ELUCIDATION OF THE MULTI-FACETED ROLES OF THE SIN (SEPTATION INITIATION NETWORK); UNDERSTANDING HOW THE SIN PROMOTES CYTOKINESIS AND INHIBITS INTERPHASE GROWTH IN THE FISSION YEAST *SCHIZOSACCHAROMYCES POMBE*.

A Dissertation Presented by

SAMRIDDHA RAY

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CROSS-TALK BETWEEN THE NDR PATHWAYS SIN AND MOR REGULATES THE TRANSITION BETWEEN MITOSIS AND INTERPHASE GROWTH IN FISSION YEAST *S. POMBE*

A Dissertation Presented By

Samriddha Ray

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

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Dannel McCollum, Ph.D., Thesis Advisor

Stephen Doxsey, Ph.D., Member of Committee

Kirsten Hagstrom, Ph.D., Member of Committee

William Theurkauf, Ph.D., Member of Committee

Satoshi Yoshida, Ph.D., Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

---

Nicholas Rhind, Ph.D., Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the School

---

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

Interdisciplinary Graduate Program

August 17, 2010

I dedicate this thesis to Maa and Baba

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ABSTRACT

Cytokinesis is the cytoplasmic division of one cell into two independent daughter cells. Precise regulation of cytokinesis during cell cycle is essential for healthy and rapid multiplication of any organism. *Schizosaccharomyces pombe* has emerged as an excellent model system to study eukaryotic cell division regulation. This rod shaped organism grows by bipolar elongation in interphase when its actin cytoskeleton is concentrated at the cell ends (poles). However, growth stops in mitosis and the actin cytoskeleton is rearranged to facilitate assembly of the contractile actomyosin ring at the cell middle. Although several studies have focused on the separate processes of growth and division, it was unclear how cells regulate the cytoskeletal remodeling during the transition between the different stages of the cell cycle. The Septation Initiation Network (SIN) is a signaling cascade essential for fission yeast cytokinesis (Balasubramanian et al., 1998; Mishra et al., 2004) and the MOR (morphogenesis) signaling pathway is essential for interphase bipolar growth (Kanai et al,2005). Interestingly, inactivation of the SIN not only failed to maintain the cytokinetic apparatus at the cell middle but also caused the redistribution of the cytoskeletal elements like actin to the cell ends that led to bipolar cell elongation similar to cells in interphase (Mishra et al., 2004). These results suggested that SIN signaling inhibits interphase bipolar growth, but it was not clear if the SIN had a direct role in inhibition of interphase growth during mitosis and this question was the major focus of this thesis. The results presented in

Chapter II show a novel cross-pathway interaction between the SIN and the MOR in the fission yeast. Our results in Chapter III suggest that some of the MOR pathway components might be important for coordination between nuclear and cytoplasmic divisions in mitosis, revealing novel roles of the pathway. In a separate study (Chapter IV) we sought to identify additional regulators of the SIN and cytokinesis through a suppressor screen and found that the nucleolar rDNA transcription machinery inhibits cytokinesis in fission yeast.

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CHAPTER I

General Introduction

Proliferating cells cycle through distinct phases of growth and cell division. In any organism, each of these phases is mediated by distinct cytoskeletal arrangements involving the essential cytoskeletal elements actin and microtubules along with their associated factors. For instance in migratory animal cells, extensive network of cytoplasmic actin and microtubules maintain cell shape and growth in interphase. Further, actin based structures like lamellipodia and filopodia are essential to form protrusions on the cell surface that aid in cell migration. In mitosis, actin undergoes drastic remodeling to form the cytokinetic ring apparatus at the cleavage furrow, which is central to the process of cell division (Eggert et al., 2006). Successful cell cycle progression also requires precise and timely remodeling of the cytoskeleton to form cell cycle stage specific structures. Several studies have focused on the mechanisms and regulation of formation of the distinct cytoskeletal structures during the different stages of growth and cell division (Etienne-Manneville, 2010; Hehnly and Stamnes, 2007; Heng and Koh, 2010). However, the mechanisms that regulate cytoskeletal remodeling during the transition between cell growth and cytokinesis and vice versa remain less clear. My thesis explores this

question in the rod shaped single celled eukaryote *Schizosacharomyces pombe* (*S.pombe*).

Figure1-1. Actin based cytoskeletal structures are essential to maintain cell shape and motility in interphase and to form the cytokinetic ring in mitosis. Actin (red) is at the growing ends to drive filopodia formation for cell migration in interphase and is at the cleavage furrow (red ring) to drive cell division.

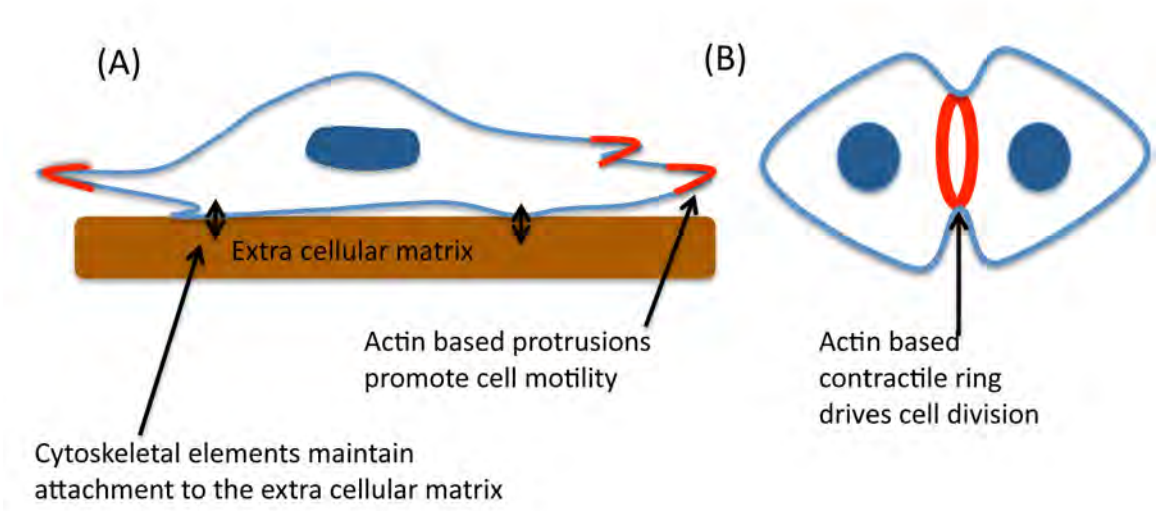


Figure 1-1: Actin remodeling during cell cycle

Multiple factors make the *S.pombe* an excellent model system to study cell cycle regulation. Its entire genome has been sequenced and there is a deletion library available for all non-essential genes, making functional analysis easier and faster. Further, it is amenable to multiple genetic and genomic manipulations, including construction of conditional mutants and efficient homologous recombination mediated tagging and knockout strategies. In addition, it is easy to apply microscopy based strategies that allow direct visualization of cytoskeletal dynamics in living cells.

S. pombe undergoes distinct cytoskeletal changes similar to cycling animal cells. During interphase, this cylindrical organism grows by directing the actin and microtubule cytoskeletons towards the cell ends (tips of the cylinder). However, growth stops when cells attain an optimum size and enter mitosis. At this time the cytoskeletal machinery is rearranged to concentrate growth at the cell middle in preparation for cytokinesis. These cell cycle dependent cytoskeletal transitions (shown in Figure 1-2; red dots/lines denote actin) during its life cycle make it a useful model to study regulation of polarized growth. Fission yeast cytokinesis involves contraction of a conserved actomyosin ring apparatus similar to that in animal cells (Sirotkin et al., 2010b). Homologs of many of the growth and cytokinesis related genes have been identified in higher eukaryotes, including humans, where they are involved in similar processes. Thus, information about basic cell cycle regulation from a simple organism like fission yeast is relevant to understand similar processes in higher eukaryotes.

Figure1-2. Distinct cytoskeletal structures are formed by shared cytoskeletal elements like actin to promote interphase bipolar growth and mitotic cell division. Actin structures form patches (red dots) at the cell tips in interphase but are reorganized to form the actomyosin ring (red ring) at the cell middle during mitosis. The ring undergoes constriction and leads to septum formation (green line) during cytokinesis followed by cell separation. The blue circles denote nuclei.

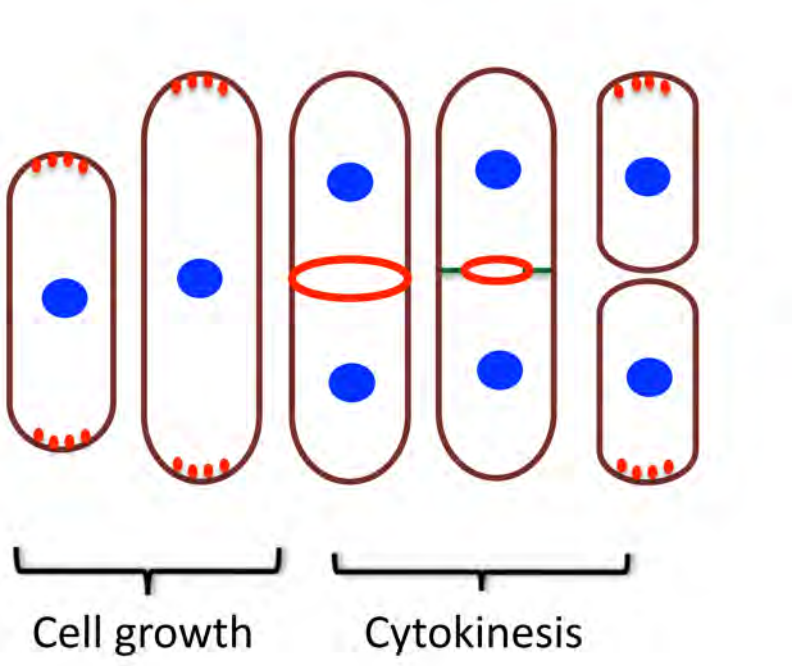


Figure 1-2: Distinct actin cytoskeletal arrangements during the fission yeast cell cycle

In the following sections in this chapter, I shall summarize our current understanding of polarized interphase cell growth and mitotic cell division during fission yeast cell cycle in terms of the roles played by essential cytoskeletal structures and conserved signaling pathways that drive these processes.

The cell cycle is a highly orchestrated process. To ensure efficient transitions between different stages cells have surveillance mechanisms called “checkpoints”. As the name suggests these are control mechanisms that ensure that a particular cell cycle process is successfully executed before the onset of the next phase (Hartwell and Weinert, 1989). For instance entry into Mitosis (M phase) is blocked if DNA is damaged or not replicated. Similarly, in the event of a delay in cytokinesis, the cytokinesis checkpoint delays initiation of further nuclear division and bipolar growth until the completion of cell division in the previous cycle (Mishra et al., 2004). The SIN signaling pathway (described later) is essential for the cytokinesis checkpoint. However, the mechanism by which the SIN maintains inhibition of bipolar growth to promote cytokinesis was unknown and was the major part of my thesis (Chapter II). Chapter IV describes the identification of some nucleolar proteins as suppressors of the cytokinesis defect in SIN compromised cells revealing a novel role of the nucleolus in cytokinesis regulation.

Regulation of polarized cell growth in fission yeast

Role of cytoskeletal elements

Both actin and microtubules are essential for polarized cell growth (Perez and Rincon, 2010; Piel and Tran, 2009). It has been shown that actin structures are perpetually associated with regions of cell growth throughout the cell cycle (Marks et al., 1986). In addition, actin depolymerizing drugs completely inhibit cell growth, and protoplast regeneration experiments show no cell wall recovery in absence of actin, suggesting that actin is essential for cell growth and cell wall deposition (Kobori et al., 1989).

The primary actin based cytoskeletal structures during interphase are actin patches and cables. Actin patches are dynamic sites of actin polymerization and act as sites of endocytosis (Gachet and Hyams, 2005; Kaksonen et al., 2003; Sirotkin et al., 2010). Actin cables are composed of bundles of actin filaments (F-actin) and serve as tracks for movement of cargoes. These cargoes include actin patches and the type V myosin, myosin Myo4/Myo52, which move along the cables and promote cell growth at specific sites in a cell cycle dependent fashion. Myo52 is essential for transport of secretory vesicles, which promote new cell wall synthesis, and hence is required for polarized growth both at cell ends and the cell middle (Motegi et al., 2001; Win et al., 2001). Consistent with their role in cell growth, the directionality of F-actin in these cables was shown to change in a cell cycle dependent fashion (Kamasaki et al., 2005).

During cell growth the majority of F-actin filaments are oriented such that the barbed end (growing end) faces the cell tips. In contrast, during cell division, barbed ends face the cell middle. The proper orientation of F-actin cables ensures spatial regulation of polarized growth during cell cycle. Cells that fail to maintain F-actin cables are either spherical or pear-shaped indicating defect in polarized growth (Figure 1-3B).

Microtubules direct the correct localization of cell growth factors. Microtubules are polymers of alpha and beta-tubulin dimers that are nucleated by γ-tubulin from perinuclear structures termed the interphase microtubule organizing centers (iMTOC). The newly formed microtubules are stabilized by bundling at their minus ends, which are the non-growing ends close to the nucleation site (Martin, 2009; Piel and Tran, 2009). Following nucleation, microtubules are oriented along the cell length and extend their dynamic plus ends towards the opposite ends of cells. These plus ends undergo rapid growth and shrinkage and serve two essential functions in cell morphogenesis and division. Firstly, interaction of these ends with the cortex generates a pushing force on the nucleus that moves the nucleus in a direction opposite to that of microtubule growth. Hence, growth of plus ends towards the opposite cell ends results in the medial placement of the nucleus, which in turn determines the site of cell division in mitosis (Piel and Tran, 2009) (see below). Secondly, plus ends carry polarity factors to cell tips that mark the sites of polarized growth on the cortex. Hence, microtubules are required to define the antipodal sites of tip

growth and hence maintain the cylindrical cell shape. Defects in microtubule organization result in mislocalization of the growth apparatus and cells do not grow straight and occasionally have a branched or “T” phenotype (Figure 1-3C).

