p ring (red). (I) Septum is misplaced in different spherical mutants. *pal1*Δ *wee1*-50 and *orb6*-25 *wee1*-50 mutants were grown in YES medium at 24°C and shifted to 36°C for 5 h. *sph2*-3 cells were cultured in YES medium at 30°C. *pal1*Δ *tea1*Δ mutants were grown in YES supplemented with 1.2 M sorbitol at 24°C and shifted to 36°C for 5 h. Scale bar, 5 μm. Except for sorbitol-grown *pal1*Δ *tea1*Δ, in which abnormal cells were counted, the phenotype in spherical cells is indicated.

Figure 4.7


A
B
C
D
pal1Δ
wt
pal1Δ tea1Δ
tea1Δ
30°C
36°C
% of spherical cell
pal1Δ uninucleate
pal1Δ binucleate
tea1Δ uninucleate
tea1Δ binucleate
tea1Δ pal1Δ uninucleate
tea1Δ pal1Δ binucleate
E
10 min
60 min
120 min
150 min
180 min
240 min
F
G
% of cells
septum in between
misplaced septum
H
Cdc4p
TAT1
DAPI
merge
I
septum in between
misplaced septum
% of cells
pal1Δ wee1-50
orb6-25 wee1-50
sph2-3
pal1Δ tea1Δ

to the actomyosin ring (Figure 4.7H). To establish that these defects in spatial regulation of cytokinesis were due to a spherical cell shape and not due to *tea1*Δ in the background, a variety of spherical mutants were scored for coordination of planes of mitosis and cytokinesis. The mutants included *pal1*Δ *wee1*-50, *sph2*-3 (Sipiczki *et al*., 2000), and *orb6*-25 *wee1*-50. The *orb6*-25 cells contain a mutation in a serine/threonine protein kinase which is related to mammalian myotonic dystrophy kinase (Verde *et al*., 1998) whereas *sph2*-3 cells have mutation in an unidentified gene. We found that the mitotic and cytokinetic planes were not coordinated in spherical cells that were genotypically *tea1*+ as well as *tea1*-, although the extent of defects varied in these strains (Figure 4.7I). Finally, defective spatial regulation of cytokinesis in the *pal1*Δ *tea1*Δ double mutant was almost completely suppressed by growth in medium containing sorbitol (Figure 4.7I). These experiments suggested that a spherical morphology does not allow for optimal spatial regulation of cytokinesis.

4.3 Discussion

The identification and characterization of a novel protein Pal1p allow us to study the role of a cylindrical morphology in spatial regulation of cytokinesis in fission yeast. We showed that Pal1p regulates morphogenesis by modulating cell wall integrity. Cells without *pal1* repolarize to establish a cylindrical-axis in a Tea1p-dependent manner. Failure of repolarization leads to mitosis and cytokinesis in spherical-shaped cells, and results in the accumulation of anueploid cells. Thus, a cylindrical morphology in fission yeast is important for spatial coordination of cytokinesis.

During the course of study to identify proteins involved in cell division, we identified a novel protein Pal1p based on the homology to its counterpart in budding yeast that localizes to division site. Pal1p-related proteins are found in fungi, but not in plants or metazoans. As with the budding yeast YDR348c gene product, Pal1p is also detected at the cell division site. Interestingly, Pal1p is in addition detected at the growing ends of the cell. The localization of Pal1p is independent of F-actin and microtubule functions. The localization of Pal1p to the growing ends was established by its restriction to the growing end in cells growing in a monopolar manner and to both ends in G2 arrested *cdc25*-22 cells that grow in a bipolar manner. The localization of Pal1p to the ectopic cell ends generated in a *tea1*Δ mutant and the localization of Pal1p to a larger region of the cell cortex in spherically shaped cells with no clear long and short axis further established that Pal1p was associated with the growth zones. Thus, Pal1p might either mark the growth sites or be a part of the growth machinery itself in addition to marking the growth zones. Pal1p also localizes to the zone of cell fusion in mating cells. Thus, Pal1p function is associated with polarized growth during the vegetative life cycle and during cell fusion before meiosis.