Figure 1-3. Actin and microtubules are involved in regulation of polarized growth and morphology. (A) Normal cell shape and growth is maintained by the correct assembly and orientation of actin and microtubules. (B) Inactivation of actin cytoskeletal structures causes round (“orb”) phenotype. (C) Inactivation of microtubule formation disrupts cell morphology, producing branched “T” shaped cells, but does not inhibit polarized growth per se.

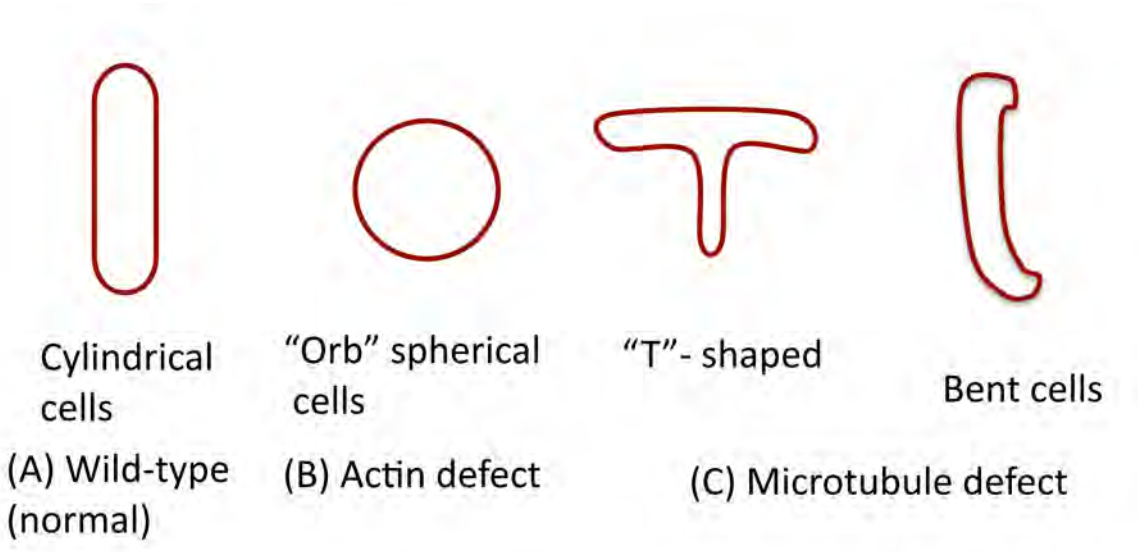


Figure 1-3: Actin and microtubules regulate polarized growth and morphology in fission yeast

The MOR Pathway

The regular cylindrical shape of fission yeasts makes them amenable for visual screens for cells with altered morphologies (Koyano et al., 2010; Snell and Nurse, 1994; Verde et al., 1995). Several of the genes identified from such screens have been cloned and characterized. Fission yeast morphology mutants fall into two broad classes. Class I mutants have a spherical (orb) shape due to their isotropic growth pattern instead of the normal polarized pattern. Hence these represent genes that are required for polarized growth. The second class of mutants, class II, includes cells that have bent or branched shapes suggesting that they failed to maintain the antipodal mode of polarized growth. The genes defined by the class II mutants have defects in microtubule architecture, growth and orientation (Verde et al., 1995) and these findings have shown that microtubules are not required for polarized growth per se.

Characterization of some of the "*orb*" genes led to the identification of a signaling pathway known as the morphogenesis network (MOR) (Kanai et al., 2005). Signaling through the MOR requires activity of the NDR kinase Orb6 and its binding partner Mob2 (Hou et al., 2003; Verde et al., 1998). Inactivation of Orb6 results in round cells and mitotic arrest implicating its role in regulating polarized growth and mitotic entry (Kanai et al., 2005). Orb6 activation is dependent on the Ste-20 like kinase Nak1 and the scaffold protein Mor2, which serves as a linker between Orb6 and Nak1 (Hirata et al., 2002; Kanai et al.,

2005). Localization and activity of both Nak1 and Orb6 are in turn dependent on Pmo25 (*S.pombe* homolog of hMO25, which is an activator of the LKB1 tumor suppressor protein kinase) making it the most upstream regulator of the pathway. Figure 1-4A is a schematic of the pathway.

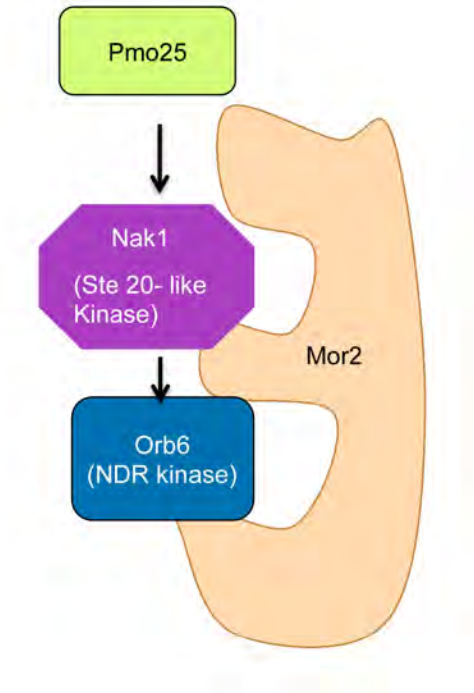
During interphase all the MOR components are localized to the cell ends consistent with their role in polarized tip elongation. Intriguingly, during mitosis, the MOR proteins disappear from cell ends and both Nak1 and Pmo25 are localized to the spindle pole body (SPB) (Kanai et al., 2005; Leonhard and Nurse, 2005). Also during mitosis, Orb6 kinase activity drops but then returns to normal as cells initiate polarized growth following cytokinesis (Kanai et al., 2005). Although currently we do not understand the implications of the mitosis specific localization of upstream MOR components it is tempting to speculate that this is a mechanism by which cells inactivate the MOR to maintain growth arrest in mitosis. This aspect of MOR signaling is discussed further in Chapter 3. We found that consistent with previous reports, Nak1 was recruited to the SPB early in mitosis, when cells have a high Cdk1 activity. However, Pmo25 was localized at the SPB in anaphase when cells have low mitotic cyclin/Cdk1 activity. The mitosis specific localization pattern of the upstream MOR components is illustrated in Figure 1-4B.

Besides interphase growth, MOR signaling has also been implicated in cell separation, which might explain the medial localization of MOR components

during cytokinesis (Hirata et al., 2002; Kanai et al., 2005). So far no targets of the MOR have been reported to explain its role in cell separation.

Figure 1-4. The MOR pathway is essential for polarized growth in interphase. (A) Schematic of the MOR pathway. (B) MOR pathway components are localized to the cell ends and cell division site during interphase and cytokinesis respectively. However two of the upstream MOR components, Nak1 and Pmo25, are alternately localized at the SPB (spindle pole bodies) during mitosis.

(A)



(B)

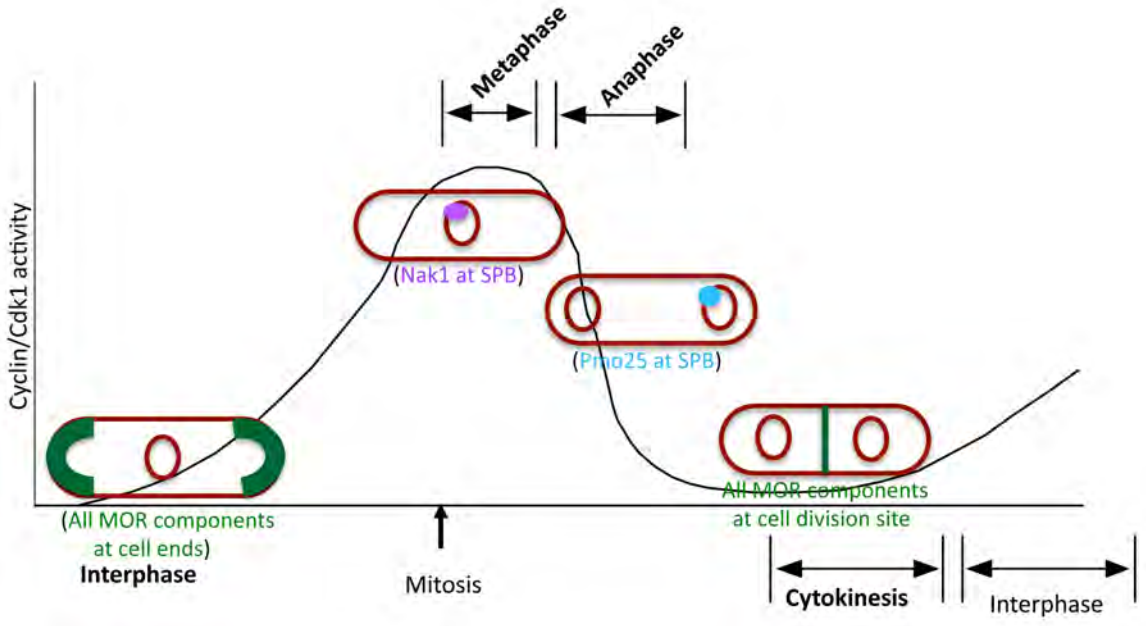


Figure 1-4: The MOR signaling pathway

It remains unclear what triggers the activation of MOR signaling during the cell cycle. A previous study has suggested that the SIN (Septation Initiation Network) pathway, which promotes cytokinesis (as described later), is essential for the full activation of MOR following cell division (Kanai et al., 2005). We however found that the SIN inhibited Orb6 kinase activity during cytokinesis suggesting that the SIN has a biphasic role in regulating MOR signaling (Chapter 2). Our knowledge about the MOR pathway is still very preliminary and further work will be required to unravel mechanisms of its regulation during the cell cycle.

To understand how MOR signaling regulates polarity it is essential to identify targets of the pathway. To that end, it has been shown that the most downstream MOR component Orb6 regulates localization of active Cdc42 (Das et al., 2009). A member of the Rho/Rac subfamily of the Ras superfamily of GTPases, Cdc42 is a universal regulator for polarized growth(Perez and Rincon, 2010). These proteins act as “molecular switches” since they are able to switch between a GTP-bound active state and a GDP bound inactive state (Figure 1-5A). The fission yeast Cdc42 is activated by the GEFs Gef1 and Scd1 and negatively regulated by the GAP Rga4 (Das et al., 2007; Hirota et al., 2003). Active Cdc42 interacts with multiple effector molecules to drive polarized growth. In fission yeast, only a small number of Cdc42 effectors have been well characterized. As depicted in Figure 1-5B, one of its essential targets is the formin For3 that promotes actin nucleation and cable formation to drive polarized growth (Feierbach and Chang, 2001; Martin et al., 2007). Another target of active

Cdc42 is the PAK kinase Pak1 that drives polarized bipolar growth via myosin phosphorylation and stabilization of the microtubule cytoskeleton (Attanapola et al., 2009; Marcus et al., 1995).

The regulators of *S.pombe* Cdc42 are localized to characteristic cortical positions that define regions of growth. Gef1 and Scd1, which promote Cdc42 activation, are localized at cell tips in interphase and at the cell middle during cell division (Rincon et al., 2007). On the contrary, Rga4 is localized to the cell sides that normally do not undergo growth (Das et al., 2007; Tatebe et al., 2008). Hence one possible mechanism by which cells promote polarized growth is by regulating the activity and localization of Cdc42 regulators. Consistent with this idea, Orb6 was shown to regulate recruitment of active Cdc42 by maintaining polarized distribution of Gef1 to the cell tips (Das et al., 2009), Figure 1-5C. The effect of Orb6 was specific to Gef1 as Orb6 mutants did not show any aberration in the localization of Scd1 or the GAP Rga4. This report identified the first downstream targets of the MOR pathway.

Figure 1-5. Cdc42 is essential for polarized growth. (A) The Cdc42 GTPase cycle. (B) Regulation of Cdc42 activity in fission yeast involves the GEFs Scd1 and Gef1, and the GAP Rga4. Active Cdc42 triggers For3 and Pak1 activity. (C) The MOR regulates localization of active Cdc42 via Gef1.

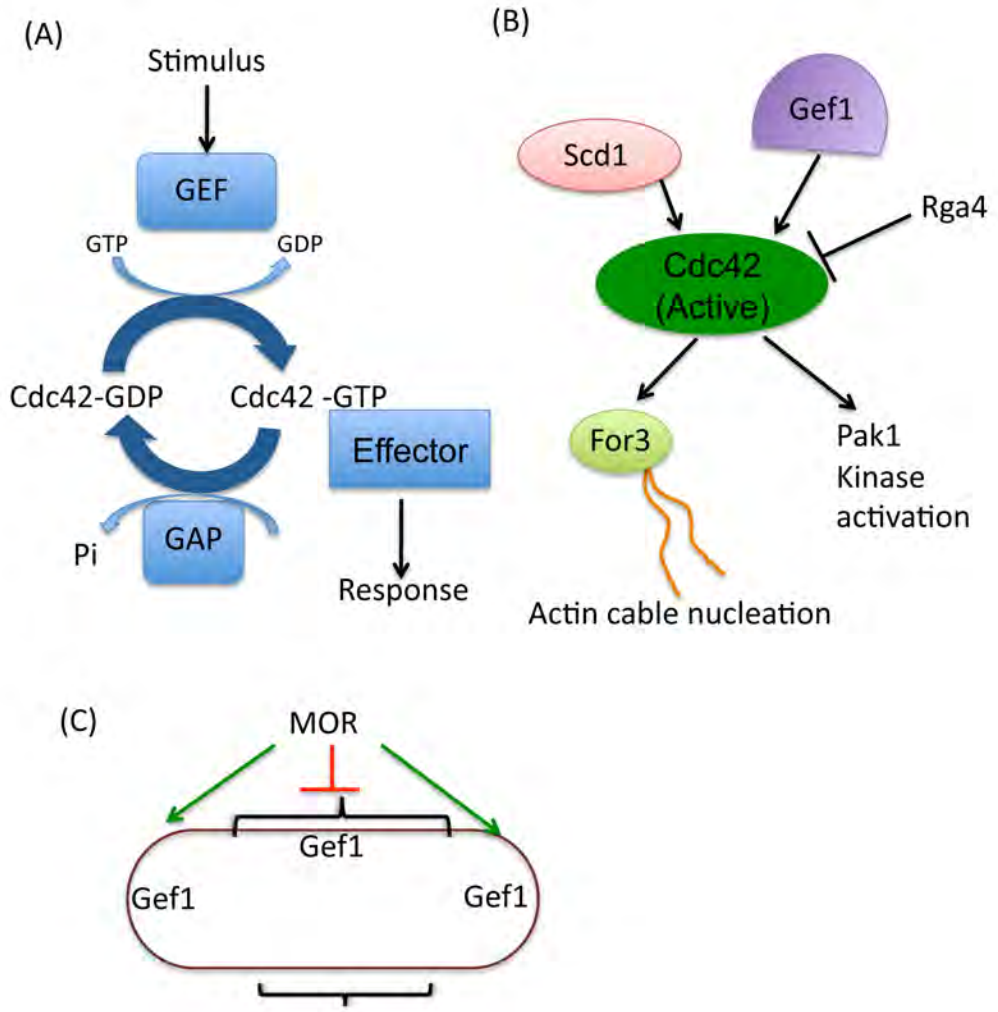


Figure 1-5: Essential role of Cdc42 in polarized growth

Regulation of cell division

Role of the cytoskeleton

Cytokinesis marks the end of mitosis and involves cytoplasmic cleavage of one cell into two independent daughter cells (Guertin et al., 2002a; Pollard, 2010). It requires formation of a centrally localized contractile ring apparatus, composed mainly of actin and myosin 2 (actomyosin ring) along with other ring components (Stark et al., 2010; Wu et al., 2006). Preparation for cytokinesis begins in early mitosis with the rearrangement of the actin cytoskeleton to reorient actin patches and cables towards the cell middle for stable ring assembly (Arai and Mabuchi, 2002).

An essential requirement of successful cell division is the precise medial placement of the ring. Screens for mutants with defective ring placement have identified Mid1 (anillin homlog) and Plo1 (polo kinase) as essential regulators of this step (Bahler et al., 1998a; Chang and Nurse, 1996; Chang et al., 1996; Sohrmann et al., 1996). In interphase Mid1 is primarily in the nucleus but upon mitotic onset Plo1 mediated phosphorylation drives it out of the nucleus into the cytoplasm. In the cytoplasm Mid1 associates with the cortex close to the nucleus and hence it is assumed that the position of the nucleus via Mid1 regulates the medial ring localization. Further, regulators of cortical endoplasmic reticulum (ER) network also contribute to the positioning of the ring by restricting diffusion

of Mid1 along the lateral cortex (Zhang et al., 2010). Additionally, negative signals by the tip regulator Pom1 inhibits Mid1 spreading to cell tips to maintain the medial localization of the cytokinetic ring (Huang et al., 2007) .

Formation of a stable ring is the first step towards cytokinesis. Several groups have focused on understanding the underlying mechanism(Coffman et al., 2009; Pollard and Wu, 2010; Stark et al., 2010; Vavylonis et al., 2008; Wu et al., 2006). Briefly, assembly of the ring begins in early mitosis with formation of a broad band of cortical spots termed as “cytokinesis nodes” around the equatorial cortex near the nucleus. These nodes are comprised of Mid1, Myosin II (Myosin motor protein), Rng2 (member of the IQGAP family), Cdc15 (F-BAR domain containing protein), Cdc12 (formin family protein) and several other proteins. Actin filaments are indispensable for ring formation. The formin Cdc12 has actin nucleating properties and is essential for actin filament formation (Kovar et al., 2003; Yonetani et al., 2008). The population of Cdc12 in the cytokinesis nodes has been proposed to initiate actin filament nucleation from cytoplasmic actin monomers in association with profilin (Cdc3). Thus Cdc12 anchors actin filaments to the cytokinesis nodes. Following formation of actin filaments from adjacent nodes, myosin II in the individual nodes captures these filaments from neighboring nodes and interaction between bundles of actin filaments and cytokinesis nodes leads to the condensation of the nodes into a contractile ring. Once assembled the ring then undergoes a “maturation phase” when additional proteins like Myo3 and Imp2 (F-BAR protein) are added to the ring from the

cytoplasm. Interestingly, Mid1 is lost from the mature ring. Thus Mid1 bearing nodes act as a scaffold for the correct placement and formation of the ring but not in subsequent steps towards cytokinesis. However, it has been observed that Mid1 deletion does not obliterate ring formation but disrupts its medial placement (Bahler et al., 1998a).

Interestingly Hachet et al (Hachet and Simanis, 2008) and Huang et al (Huang et al., 2008) have shown that a Mid1 independent pathway can also lead to successful ring formation in mitosis. In the absence of Mid1, ring formation initiated with strands of ring elements comprising actin cables, Cdc12, Cdc15 and Myo2. Eventually these strands coalesced into stable rings that underwent constriction and septum formation. Thus it is believed that there are two parallel, Mid1 dependent and independent, mechanisms for formation of the ring apparatus. Activation of the SIN signaling pathway in early anaphase (described below) is essential for functional ring assembly and cytokinesis since in SIN mutants the ring disassembles and cells continue with nuclear division in absence of cell division. Intriguingly, in absence of Mid1, activation of SIN signaling is sufficient for ring assembly and cytokinesis, suggesting a role of the SIN in early mitosis (Hachet and Simanis, 2008; Roberts-Galbraith and Gould, 2008). Future work will clarify how the SIN promotes ring assembly in early mitosis.

Following completion of ring formation in late anaphase, cells initiate ring constriction with concomitant formation of primary septum along with flanking

secondary septa. The primary septum is composed mainly of linear 1,3-beta-glucan and requires Cps1/Bgs1, the 1,3-beta-glucan synthase for its assembly (Cortes et al., 2002; Cortes et al., 2007; Humbel et al., 2001) .The secondary septa is primarily composed of branched 1,3-beta-glucan, alpha-galactomannan and 1,3-alpha-glucan that require the glucan synthases Bgs4 and Ags1/Mok1 (Cortes et al., 2005; Humbel et al., 2001; Vos et al., 2007). Following septa assembly, cells undergo dissolution of the primary septum by Agn1 (Dekker et al., 2004; Garcia et al., 2005) and Eng1 (Martin-Cuadrado et al., 2003) to trigger cell separation. The secondary septum becomes the cell wall of the progeny following cell separation (Sipiczki, 2007).

The SIN pathway

Identification of cell division regulators was largely dependent on visual screens for mutants with cell division defects (cdc). As reported by several studies pioneered by Paul Nurse (Nurse et al., 1976) and subsequently by F.Chang and Nurse (Chang et al., 1996; Sohrmann et al., 1996) and Balasubramanian et al (Balasubramanian et al., 1998), the mutants in cytokinesis can be classified into three separate classes: a) misplaced cytokinetic ring, b) ring formation defect, and c) defective ring constriction and/or septum deposition. The class a) and b) mutants led to the identification of proteins involved in proper placement and formation of the actomyosin ring apparatus. Biochemical characterization and genetic interaction studies of the genes corresponding to class c) mutants identified a signal transduction pathway that was termed the Septation Initiation Network (SIN) since mutants showed defect in septum initiation.

At the molecular level, the SIN is a SPB (spindle pole body; functionally equivalent to centrosomes of animal cells) localized signaling cascade that is triggered by a Ras family GTPase Spg1 (Schmidt et al., 1997). Like any other GTPase, Spg1 switches between an active GTP state and an inactive GDP state. The GAP (GTPase-activating protein) complex comprised of Cdc16 (catalytic subunit) and Byr4 (scaffolding subunit) regulates the nucleotide state of Spg1 (Cerutti and Simanis, 1999; Krapp et al., 2008) in a cell cycle dependent manner. In interphase Spg1 remains inactive but is activated in early mitosis. Active Spg1 then recruits the Cdc7 kinase to the SPB (Fankhauser and Simanis, 1994).

In anaphase Spg1 is asymmetrically inactivated by association with the GAP complex at the old SPB concomitant with cyclin destruction and Cdk1 inactivation (Cerutti and Simanis, 1999; Grallert et al., 2004). Since Cdc7 is recruited by active Spg1, it is asymmetrically localized at only the new SPB in anaphase (Sohrmann et al., 1998). Concomitant with cyclin destruction, the Ste-20 like kinase Sid1 in association with its binding partner Cdc14 is also recruited to the new SPB (Guertin et al., 2000). The Sid1 binding partner Cdc14 is not orthologous to the *Saccharomyces cerevisiae* Cdc14 phosphatase. The Sid1/Cdc14 complex at the SPB promotes activation of the most downstream SIN component, the NDR kinase Sid2 (Sparks et al., 1999). Finally, Sid2 and its binding partner Mob1 associate with the ring to trigger ring constriction and septum deposition in late anaphase/telophase (Hou et al., 2004; Sparks et al., 1999).

Recruitment of Sid1 and subsequent signaling through the SIN is inhibited by high Cdk1 activity suggesting that activation of the pathway is cell cycle regulated and SIN activity is inversely correlated with cyclin /CDK activity. This regulation is important to ensure cytokinesis is coordinated with nuclear division. SPB localization of the signaling components of the SIN pathway is dependent on SPB structural proteins Sid4 (Tomlin et al., 2002), Cdc11 (Krapp et al., 2001; Morrell et al., 2004; Tomlin et al., 2002) and Ppc89 (Rosenberg et al., 2006) since inactivation of one or more of these SPB proteins cause delocalization of

SIN components from the SPB and loss of SIN signaling. Figure 1-6 shows the localization and activity of the SIN during the fission yeast cell cycle.

Figure 1-6. Cell cycle dependent localization and activity of the SIN (Septation Initiation Network) components. The SIN is activated after destruction of mitotic cyclin and inactivation of CDK1. All the SIN components are localized at the daughter SPB during cytokinesis except the most downstream kinase Sid2 and its binding partner Mob1 that are also at the ring during its constriction.

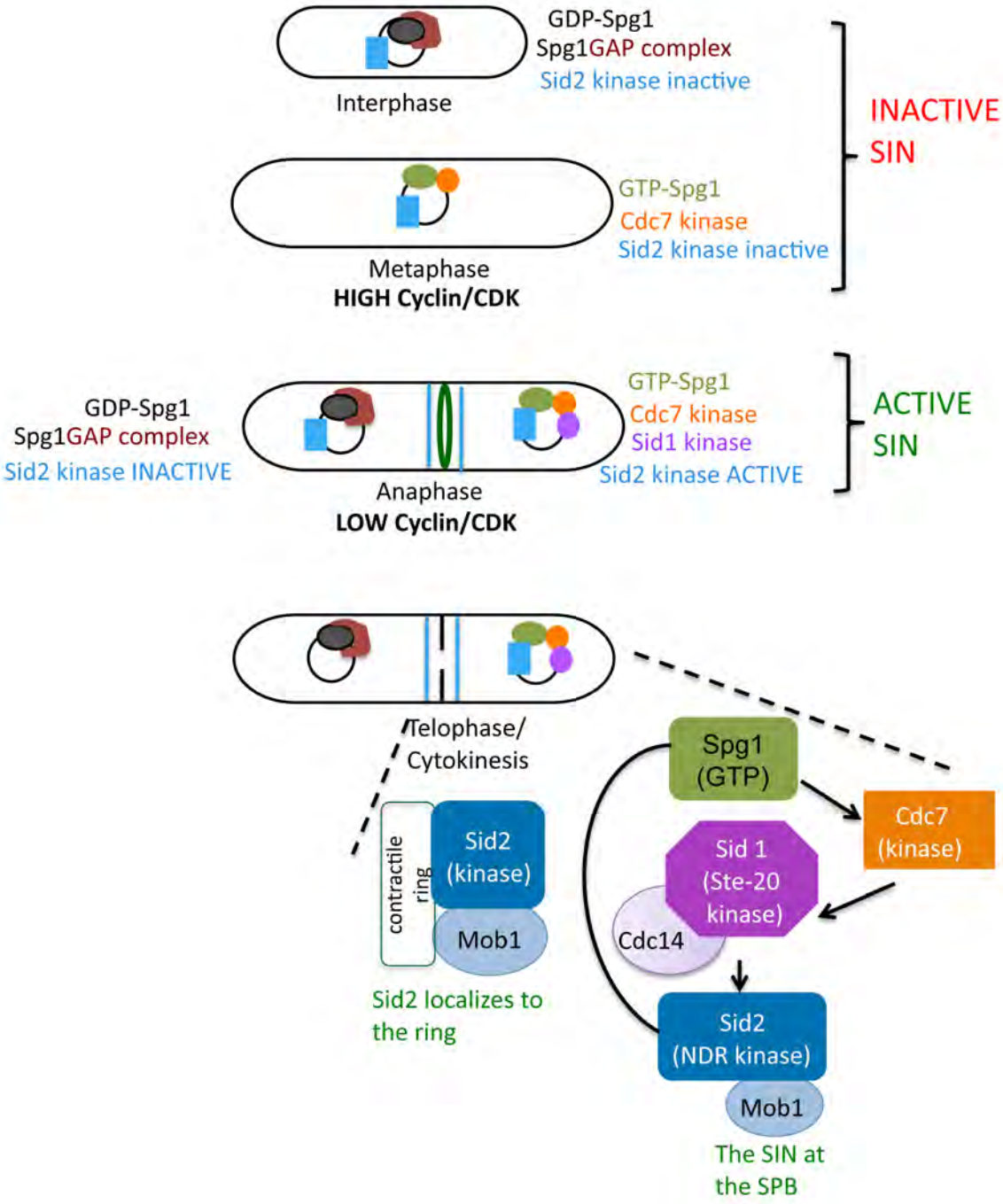


Figure 1-6: The Septation Initiation Network (SIN) and cell cycle.