Cells deleted for *pal1* display morphological abnormalities, suggesting a role for Pal1p in the maintenance of cylindrical cell morphology. Cells deleted for *pal1* are largely pear-shaped, although spherical morphology is also commonly observed. What role might Pal1p play in cellular morphogenesis? The cell wall of budding and fission yeast cells plays a key role in the establishment of proper cell morphology. Furthermore, several spherical mutants define proteins important for cell wall metabolism in fission yeast

(Ribas *et al*., 1991; Arellano *et al*., 1996; Hochstenbach *et al*., 1998; Katayama *et al*., 1999; Martin *et al*., 2003). We have shown abnormalities in the cell wall of *pal1*Δ cells by electron microscopy. Excessive deposition of cell wall material was revealed when thin sections were examined by electron microscopy. Although excessive cell wall deposition is observed in *pal1*Δ cells, it is likely that the cell wall is structurally defective. This is due to our observation that the deleterious consequences resulting from loss of Pal1p are effectively rescued by exogenous addition of sorbitol.

Although Pal1p is important for establishment of a cylindrical morphology, the proportion of *pal1*Δ cells that are spherical is low (~5%). Interestingly, spherical *pal1*Δ cells with two nuclei are only rarely observed. These observations suggested that upon loss of Pal1p function, a second pathway might compensate for its loss and allow polarization. Consistent with this, the majority of *pal1*Δ cells are pear-like in appearance. The shape change from spherical to pear-like depends on Tea1p and occurs by formation of a "tip" at or near the new end generated by the previous septation event. In the absence of Tea1p, which is known to be important for new end growth, spherical *pal1*Δ cells are unable to carry out tip growth and over several generations the vast majority of cells accumulate and die with a spherical morphology. A similar mechanism also operates to alter the morphology of spherical cells of other orb mutants such as *orb3*-167, *ras1*Δ, and *sph2*-3, suggesting that the shape correction is not unique to *pal1*Δ. Several *orb* mutants also exhibit synthetic lethal interactions with *tea1*Δ mutants (Papadaki *et al*., 2002). Thus, we propose that Tea1p becomes essential in spherical cells and that "tip" formation is the equivalent of new end growth in such spherical cells. Our studies are consistent with the

proposal of multiple pathways of cell polarization (Feierbach *et al*., 2004) and with recent studies of Sawin and Snaith (2004), suggesting a role for Tea1p in resetting polarity.

Previous studies have shown that Tea1p and microtubules are important for proper antipodal growth of fission yeast cells (Snell and Nurse, 1994; Verde *et al*., 1995; Mata and Nurse, 1997; Sawin and Nurse, 1998), although they are not required for establishment of the cylindrical morphology per se (Sawin and Snaith, 2004). It is likely that Tea1p plays a major role in targeting the growth machinery to the tips generated in spherically born *pal1*Δ cells. An attractive possibility is that stochastic accumulation of Tea1p at the cell cortex to "critical" levels might allow F-actin and cell wall assembly, leading to tip growth. Although it seems likely that other molecules involved in microtubule-based polarization, such as Tip1p, Mal3p, Tea2p, Pom1p, Tea3p, and Tea4p (Bahler and Pringle., 1998; Brunner and Nurse, 2000; Arellano *et al*., 2002; Browning *et al*., 2003; Busch and Brunner, 2004; Martin *et al*., 2005) should become essential when cylindrical morphology is compromised, future studies should establish if this is indeed the case.

We have shown that *pal1*Δ *tea1*Δ double mutants are unable to form colonies under conditions in which both parents are capable of colony formation. Double mutants of the genotypes *scd1*Δ *tea1*Δ and *ras1*Δ *tea1*Δ have also been shown to display a synthetic lethal defect, although the basis of this lethality has not been fully characterized (Papadaki *et al*., 2002). We have shown that the spherical *pal1*Δ *tea1*Δ cells are unable to spatially coordinate mitosis and cytokinesis frequently, leading to the formation of a