SIN activation in anaphase is essential for completion of the ring assembly and to promote ring constriction and cytokinesis at the end of mitosis (Roberts-Galbraith and Gould, 2008). Intriguingly, the SIN is inactivated concomitantly with the completion of cytokinesis. Regulators of SIN signaling include Plo1 (Mulvihill and Hyams, 2002), Etd1 (Garcia-Cortes and McCollum, 2009), Dma1 (Guertin et al., 2002b) and Dnt1 (Jin et al., 2007). While Plo1 and Etd l are positive regulators of the SIN, Dma1 inhibits SIN activation and delays cytokinesis in the event of a spindle assembly defect possibly by inhibiting Plo1 (Guertin et al., 2002b). The identification of the nucleolar protein Dnt1 as a SIN inhibitor revealed a novel function of nucleolar components in the regulation of cytokinesis (Jin et al., 2007) (Chapter III). Etd1 promotes SIN activation in anaphase and subsequent degradation of Etd1 was found to be essential for inactivation of the pathway following cytokinesis (Garcia-Cortes and McCollum, 2009; Lattmann et al., 2009). Further work will help to understand the molecular details and significance of the role of Etd1 in the process. Inactivation of the SIN causes cytokinesis failure but cells continue with bipolar growth and nuclear division in the absence of cytokinesis (Krapp and Simanis, 2008). In contrast, ectopic activation of the pathway triggers septum formation in interphase arrested cells (Fankhauser et al., 1993; Minet et al., 1979). Thus although ring formation has been thought to be dependent on mitosis-specific factors, activation of the SIN alone can produce functional rings. Further investigation into this phenotype suggested that the SIN

is essential for both stages of cytokinesis; completion of actomyosin ring assembly as well as constriction.

Multiple roles of the SIN in regulation of fission yeast cytokinesis

1) Ring stability and cytokinesis

Several lines of evidence suggest that the SIN is essential for assembly and maintenance of the actomyosin ring. In the Mid1 mediated ring assembly pathway described earlier, the ring maturation step fails in SIN mutants, however, in the absence of Mid1, SIN signaling alone can drive functional ring assembly and cytokinesis. Hence SIN signaling is crucial for both Mid1 dependent and independent pathways of ring assembly. One possible target of the SIN is the F-bar protein Cdc15, which is a core ring component essential for ring stability and cytokinesis (Carnahan and Gould, 2003; Fankhauser et al., 1995; Roberts-Galbraith et al., 2010). Hachet et al (Hachet and Simanis, 2008) showed that Cdc15 recruitment to the ring was severely compromised in the absence of SIN signaling. Consistent with Cdc15 being a target of the SIN, depletion of Cdc15 results in SIN phenotype, characterized by ring disassembly in anaphase and failure to form a septum (Fankhauser et al., 1995). Cdc15 is a phosphoprotein and its phosphorylation state regulates its localization during the cell cycle. In interphase, it is hyperphosphorylated and remains tip localized. However,

dephosphorylation by Clp1 (Cdc14 like protein; fission yeast homolog of budding yeast Cdc14) phosphatase promotes its medial localization in mitosis (Clifford et al., 2008; Wachtler et al., 2006). Besides CDK target sites, Cdc15 also has Sid2 phosphorylation (RXXS) sites and it has been shown that phosphorylation of Cdc15 on both Cdk and RXXS sites inhibits its ability to recruit Cdc12 towards formation of a stable ring apparatus. Conversely, Cdc15 mutants in which all possible phospho-sites were abolished showed precocious medial localization and ring assembly in interphase. These observations about phosphoregulation of Cdc15 lead to the apparent paradox that despite being a downstream effector of the Sid2 kinase Cdc15 is dephosphorylated when SIN is active (Roberts-Galbraith et al., 2010). Our results in Chapter II showing that active SIN inhibits Orb6 activity might resolve this ambiguity since Orb6 also phosphorylates RXXS sites.

Hypophosphorylation of Cdc15 causes ectopic ring formation, however, these rings are not functional and show no signs of constriction and septum assembly (Fankhauser et al., 1995; Roberts-Galbraith et al., 2010). This suggests that SIN signaling is essential for cytokinesis and SIN has other targets at the ring. Previous studies have shown that precocious activation of the SIN in interphase causes ectopic recruitment and activation of the formin Cdc12 to the ring, which then undergoes constriction and septum formation (Hachet and Simanis, 2008; Schmidt et al., 1997). Further, overexpression of a mutant version of Cdc12 that retained only the actin polymerization driving domains was able to

promote ring assembly and triggered ectopic cytokinesis out of interphase (Yonetani and Chang, 2010). These results suggested that Cdc12 likely functions downstream of the SIN to trigger cytokinesis.

2) Cytokinesis checkpoint

In order for cells to undergo faithful segregation of the duplicated genome it is essential that cytokinesis is coordinated with nuclear division. To that end, it has been observed that activation of the SIN in anaphase occurs after completion of chromosome segregation and inactivation of Cdk1 (Garcia-Cortes and McCollum, 2009; Guertin et al., 2000). This coordination inhibits aberrant nuclear cleavage by ectopic or premature septation. However, the flip side of this mechanism is that the SIN is sensitive to CDK activity. Thus in the event of a delay in cytokinesis, either due to errors in ring assembly or in septum formation, the rising CDK activity of the next cell cycle would inhibit SIN function unless the SIN has a mechanism to inhibit Cdk1 until cytokinesis is complete. Interestingly, over-expression of the SIN component Cdc14 was found to cause G2/M arrest in a Wee1 dependent manner suggesting that activation of the SIN can inhibit CDK activity (Fankhauser and Simanis, 1993). Wee 1 is a mitotic inhibitor that maintains inhibitory phosphorylation on Cdc2 (the gene expressing Cdk1) (Gould and Nurse, 1989; Russell and Nurse, 1987). Further, multiple studies on cell division regulation found that in presence of SIN signaling, cells underwent a Wee1 dependent nuclear division delay when cytokinesis was perturbed due to a septum formation defect (Liu et al., 2000; Liu et al., 1999; Nurse et al., 1976)

These studies suggested that fission yeast cells have a SIN-mediated, Wee1-dependent “cytokinesis checkpoint” mechanism that ensures that cells do not enter the next nuclear cycle until the completion of cytokinesis of the previous cycle (Liu et al., 2000). These studies also underscored the role of SIN signaling in coordination of cell division with nuclear division. Subsequent studies showed involvement of the Clp1 phosphatase in maintenance of this checkpoint (Cueille et al., 2001; Mishra et al., 2004; Trautmann et al., 2001).

Clp1 is a nucleolar protein that exits into the cytoplasm during mitosis and is maintained there until the completion of cytokinesis. Following cytokinesis Clp1 goes back to the nucleolus (Trautmann et al., 2001). Interestingly, cytoplasmic retention of Clp1 is dependent on the SIN. Further studies suggested that Sid2 mediated phosphorylation of Clp1 results in its binding to the 14-3-3 protein Rad24, which maintains its cytoplasmic localization until the completion of cytokinesis (Chen et al., 2008). Cytoplasmic Clp1 inhibits CDK1 activity by inactivation of the mitosis inducing phosphatase Cdc25 and hence maintains persistent SIN signaling (Esteban et al., 2004; Wolfe and Gould, 2004). These studies suggested that the SIN and Clp1 regulate each other’s activity via a positive feedback mechanism that becomes critical for successful cell division when cytokinesis is delayed (Mishra et al., 2004; Trautmann et al., 2001).

3) Inhibition of bipolar growth

Besides the G2/M arrest, the cytokinesis checkpoint also ensures that cells maintain the medial contractile ring to promote completion of cytokinesis (Mishra et al., 2004). However, it was observed that inactivation of Clp1 and/or the SIN in the *cps1-191* background (Cps1 is the primary septum synthesizing enzyme whose mutation causes cytokinetic delay) caused disassembly of the medial actomyosin ring and re-localization of actin structures back to the cell tips, which was reminiscent of interphase cells undergoing bipolar growth. This tip localization of actin was also observed in the SIN mutants, which fail in cytokinesis but continue with bipolar elongation. These observations suggested that the SIN is required to prevent bipolar growth during cytokinesis (Figure 1-7).

Figure 1-7. Dual role of the SIN in cytokinesis and interphase bipolar growth. The SIN promotes cytokinesis but inhibits bipolar cell elongation in mitosis.

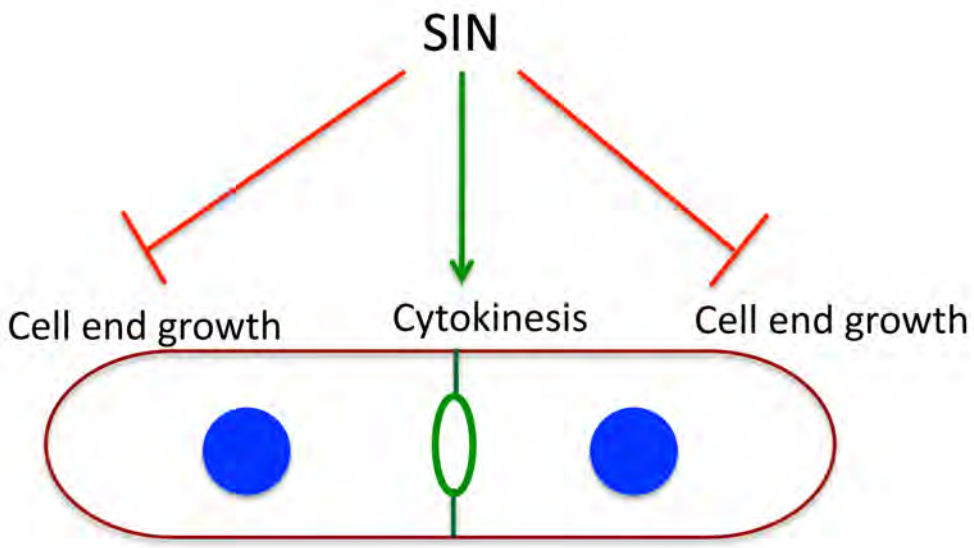


Figure 1-7: Dual role of the SIN in mitosis

NDR pathways and functions in other eukaryotes

As described earlier, signaling through the SIN and MOR pathways requires activation of the NDR kinases Sid2 and Orb6 respectively. Hence the SIN and MOR represent separate NDR pathways in fission yeast. NDR kinases are Ser/Thr kinases that have been classified as a subgroup of the AGC (protein kinase A (PKA)/PKG/PKG-like) class of protein kinases (Pearce et al., 2010). NDR kinases have been implicated in diverse cellular processes ranging from polarized growth and morphogenesis to cell division and proliferation in distant eukaryotes ranging from yeasts to humans (Hergovich and Hemmings, 2009; Hergovich et al., 2006). The NDR kinases are conserved at several levels. First, the primary structure of NDR kinases is highly conserved from yeasts to humans. Second, sequence alignments followed by functional studies have suggested that the key regulatory phosphorylation sites and catalytic domains are conserved at the primary sequence level among the different NDR kinases identified so far (Hergovich et al., 2006; Jansen et al., 2006; Millward et al., 1999; Pearce et al., 2010). Third, genetic and biochemical studies have shown that in all the eukaryotes studied so far, complete activation of the NDR kinases requires Ste-20 like upstream kinases and association with MOB proteins (Figure 1-8). Fourth, homologs of the fission yeast NDR proteins in other eukaryotes are involved in regulation of similar cellular processes (as described below). These homologies in activation mechanisms and cellular functions open up the possibility that

studies of these kinases in lower eukaryotes like fission yeast could be applicable to understand their roles in higher eukaryotes like humans.

In the budding yeast *S.cerevisiae,* NDR kinases Dbf2 and Cbk1 are the most downstream targets of homologous signaling cascades termed the MEN (Mitotic Exit Network) and the RAM (Regulator of Ace2 activity and cellular morphology) pathways. The MEN is required for mitotic exit (Seshan and Amon, 2004) and cytokinesis (Meitinger et al., 2010) while the RAM pathway is essential for polarized growth and cell separation (Kurischko et al., 2008; Mazanka et al., 2008; Nelson et al., 2003). The role of the MEN in mitotic exit involves the Cdc14 phosphatase since Cdc14 triggers degradation of mitotic cyclins (Queralt and Uhlmann, 2008; Stegmeier and Amon, 2004). Similar to its fission yeast counterpart Clp1, Cdc14 in budding yeast is also in the nucleolus in interphase where it is kept inactive by Net1/Cfi1 (Stegmeier and Amon, 2004). Cdc14 is released into the nucleus by the FEAR (Cdc14 early Anaphase Release) network and is subsequently driven into the cytoplasm by the MEN (D'Amours and Amon, 2004; Stegmeier and Amon, 2004). This situation is different from fission yeast since SIN is not required for the release of Clp1 from the nucleolus in early mitosis but is essential for the cytoplasmic retention of active Clp1 (Trautmann et al., 2001). The nucleolar anchor for Clp1 is still a mystery. Although Dnt1 has strong sequence and partial functional similarities with Net1, our studies in Chapter IV have negated its role as the Net1 homolog for fission yeast (Jin et al., 2007).

Cdc14 in budding yeast is essential for nucleolar segregation and has been shown to inhibit RNA Polymerase (Pol) 1 mediated rDNA transcription in anaphase to facilitate efficient rDNA condensation and faithful nucleolar segregation in mitosis (Clemente-Blanco et al., 2009). In fission yeast Clp1 is not essential for rDNA segregation (Chen et al., 2006b), however, we found that the rDNA transcription machinery in fission yeast regulates SIN functions independent of Clp1 (Chapter IV). These results point to a novel role of the fission yeast nucleolus in regulation of the SIN and cytokinesis.

In *S.cerevisiae* the NDR pathway homologous to the MOR cascade in *S.pombe* involves the Cbk1 driven signaling cascade called RAM (Krapp et al., 2004). RAM is essential for polarized growth (Kurischko et al., 2008) and the daughter cell specific distribution of the transcription factor Ace2 that promotes mother-daughter cell separation (Mazanka et al., 2008; Mazanka and Weiss, 2010). Since MOR mutants fail to separate after septum formation in cytokinesis, it is reasonable to speculate that the MOR is also essential for cell separation in fission yeast. Interestingly, *S.pombe* Ace2 is also required for cell separation at the end of mitosis (Alonso-Nunez et al., 2005). Based on the studies in budding yeast, it remains a possibility that the MOR acts through Ace2.

NDR kinases in higher eukaryotes

Clearly defined signaling pathways homologous to the SIN/MEN and the MOR/RAM have not been identified in higher eukaryotes. Yet homologs of some essential components of these pathways have been identified in higher eukaryotes, including humans, where they are involved in similar physiological functions. For instance the mammalian homologs of Sid2/Dbf2 are LATS1/2 and homologs of Orb6/Cbk1 in mammals are NDR1/2. LATS1/2 and NDR1/2 are involved in the regulation of cytokinesis/mitotic exit and cell growth respectively, similar to their counterparts in yeast (Hergovich et al., 2008; Vichalkovski et al., 2008; Zhao et al., 2008). NDR1 has also been implicated in regulation of mitotic spindle assembly and chromosome segregation (Chiba et al., 2009; Oh et al., 2010). In the nematode *C.elegans*, the NDR kinase SAX1 is essential for neuronal cell shape and migration(Gallegos and Bargmann, 2004). *Drosophila melanogaster* also has two NDR proteins, Trc (tricornered) and Warts/Lats, which are essential for regulation of morphogenesis and polarized growth in neurons and epidermis (Emoto et al., 2004; Emoto et al., 2006; Geng et al., 2000). Furthermore, Warts is essential for the Hippo signaling pathway that regulates cell growth and proliferation (Dong et al., 2007; Justice et al., 1995).

Consistent with the essential roles of NDR kinases in cell growth and division, inactivation of these kinases or their regulators in flies and mammals lead to tissue overgrowth, often associated with cancer (Hergovich et al., 2008).

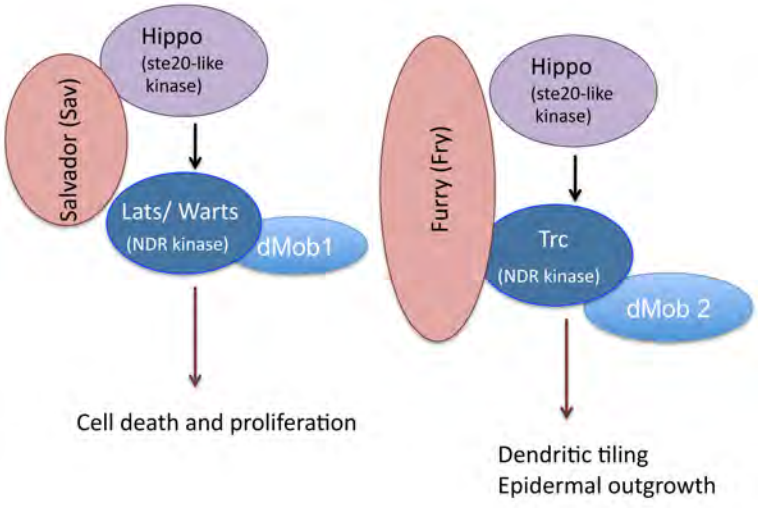
Hence detailed understanding of the regulation of these proteins and their functions is critical to understand how normally dividing cells maintain the balance between growth and division and what goes awry in disease states like cancer. Although several studies have led to the identification of the roles of homologous NDR kinases and their regulators in different eukaryotes, it remains unclear if the separate NDR pathways in any organism influence each other's functions and/or regulation to coordinate the different processes they are involved in. Our study (Chapter II) provides the first evidence for existence of a cross-talk between two separate NDR pathways that helps to coordinate the switch between cytokinesis and polarized growth in fission yeast.

Some evidence in flies and humans present suggest possible links between separate NDR kinase pathways in higher eukaryotes. In flies, although Warts and Trc act through separate pathways (Figure 1-8A) for their respective functions, it is striking that the upstream Ste-20 like kinase Hippo acts through both Warts and Trc to regulate dendritic tiling in the neurons (Emoto et al., 2006) suggesting a functional coordination between the separate pathways. Initially it was believed the mammalian Ste20 (MST) kinases MST1/2 activates the tumor suppressor LATS1/2 (Chan et al., 2005; Hao et al., 2008; Zhao et al., 2007) while MST3 promotes activation of NDR1/2 (Stegert et al., 2005). But recent work has found that MST1 mediated activation of NDR1 is essential for regulation of T cell growth and apoptosis (Cornils et al., 2010; Vichalkovski et al., 2008). Further, the mitosis specific functions of NDR1 are dependent on MST1/2 (Chiba et al., 2009;

Oh et al., 2010) (Figure 1-8B). We are just beginning to understand the mechanisms by which the different NDR kinases regulate mammalian cell division and growth. Extensive future research in this field will help to further understand if separate NDR pathways have cross-talk interactions similar to those reported in our study and their significance in mammalian cell cycle and development.

Figure 1-8. Schematic of NDR pathways in flies and humans. Similar to their yeast counterparts, activation of the NDR kinases in higher eukaryotes requires upstream Ste20-like kinases and association with MOB proteins. Furthermore, these proteins are involved in similar cellular processes. (A) In flies, NDR kinases Warts/Lats and Trc are activated by the Ste20-like kinase Hippo and require the binding proteins Mob1/2. Scaffolding proteins Fry and Sav facilitate the activation by promoting association of NDR kinases with MST kinases. (B) The mammalian NDR kinases LATS1/2 and NDR1/2 are activated by the mammalian Ste20-like (MST) kinases in association with the binding partners human MOB1/2 and the scaffolding proteins WW45 and human Furry.

(A) NDR pathways in Drosophila melanogaster (flies).



(B) NDR pathways in homo sapiens (humans).

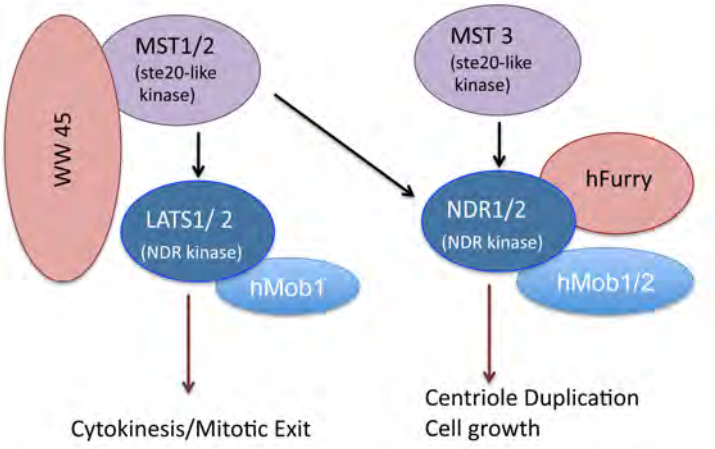


Figure 1-8: NDR pathways in flies and humans

CHAPTER II

The mitosis to interphase transition is coordinated by crosstalk between the SIN and MOR pathways in *S. pombe*

Declaration of figure contributions:

Figure 2-3 was contributed by Kazunori Kume and Dai Hirata (University of Hiroshima, Japan).

Figures 2-4C,D; 2-5; 2-6; 2-7A were contributed by Sneha Gupta (Dan McCollum Lab).

Summary

The mechanisms that regulate cytoskeletal remodeling during the transition between mitosis and interphase are poorly understood. In fission yeast the MOR pathway promotes actin polarization to cell tips in interphase, whereas the SIN signaling pathway drives actomyosin ring assembly and cytokinesis. We show that the SIN inhibits MOR signaling in mitosis by interfering with Nak1 kinase-mediated activation of the most downstream MOR component the NDR-family kinase Orb6. Inactivation of the MOR may be a key function of the SIN since attenuation of MOR signaling rescued the cytokinetic defects of SIN mutants and allowed weak SIN signaling to trigger ectopic cytokinesis. Furthermore, failure to inhibit the MOR is toxic when the cell division apparatus is compromised. Together our results reveal a mutually antagonistic relationship between the SIN and MOR pathways, which is important for completion of cytokinesis and coordination of cytoskeletal remodeling at the mitosis to interphase transition.

Introduction

Eukaryotic cells undergo major reorganizations of their cytoskeleton during cell cycle transitions from interphase to mitosis and vice versa. Because cells reorganize many shared cytoskeletal elements such as actin in the transition between interphase and mitosis they must have mechanisms to ensure both a rapid and decisive switch between the two states. Although there has been considerable study of how cells assemble the various cytoskeletal structures during each cell cycle stage, it remains unclear how the transition between stages is coordinated.

The fission yeast *Schizosaccharomyces pombe* has proven to be an excellent model to study cell cycle dependent cytoskeletal arrangements (Martin, 2009; Roberts-Galbraith and Gould, 2008). This cylindrical eukaryote grows from both ends in interphase by polarization of actin patches and cables towards the growing tips of the cell (Snell and Nurse, 1993). However, the polarized actin distribution is lost with the onset of mitosis when growth ceases and actin is re-localized to the cell middle to form the actomyosin contractile ring. A conserved signaling network called the MOR is essential for polarized growth in interphase, since MOR mutants have depolarized actin and become spherical in morphology. The MOR pathway is comprised of several proteins including Pmo25 (MO25 in humans (Kanai et al., 2005; Kume et al., 2007; Mendoza et al., 2005)), Nak1 (a GC kinase (Kanai et al., 2005; Leonhard and Nurse, 2005)), and the homolog of

the *drosophila* Furry protein called Mor2 (Cong et al., 2001; Hirata et al., 2002), all of which are required for the activity of the NDR kinase Orb6 (Imai et al., 2004; Kanai et al., 2005; Tamaskovic et al., 2003; Verde et al., 1998), the most downstream component of the pathway.

Interestingly, Sid2, the other NDR-family kinase in fission yeast, acts at the end of the SIN signaling pathway (Sparks et al., 1999). The SIN is essential for stable actomyosin ring formation, ring constriction, and septum formation during cytokinesis (Krapp and Simanis, 2008). Although the two NDR-kinase pathways, MOR and SIN, are essential for separate cytoskeletal configurations, the mechanism that controls the switch between the two states remains unknown. Interestingly, an earlier study suggested that the SIN inhibits actin polarization towards cell tips (Mishra et al., 2004), however the mechanism of SIN action was unclear.

In this study we show that the SIN can directly inhibit polarized growth by blocking MOR pathway signaling. Our data further suggest that this cross-talk between the SIN and MOR pathways is important to coordinate cytoskeletal rearrangements during the transition from mitosis to interphase.

Results

SIN activation in interphase arrests nuclear division and inhibits the polarity machinery

To examine how the SIN affects interphase polarity and cell cycle progression we ectopically activated the SIN in interphase. The SIN can be turned on by inactivation of Cdc16, which is the GAP for the Spg1 GTPase (Fankhauser et al., 1993; Schmidt et al., 1997). If the SIN is activated in asynchronous cells, the cells stop polarized growth, since they cease to elongate, and arrest as mononucleate or binucleate cells with multiple septa (Fankhauser et al., 1993; Minet et al., 1979). The disruption of polarized growth in these cells could be an indirect effect caused by ectopic septa formation, which could interfere with growth at cell ends or cause cell death by cutting the nuclei in half. To remove potential complications due to formation of multiple septa, the SIN was activated using the *cdc16-116* temperature sensitive mutant in either the *cdc3-124* or *cdc15-140* temperature sensitive mutant background. The *cdc3-124* and *cdc15-140* mutations block cytokinesis and septum formation by disrupting actomyosin ring assembly but do not interfere with SIN signaling (Balasubramanian et al., 1994; Fankhauser et al., 1995; Sparks et al., 1999). The *cdc16-116 cdc3-124* and *cdc16-116 cdc15-140* strains were shifted to restrictive conditions for 4 hours (almost two cell cycles) and were scored for cell length and number of nuclei. Because the *cdc3-124* and *cdc15-140* mutations block cytokinesis the *cdc16-116 cdc3-124* and *cdc16-116 cdc15-140* cells should be tetranucleate and twice the

length of normal wild type cells if nuclear division and cell growth proceeded normally. Interestingly, the *cdc16-116 cdc3-124* and *cdc16-116 cdc15-140* double mutant cells arrested as both mono and binucleate cells and failed to elongate past the size of asynchronous wild-type cells (Figure 2-1A, Table 2-1). These cells also displayed an increase in cell diameter consistent with a disruption in polarized growth (Table 2-1). FACS analysis showed that the cells were arrested with 2C and 4C peaks of similar size to the mono and binucleate peaks, consistent with each nuclei arresting with a G2 DNA content (Figure 2-2). Thus SIN activation blocked cell elongation and mitotic entry in interphase cells.

Cell elongation in interphase requires polarization of actin patches to the cell tips. To test if the block in cell elongation upon SIN activation is due to inhibition of the interphase polarity machinery, we looked at the actin distribution in the *cdc3-124 cdc16-116* and *cdc15-140 cdc16-116* cells. Upon shift to the restrictive temperature the double mutant cells had a dispersed actin cytoskeleton (Figure 2-1B and Figure 2-2B,C) suggesting that SIN signaling disrupted interphase polarity. To rule out the possibility that Cdc16 inactivation could trigger an alternative pathway besides the SIN or that the *cdc3-124* ring mutation disrupts interphase polarity, we inactivated the most downstream SIN component Sid2 using the *sid2-250* mutation. The *cdc3-124 cdc16-116 sid2-250* cells showed no block in cell elongation and nuclear division at the restrictive temperature and became long and multi-nucleate with actin polarized at the cell

tips (Figure 2-1A,B, Figure 2-2B,C and Table 2-1), confirming that the effects of Cdc16 inactivation are caused by ectopic activation of SIN signaling.

The G2 arrest observed after SIN activation is presumably mediated by inhibitory phosphorylation on Cdk1 since the nuclear division arrest was lost when the Cdk1 inhibitory kinase Wee1 was inactivated (Figure 2-1A). Interestingly, examination of *cdc3-124 cdc16-116 wee1-50* cells at the restrictive temperature showed that although the *wee1-50* mutation overcame the nuclear division block caused by SIN signaling, these cells failed to elongate and had depolarized actin (Table1 and Figure 2-1B and Figure 2-2B,C) showing that the *wee1-50* mutation did not overcome the SIN-mediated inhibition of polarized growth. Thus SIN inhibition of nuclear division can be uncoupled from its inhibition of interphase actin organization.

Table 2-1. Activation of the SIN inhibits polarized cell growth.