septated-binucleate cell in which both nuclei are placed on one side of the septum. Such defects are observed in spherical cells that are genotypically *tea1+* (*pal1*Δ *wee1*-50, *sph2*-3, *orb6*-25 *wee1*-50) as well as *tea1-* (*pal1*Δ *tea1*Δ and *orb3*-167 *tea1*Δ). Furthermore, <5% of cytokinetic events are spatially impaired in *pal1*Δ *tea1*Δ cells, whose morphology is suppressed in medium containing the osmolyte sorbitol. We propose that it is the spherical shape, rather than the absence of Tea1p that leads to the observed defects in spatial regulation of cytokinesis, although a role for Tea1p in ring positioning in certain genetic backgrounds cannot be ruled out, given the low percentage of cells with spatial defects in cytokinesis in sorbitol suppressed *pal1*Δ *tea1*Δ mutants. It is likely that the lethality of spherical mutants defining essential genes (such as *mor2*, *orb6*, *mob2*, *bgs3*, etc.) might in part result from defective spatial coordination of cytokinesis, in addition to possible defects in cell wall assembly (Verde *et al*., 1998; Hirata *et al*., 2002; Hou *et al*., 2003; Martin *et al*., 2003). Interestingly, mutants defining genes important for division site placement, such as *mid1*Δ and *pom1*Δ (Chang *et al*., 1996; Sohrmann *et al*., 1996; Bahler and Pringle, 1998) are viable. It is possible that the cylindrical morphology of cells of the division site selection mutants (such as *mid1*Δ and *pom1*Δ) might largely ensure that mitotic events lead to the production of daughters with one nucleus each.

We have shown that spherical *pal1*Δ cells polarize in interphase, because polarizing cells contain an interphase array of microtubules and cyclin B (Cdc13p) is detected in the nucleus, as opposed to its presence on the spindle (metaphase) or lack of detectable localization (anaphase). We have also shown that shortening of G2 utilizing a *wee1* mutation, leads to an increase in the number of spherical mitotic cells and lethality.

Previous studies have shown that spherical *S. pombe* mutants, such as *mor2*, and *orb6* (Hirata *et al*., 2002; Verde *et al*., 1998) are delayed in G2 in a Wee1p-dependent manner. This has been proposed to signify a morphogenesis checkpoint, although the functional end point of this checkpoint mechanism has not been fully explored. An attractive possibility is that some aspect of morphogenesis inactivates Wee1p, leading to entry into mitosis as has been proposed from studies in budding yeast (Lew, 2003). The formation of a tip might represent such a morphogenetic event that relieves the G2 delay. Future studies should assess if *pal1*Δ cells are delayed in G2 until tip formation and if tip formation in turn represents the functional end point upon morphogenetic checkpoint activation.

Chapter 5: Conclusion and future directions

Faithful inheritance of genetic material requires proper spatio-temporal regulation of cytokinesis. Cells utilize multiple mechanisms to ensure correct positioning of their division planes and to coordinate mitosis and cytokinesis. Mis-regulation of cytokinesis leads to genome instability, which is a hallmark of cancer cells.

In recent years, fission yeast has become an attractive organism for the study of cytokinesis. Much progress has been made in understanding the molecular components and the assembly of an actomyosin-based contractile ring. How the assembly and constriction of the actomyosin ring are coordinated with respect to cell geometry and the timing of mitosis represents an important question. This thesis touches on these topics and provides insights into aspects of spatio-temporal control of cytokinesis.

Temporal regulation of cytokinesis

In fission yeast, SIN signaling is central to the regulation of cytokinesis. It is required for actomyosin ring maintenance and septum assembly, and promotes cell division after completion of mitosis. SIN signaling is turned on during cytokinesis, and is inactivated after the completion of cell division. Much is known about how SIN signaling is activated and regulated during cytokinesis. How this signaling pathway is maintained in an inactive state after completion of cell division is largely unknown.