Strain genotype	Number of nuclei (n)	Mean Cell length(µm) +/- SD	Mean Cell Diameter (µm) +/- SD
wild type	n=1 n=2	11.9 +/- 1.8 14.9 +/- 0.817	2.93+/-0.2 2.95+/-0.35
cdc3-124 cdc16-116	n=1 n=2	10.66+/- 2 13.75 +/-2	4.62+/-0.46 4.7+/-0.5
cdc15-140 cdc16-116	n= 1 n=2	10.2 +/- 2 15+/- 1	6.46+/- 1.26 6.086+/- 1.24
cdc3-124 cdc16-116 wee1-50	n=1 n=2 n=4	6.65 +/- 0.85 7.25 +/- 1.4 9.6 +/- 1.6	4.81+/-0.45 4.43+/-0.54 4.47+/-0.61
cdc3-124 cdc16-116sid2-250	n=1 n=2 n=4	N/A 21.2+/- 1.75 26 +/- 4	N/A 3.47+/-0.36 3.6+/-0.434
cdc3-124 cdc16-116 sid2-250 orb6-25	n=1 n=2 n=4	7.86 +/- 1.5 13.23+/- 3 14.38+/- 1	5.04+/-0.41 5.3+/-0.365 8.23+/-1.13

Average length and diameter measurements for cells with indicated genotype and the given number of nuclei are shown. (SD: standard deviation).

Measurements are not shown (N/A) if the strain did not arrest with a significant number of cells with the indicated number of nuclei. Atleast 100 cells were counted for each strain.

Figure 2-1. SIN activation arrests nuclear division and inhibits the interphase polarity machinery. (A) Cells of the indicated genotypes were grown at 25°C then shifted to 36°C for 4 hours, methanol fixed, stained with DAPI, and scored for number of nuclei (n). The experiment was done in triplicate and error bars denote the standard deviation (SD).

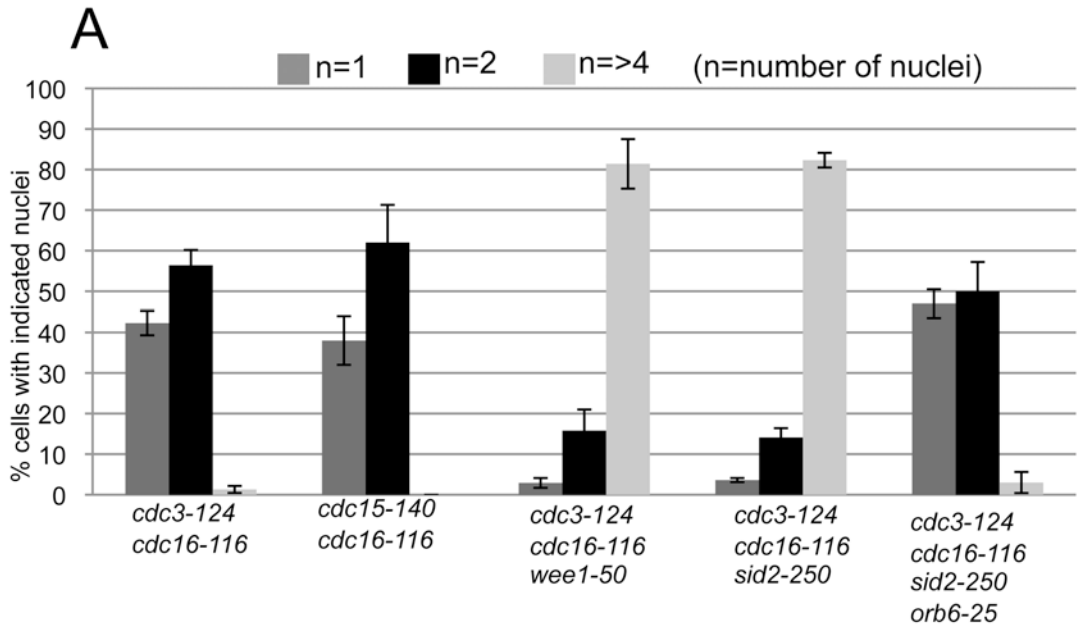


Figure 2-1A: SIN activation arrests nuclear division

Figure 2-1. (B) Cells of the indicated genotypes were shifted to 36˚C for 4 hours then stained with Alexafluor 488-phalloidin and DAPI to visualize actin and DNA respectively. Dashed dividing lines separate individual images for montage presentations.

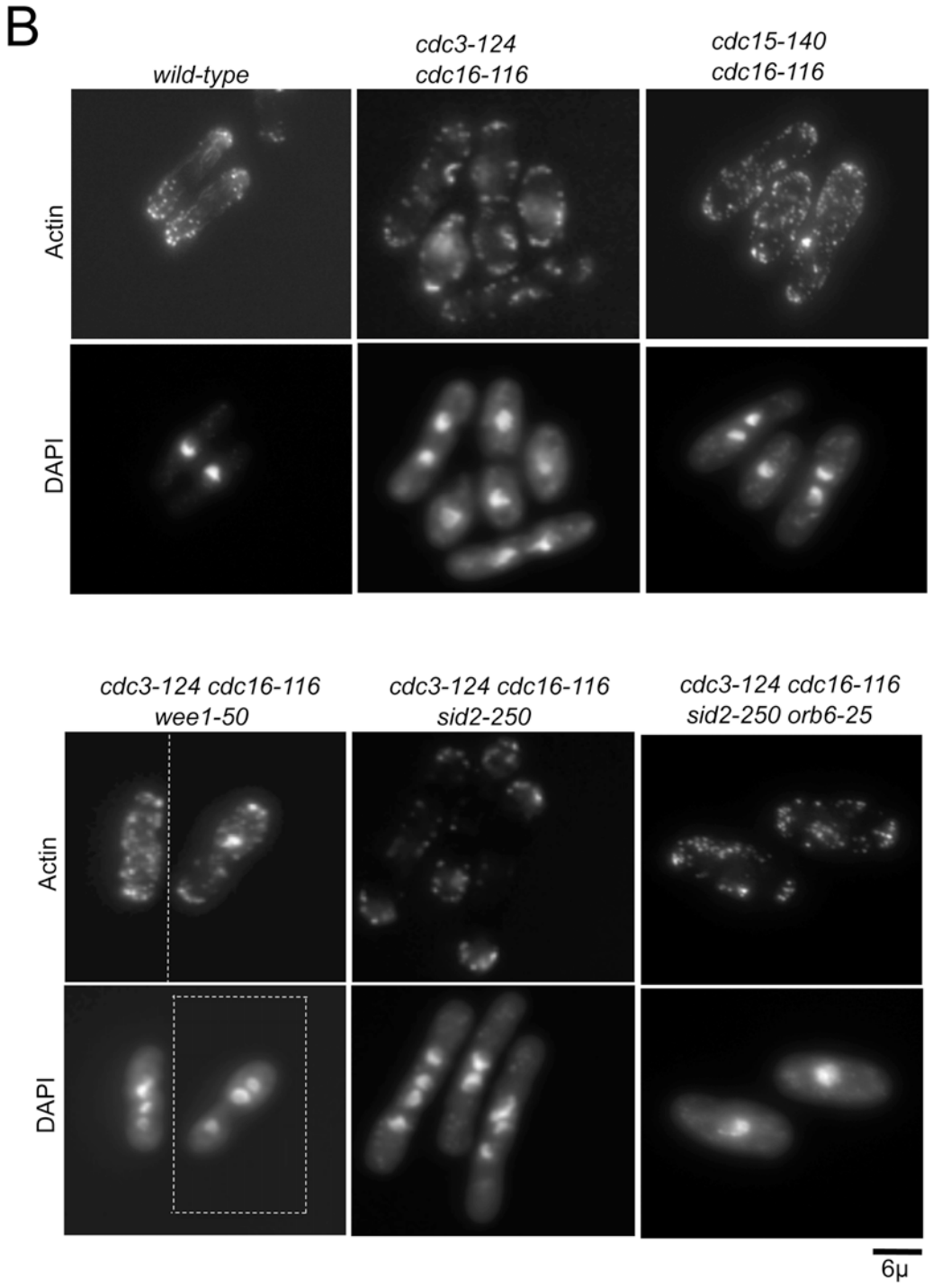


Figure 2-1B: SIN activation inhibits interphase polarity

Figure 2-2. (A) SIN activation inhibits G2/M transition. The indicated strains were grown at 25°C and then shifted to 36°C for 4 hours and analyzed for DNA content (C) by flow cytometry before (i) and after (ii) shift to 36˚C. (B) Quantification of the actin staining shown in Fig1B. The diagram shows how each *S. pombe* cell was divided into quarters by length, the left most quarter (L), the middle 2 quarters (M) and right most quarter (R). Fluorescence intensity of F-actin staining at the cell ends (L and R) versus the cell middle (M) was measured. (C) Line graph plots for the sum total fluorescence intensity associated with the F-actin structures in the L, M and R sections was plotted. Each line represents the actin patch distribution in a single cell. At least 20 cells were analyzed for each genotype depicted using the Slidebook 5.0 software (Intelligent Imaging Innovations Inc). Consistent with the polarized actin distribution, wild-type and *cdc3-124 cdc16-116 sid2-250* cells showed a dip in fluorescence intensity in the middle (M) relative to the left (L) and right (R) ends giving a “V” plot in contrast to *cdc3-124 cdc16-116* and *cdc3-124 cdc16-116 wee1-50* cells that exhibited a dispersed actin distribution resulting in an “inverted V” plot. Note that *cdc3-124 cdc16-116 sid2-250* cells show some cells with medial actin staining since these cells continue through the cell cycle and still reorganize actin in the cell middle in early mitosis.

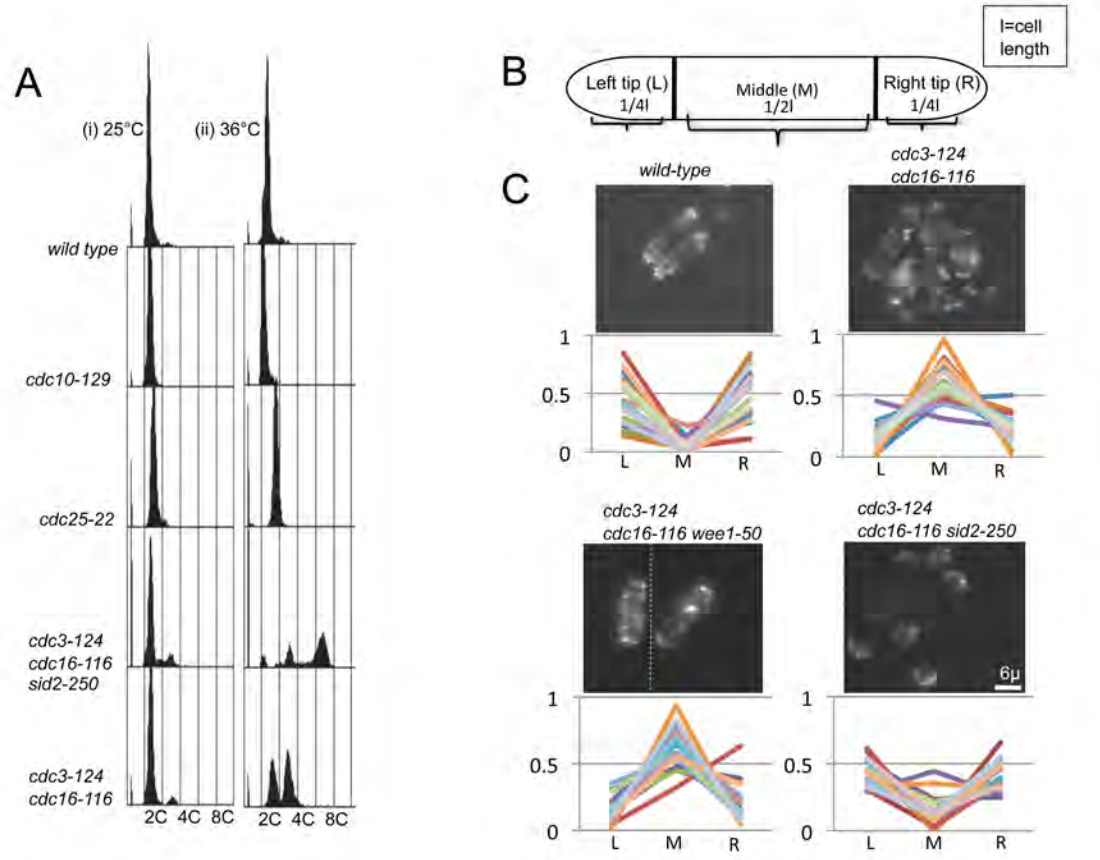


Figure 2-2: SIN activation blocks nuclear division and interphase growth

The SIN inhibits the activity of the MOR pathway kinase Orb6

The phenotype caused by ectopic activation of the SIN is very similar to that observed in MOR pathway loss of function mutants, which arrest with depolarized actin, fail to elongate, and undergo a Wee1 dependent G2 arrest (Hirata et al., 2002; Kanai et al., 2005). In fact inactivation of the most downstream MOR pathway component Orb6 caused a block in cell elongation and nuclear division along with an increase in cell diameter in *cdc3-124 cdc16-116 sid2-250* cells similar to that in the *cdc3-124 cdc16-116* double mutant (Figure 2-1A and Table1). Consistent with the block in cell elongation, the actin cytoskeleton was depolarized in the *cdc3-124 cdc16-116 sid2-250 orb6-25* cells (Figure 2-1B). These results suggested that the SIN might disrupt interphase polarity by inhibiting the MOR pathway.

The most downstream component of the MOR pathway is the NDR-family kinase Orb6, allowing the kinase activity of Orb6 to serve as a read-out for the functional status of the MOR pathway. To directly test if SIN activation blocks MOR signaling, we examined the kinase activity of Orb6, after ectopic activation of the SIN using the *cdc16-116* mutation. As before, the *cdc15-140* mutation was included to overcome complications due to constitutive septation in the *cdc16-116* cells. In contrast to asynchronous wild-type and G2 arrested *cdc25-22* cells, the *cdc16-116* cells, which also arrest in G2, showed reduced Orb6 activity, with or without the *cdc15-140* mutation (Figure 2-3A). We next examined if SIN

activation had a similar effect in interphase-arrested cells. Interestingly, although *cdc15-140 cdc25-22* cells maintained high Orb6 activity consistent with their bipolar growth in interphase, SIN activation in this mutant background using the *cdc16-116* mutation inhibited Orb6 activity (Figure 2-3A). Hence, SIN activation in interphase results in loss of Orb6 kinase activity and blocks MOR signaling.

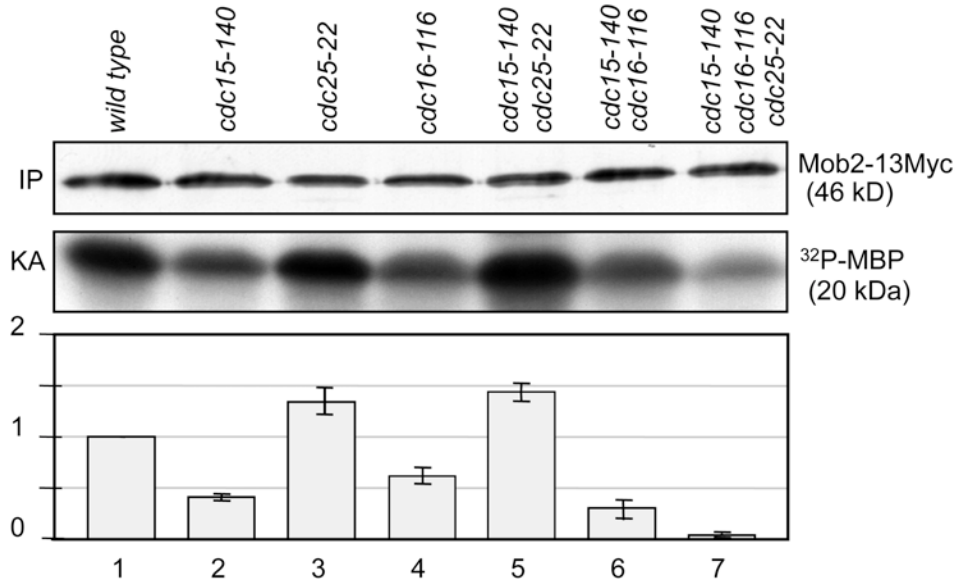
We next examined the relationship between the SIN and Orb6 activity under more physiological circumstances. In wild-type cells, Orb6 kinase activity peaks in G2 phase and then decreases as cells go through mitosis and septation (Kanai et al., 2005). Interestingly, the interphase peak in Orb6 activity following cell division was shown to require the activity of some of the SIN components such as Cdc7 in the previous mitosis (Kanai et al., 2005), apparently contradicting our results showing that ectopic SIN activation inhibits Orb6. However careful examination of Orb6 kinase activity in synchronous *cdc7-24* mutant cells resolved the apparent discrepancy. Consistent with the earlier results, Cdc7 was required for the large peak in the Orb6 activity following completion of mitosis (Figure 2-3C, compare 200-240 min. time points in WT and *cdc7-24* cells). However, *cdc7-24* cells maintained moderate levels of Orb6 activity consistent with the continued polarized growth observed in *cdc7-24* mutants. Thus the SIN is not essential for Orb6 activity per se but is required for the peak in activity associated with the onset of bipolar growth in the following cell cycle. We next examined Orb6 activity in synchronous *cdc15-140* mutants. The c*dc15-140* mutant cells have defects in actomyosin ring assembly, which

triggers the cytokinesis checkpoint, causing them to arrest as binucleate cells with activated SIN (Liu et al., 2000; Mishra et al., 2004). Interestingly, these cells maintained very low Orb6 kinase activity consistent with the SIN inhibiting Orb6 (Figure 2-3A, and 2-3C (160-240 min. in *cdc15-140*)). If Cdc7 is inactivated in these cells, they regained moderate levels of Orb6 kinase activity (Figure 2-3C, compare 160-240 min. time points in *cdc15-140 cdc7-24* and *cdc15-140* cells) but lacked the peak in the following interphase (Figure 2-3C, compare 200-240 min. time points in *cdc15-140 cdc7-24* and WT cells). Thus, SIN has a dual role in regulation of the MOR pathway. The SIN suppresses Orb6 activity during cytokinesis but is required for its later increase as cells begin new end growth in the next cell cycle following cytokinesis.

Figure 2-3. The SIN has a dual role in regulation of the Orb6 kinase activity. (A) Ectopic activation of the SIN in interphase inhibits Orb6 kinase activity. All strains expressed *mob2-13Myc* from the chromosomal promoter. Cells were grown at 25°C, then shifted to 36°C for 3 hours. Cell extracts were prepared and the Orb6-Mob2-13Myc complex was immunoprecipitated using anti-Myc antibodies. Immunoprecipitates were split, with one half immunoblotted with anti-Myc antibodies, and the other half used to assay Orb6 kinase activity using MBP as an artificial substrate. The kinase activities (KA) were quantified using Image J software and normalized to the amount of Mob2-13Myc (IP) and the background kinase activity in untagged control cells. The activity relative to wild-type cells is shown. Error bars denote SD from three separate experiments.

(B) Control for Orb6 kinase assay**.** Immunoprecipitation with anti-Myc antibodies from wild-type untagged Mob2 strain (IP) did not show any Orb6 kinase activity (KA), kinase assay was performed as in (A).

A



B

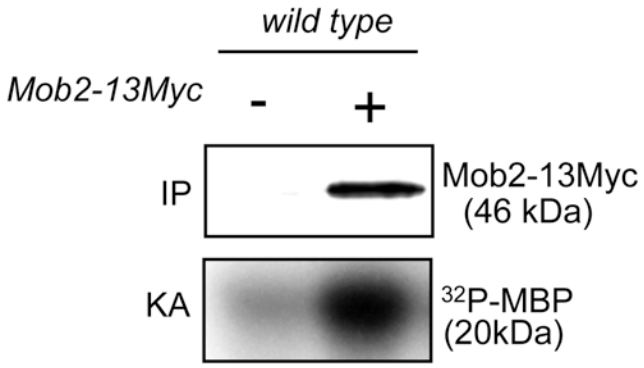


Figure 2-3: The SIN inhibits Orb6 activity in mitosis

Figure 2-3(C) The SIN inhibits Orb6 kinase activity in mitosis but promotes its full activity in the following interphase. Early G2 cells of the indicated strains expressing Mob2-13Myc from the chromosomal locus were grown at 25°C, synchronized by elutriation, then shifted to 36°C at time zero, and portions were collected at the indicated times and assayed for Orb6 kinase activity. This experiment was repeated at least once with similar results.

C

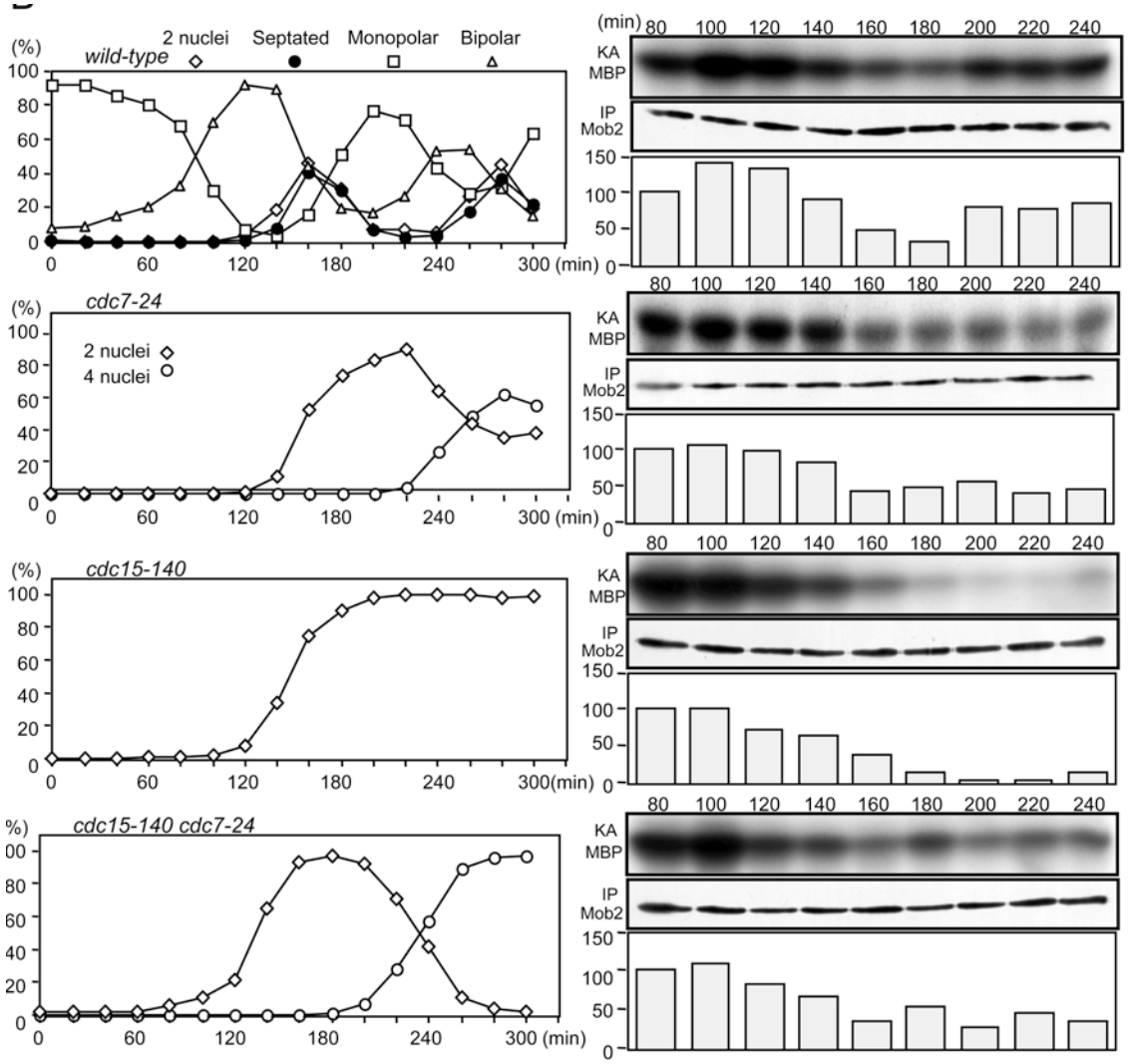


Figure 2-3C: The SIN inhibits Orb6 activity in mitosis but promotes Orb6 activation after cytokinesis

The SIN inhibits the MOR pathway by blocking Nak1 mediated activation of Orb6

We next wanted to understand the mechanism by which the SIN inhibits Orb6 kinase activity. Similar to other members of the NDR kinase family, Orb6 kinase is activated by the GC-kinase Nak1 (Hergovich et al., 2006; Kanai et al., 2005). Thus the SIN could either inhibit the activity of Nak1, or it could inhibit the ability of Nak1 to activate Orb6. A previous study suggested that the SIN does not inhibit Nak1 since Nak1 activity remained high during cytokinesis when the SIN is active (Kanai et al., 2005). To test this possibility in another way, we examined Nak1 kinase activity in *cdc3-124 cdc16-116* cells, which arrest with active SIN. Consistent with earlier results, upon SIN activation there was no significant reduction in Nak1 kinase activity (Figure 2-4A). This result suggested that the SIN did not alter Nak1 kinase activity per se but possibly disrupted the ability of Nak1 to activate Orb6.

The exact mechanism by which Nak1 activates Orb6 remains unclear. Although interaction between the 2 proteins was not observed by co-immunoprecipitation, a two-hybrid interaction was observed (Kanai et al., 2005), suggesting a physical association between the two proteins. This raised the possibility that the SIN might interfere with Nak1-mediated activation of Orb6 by preventing association of the two proteins. To test this hypothesis, we constructed a Nak1-Orb6 fusion (Figure 2-4B). The Nak1-Orb6 fusion was able

to rescue the growth defects of both the Nak1 and Orb6 single mutants (Table 2-2) suggesting that the individual proteins in the fusion retained functionality. The fusion did not rescue a mutant in the upstream regulator Pmo25, consistent with the known dependence of Nak1 activity on Pmo25 (Kanai et al., 2005). Interestingly, the fusion, but not the Nak1 and Orb6 proteins individually or in combination, completely rescued a mutation in the scaffold protein Mor2 suggesting that the key function of Mor2 is to bring Nak1 and Orb6 together (Table 2-2).

To test whether the Nak1-Orb6 fusion could bypass the block in cell elongation when the SIN is activated, we expressed the Nak1-Orb6 fusion in *cdc3-124 cdc16-116* background. Unlike *cdc3-124 cdc16-116* cells carrying the vector control plasmid, which did not elongate, the *cdc3-124 cdc16-116* cells containing the Nak1-Orb6 fusion plasmid were able to undergo significant elongation (Figure 2-4C). The *cdc3-124 cdc16-116* cells expressing the Nak1-Orb6 fusion were able to polarize actin to the cell tips, but also showed medial actin distribution consistent with the cells trying to carry out both the SIN and MOR actin polarization programs (Figure 2-4D). Expression of Nak1 or Orb6 alone or co-expression of both Nak1 and Orb6 in the *cdc3-124 cdc16-116* cells did not bypass the cell elongation and actin polarization defects observed in these cells (Figure 2-4C,D), showing that fusion of Nak1 and Orb6 was required to bypass the SIN mediated inhibition of polarized growth. Furthermore, the kinase activity of Nak1 was required in the fusion because a kinase inactivating

mutation in Nak1 blocked the ability of the fusion to drive polarized growth when the SIN is active (Figure 2-4C,D). Interestingly, the Nak1-Orb6 fusion also partially overrode the block in nuclear division caused by SIN activation as seen by the reduction in mononucleate and increase in bi- and tetranucleate cells compared to controls at the 7 hour time point (Figure 2-4C). Because either MOR inactivation or SIN activation blocks both nuclear division and cell elongation, the ability of the Nak1-Orb6 fusion to partially bypass both of these blocks when the SIN is active suggests that the SIN might block both nuclear division and cell growth by inhibiting the MOR. The failure of the fusion to completely bypass the SIN mediated block in growth and nuclear division suggests that the SIN can still partially inhibit the MOR in the presence of the fusion, or the SIN can affect nuclear division and cell growth through an additional mechanism besides inhibition of the MOR.