It appears that inactivation of SIN signaling is an important step in cytokinesis, as continuous maintenance of SIN signaling results in uncontrolled cytokinesis. Mutants with hyperactive SIN signaling assemble multiple actomyosin rings with no intervening mitotic events (Fankhauser *et al*., 1993; Minet *et al*., 1979). These mutants are also incapable of polarized growth at the cell ends. Furthermore, inactivation of SIN signaling might be required to shut-off a cytokinesis checkpoint, which keeps cells in a cytokinesis-competent phase when there is a mild perturbation of actomyosin ring (Liu *et al*., 2000; Mishra *et al*., 2004). Once the cytokinesis checkpoint is activated, the cell cycle progression is halted in a cytokinesis-competent phase to repair the defects (Mishra *et al*., 2004). Inactivation of SIN signaling might allow resumption of the cell cycle after the checkpoint has fulfilled its task.

The findings in chapter III of this study provide a mechanism to restrain the activity of SIN signaling after its activation during mitosis. An APC/C subunit Nuc2p acts negatively on SIN signaling upon completion of cell division to prevent inappropriate cytokinesis. Since the SIN signaling is activated by the small GTPase Spg1p, it is not surprising that Nuc2p acts on the regulators of Spg1p to control the pathway. Genetic evidence suggests that Nuc2p might inactivate SIN signaling by regulating Byr4p-Cdc16p, a two-component GAP. Nuc2p may promote the recruitment and/or maintenance of Byr4p-Cdc16 at the SPB in order to inactivate Spg1p after completion of cytokinesis. Nuc2p could also influence the activity of Byr4p-Cdc16p to modulate SIN signaling. Further biochemical analysis of Nuc2p and Byr4p-Cdc16p should reveal the interplay between these molecules.

Nuc2p is an evolutionarily conserved protein with homologues found in most eukaryotes. Whether the SIN-regulatory function of Nuc2p in cytokinesis is conserved in other organisms remains to be determined. Several homologues of SIN components such as Plo1p, Dma1p, Spg1p, and Sid2p have been identified in multi-cellular organisms (Champion *et al*., 2004; Tuttle *et al*., 2007). However, it is currently unknown if homologues of SIN in animal cells regulate cytokinesis. Future studies should test these possibilities.

Spatial regulation of cytokinesis

Proper positioning of the division plane not only ensures faithful inheritance of genetic material, but also generates cellular diversity. In certain cell types such as *Drosophila* neuroblasts, the cleavage furrow is asymmetrically-positioned to generate two daughter cells with different size and cell fate (Wodarz, 2005). The position of the cleavage furrow in these cells is determined by the mitotic spindle, whose orientation is regulated by the asymmetrically-localized factors at the cell cortex (Wodarz, 2005). Interestingly, in the absence of these cortical factors, cell geometry provides a default positional determinant to specify the division plane. Several cell types, such as cultured rat epithelial cells, frequently position the division plane perpendicular to the longitudinal axis (Gray *et al*., 2004; O'Connell and Wang, 2000). Whether the cell geometry also plays an important role in spatial control of cytokinesis in fission yeast is addressed in this thesis.

The findings in chapter IV of this study provide evidence that a cylindrical morphology in fission yeast is necessary in positioning an actomyosin ring that is orthogonal to the nuclear division axis. Characterization of a novel protein Pal1p involved in morphogenesis shows that Pal1p is required for the establishment and/or maintenance of a cylindrical-morphology. Interestingly, *pal1*Δ mutants have spherical morphology in interphase and establish a partially-polarized shape in mitosis. A kelch-repeat containing protein Tea1p is required to establish a cylindrical axis in spherical *pal1*Δ cells. Failure to re-polarize prior entry into mitosis in *pal1*Δ *tea1*Δ mutants results in mis-coordination of the planes of mitosis and cytokinesis. Thus, the cylindrical morphology of fission yeast provides optimal spatial control of cytokinesis.

It is currently unknown how the division plane is mis-placed in spherically-shaped cells. It is possible that the deformation of cell morphology perturbs the organization of interphase microtubule arrays which are required in positioning of the division plane in fission yeast. Alternatively, perturbation of cell morphology might disrupt proper distribution of molecules required to anchor the actomyosin ring to the right place. Which mechanism is more applicable awaits future investigation.

Collectively, work described in this thesis provides an understanding of the regulation of cytokinesis in fission yeast. It will be interesting to see if the insights gained from this study are applicable in higher eukaryotes that also utilize the actomyosin ring for cytokinesis.

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