Table 2-2 Rescue of MOR pathway mutants by the Nak1-Orb6 fusion.

Strains	vector	Nak1	Orb6	Nak1-Orb6
pmo25-35	-	-	n/a	-
nak1-167	-	+	-	+
orb6-25	-	n/a	+	+
mor2-786	-	-	-	+

The ability of the Nak1-Orb6 fusion to rescue (+) or not rescue (-) MOR pathway mutants at the restrictive temperature of 36°C is shown. (N/A) indicates condition that was not tested.

Figure 2-4. Fusion of Nak1 to Orb6 bypasses SIN inhibition of cell elongation. (A) The SIN does not inhibit Nak1 activity. Wild-type and *cdc3-124 cdc16-116* cells expressing Nak1-3GFP from the chromosomal promoter were grown at 25°C then shifted to 36°C for 3 hours before harvesting. Cell extracts were prepared and Nak1-3GFP was immuno precipitated with anti-GFP (Invitrogen) antibody. The immunoprecipitates were split, with one portion used for western blotting using GFP antibodies (Santa-Cruz) (IP), and the other portion for in vitro kinase assays using alpha-casein (Sigma) as an artificial substrate (Leonhard et al., 2005). The kinase activity (KA) was measured using a Phosphor imager (Molecular Dynamics), quantified using the ImageQuant software, and normalized to the amount of Nak1 (IP). The activity relative to wild-type cells is shown. The difference in the normalized Nak1 kinase activity between the wild-type and the *cdc3-124 cdc16-116* cells was not significant based on t-test analysis from 3 different experiments. Error bars denote SD of the relative KA. (B) Schematic representation of the Nak1-Orb6 fusion construct expressed from the medium strength thiamine repressible promoter, nmt41. (C) *cdc3-124 cdc16-116* cells carrying the indicated plasmids were induced for 19 hours in media lacking thiamine and shifted to 36°C, then cells were collected at 2, 4, and 7 hours (hrs). The plasmids used were the pRep41 vector, or the pRep41 vector carrying Nak1, Orb6, the Nak1-Orb6 fusion (fusion), the Nak1 kinase dead-Orb6 fusion (Nak1*-Orb6), or the Nak1 and Orb6 genes on separate plasmids (Nak1 + Orb6). Cells were then stained with DAPI and scored for cell length

measurements (upper panel) and nuclei count (n) (lower panel). Error bars in the cell length plot denote SD of the length measurements obtained from three separate experiments. Average cell length was compared between cells with vector control and those with the different transgenes as indicated in the figure at each time-point. Statistically significant increase in cell length (based on p-value calculation by t-test analysis) was observed only in cells expressing the fusion construct, * p-value =0.0118, ** p-value=0.0001. At least 100 cells were analyzed for each time-point. For clarity, the SD values for the nuclear count plot are shown in Figure 2-5. (D) *cdc3-124 cdc16-116* cells with the indicated transgenes were grown as in (C) and then processed for actin staining. Montage of representative cells (separated by dashed dividing lines) with actin (phalloidin staining) and nuclei (DAPI staining) staining are shown for the 7 hour time-point.

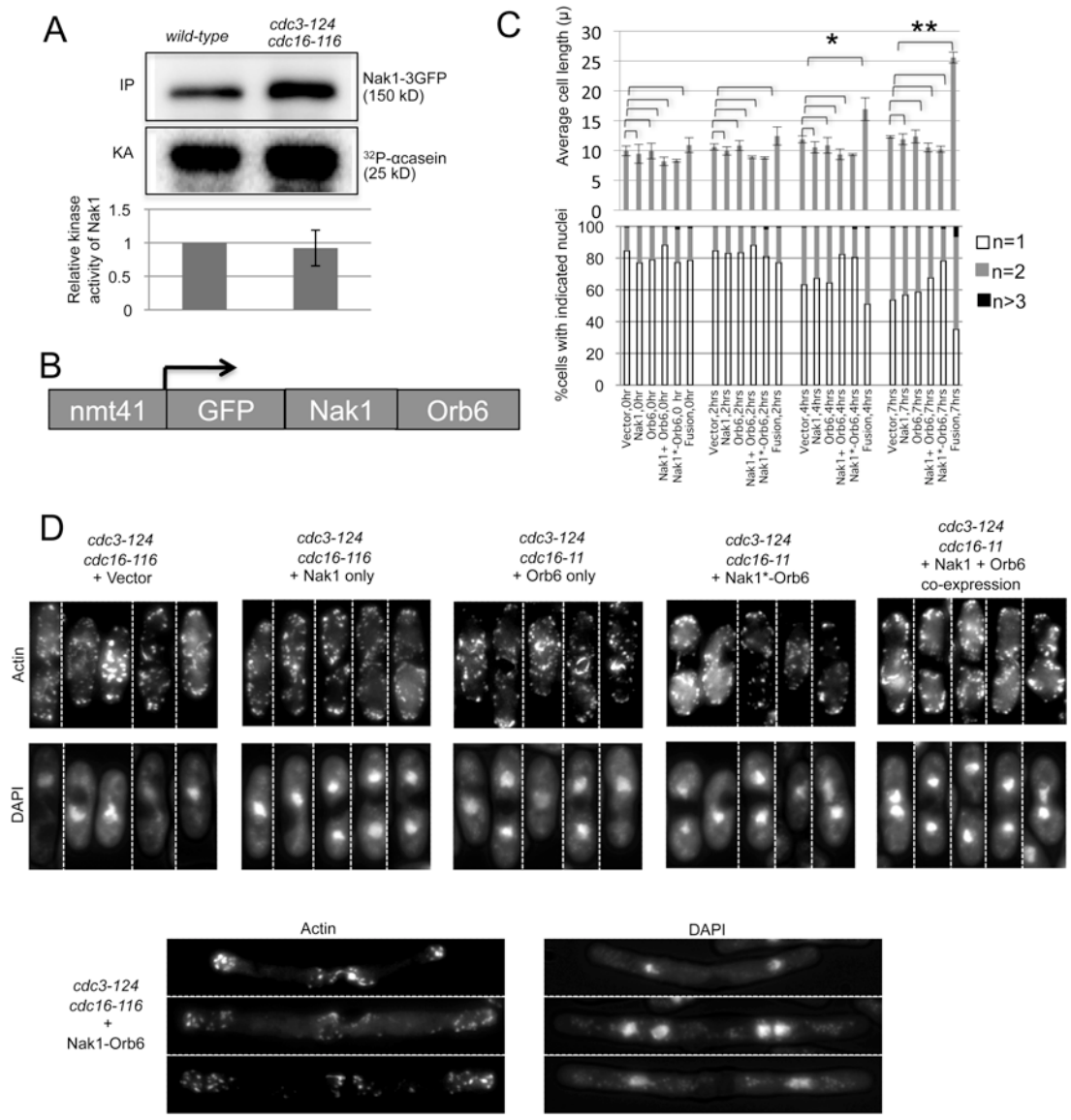


Figure 2-4:Nak1-Orb6 fusion bypasses the SIN inhibition of growth

Figure 2-5. *cdc3-124 cdc16-116* cells expressing the indicated transgenes were scored for nuclear count as described for Figure 2-4C. This figure shows the same data as in Figure 2-4C, but includes the error bars, which had been left out for clarity. Error bars show the SD for three separate experiments.

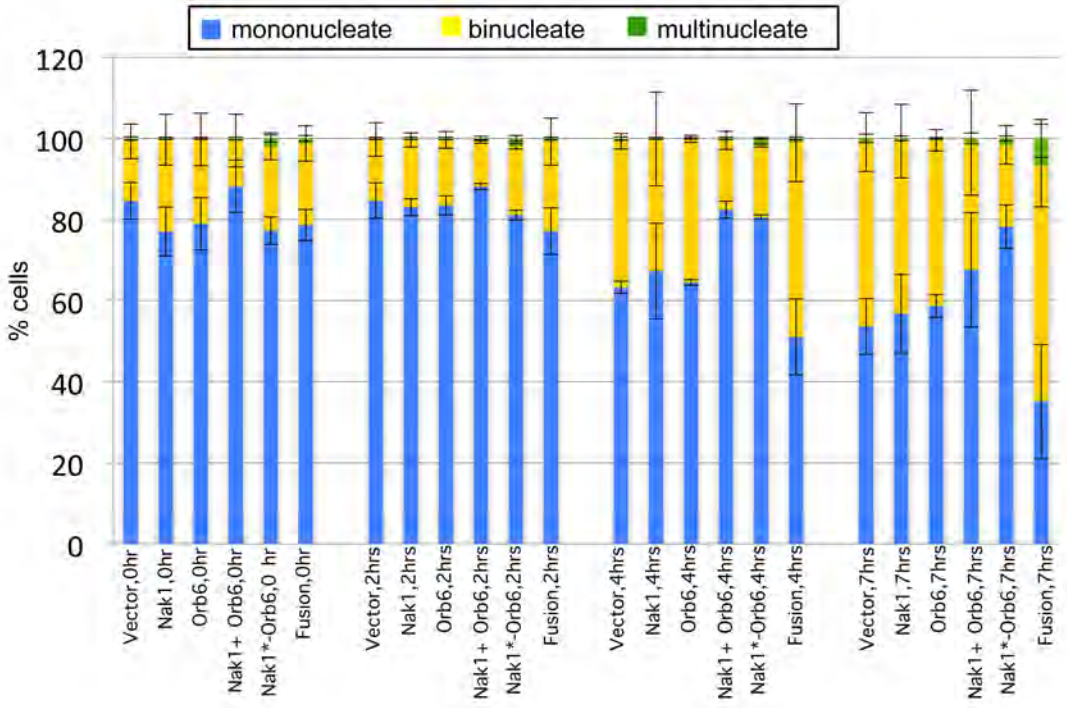


Figure 2-5: Quantification of the rescue by the Nak1-Orb6 fusion

Failure to inhibit MOR pathway signaling interferes with cytokinesis

The previous results showed that the SIN blocks polarized growth during cytokinesis by inhibiting the MOR pathway. We next wanted to address why the SIN inhibits the MOR. One possibility is that the SIN might inhibit the MOR to keep it from interfering with cytokinesis by titrating shared cytoskeletal elements such as actin away from the cell division site and towards the cell tips. To test whether loss of SIN inhibition of the MOR caused defects in cytokinesis, we examined the effects of expressing the Nak1-Orb6 fusion in different *S. pombe* strains. The fusion protein caused a very slight increase in cell length (Figure 2-6), and surprisingly, expression of the Nak1-Orb6 fusion had only mild effects on cytokinesis in wild-type cells (not shown), perhaps because expression of the fusion does not totally bypass SIN inhibition. To see if expression of the Nak1-Orb6 fusion might interfere with cytokinesis in a more sensitized background, we expressed the fusion in cells with compromised SIN signaling. Interestingly, expression of the fusion was lethal when expressed in the temperature sensitive SIN mutant *sid2-250* at the semi-permissive temperature of 29°C (Figure 2-7A). This result suggested that MOR inhibition becomes essential when cytokinesis is partially compromised. To further test this hypothesis we used an alternative way to interfere with the cell division machinery. Low doses of the actin-depolymerizing drug, Latrunculin B (Lat B) causes a cell division delay in wild-type cells (Trautmann and McCollum, 2005). During the delay the SIN remains

active causing an arrest in polarized growth and nuclear division until cytokinesis is complete (Mishra et al., 2004). We found that expression of the Nak1-Orb6 fusion in wild-type cells treated with low doses of Lat B is lethal (Figure 2-7B). Examination of similarly treated wild-type cells in liquid culture showed that wild-type cells with the vector control initially accumulate binucleate cells because of the delay in cytokinesis, but are eventually able to divide as judged by their ability to maintain a population of mononucleate cells and failure to accumulate multinucleate cells (Figure 2-7C,D). In contrast, wild-type cells expressing the fusion protein are unable to complete cytokinesis as seen by the loss of mononucleate cells and the accumulation of multinucleate cells (Figure 2-7C,D). This phenotype could be caused by loss of SIN signaling, which also causes cells to fail cytokinesis and become multinucleate when cytokinesis is delayed (Mishra et al., 2004), or the Nak1-Orb6 fusion could be interfering with the cytokinetic apparatus keeping cells from completing cytokinesis. We do not think that expression of the fusion protein is interfering with SIN signaling, since the cells expressing the fusion maintained similar levels of SIN activity as those with the control vector as judged by the presence of the SIN kinase Cdc7 at the SPB.

We also used another approach to test whether the Nak1-Orb6 fusion was affecting SIN signaling. We completely blocked cytokinesis using the *cdc3-124* profilin mutant, which cannot form actomyosin rings. After shift to restrictive temperature, these cells undergo a prolonged SIN-dependent arrest as binucleates but eventually leak past the nuclear division arrest and become

multinucleate (Trautmann et al., 2001). Expression of the Nak1-Orb6 fusion in these cells did not significantly interfere with the ability of these cells to arrest as binucleates suggesting that it was not affecting SIN signaling (Figure 2-7E), which is required to maintain the nuclear division arrest. Furthermore, examination of the kinase activity of the most downstream component of the SIN pathway, Sid2, did not show a significant change when the Nak1-Orb6 fusion was expressed (Figure 2-7F). Along similar lines, we did not observe any evidence for persistent SIN signaling when the MOR was inhibited in *orb6-25* mutants (Figure 2-8). Together these results suggest that the MOR pathway may interfere with actomyosin ring assembly and constriction downstream of the SIN, and that MOR inhibition is essential when the cell division machinery is compromised and cytokinesis is delayed.

Figure 2-6. Wild-type cells expressing either the empty vector or the Nak1-Orb6 fusion were grown in media lacking thiamine for 30 hours and samples collected at the indicated times for cell length measurements (0 hr denotes the start point). The statistical significance of cell length difference between vector containing and fusion containing cells was analyzed using t-test analysis; the obtained p-values with their corresponding significance are shown. *p-value=0.0051(very significant), #p-value=0.1306(not significant), **p-value=0.0001(extremely significant), ***p-value=0.0001(extremely significant), ****p-value=0.0001(extremely significant).

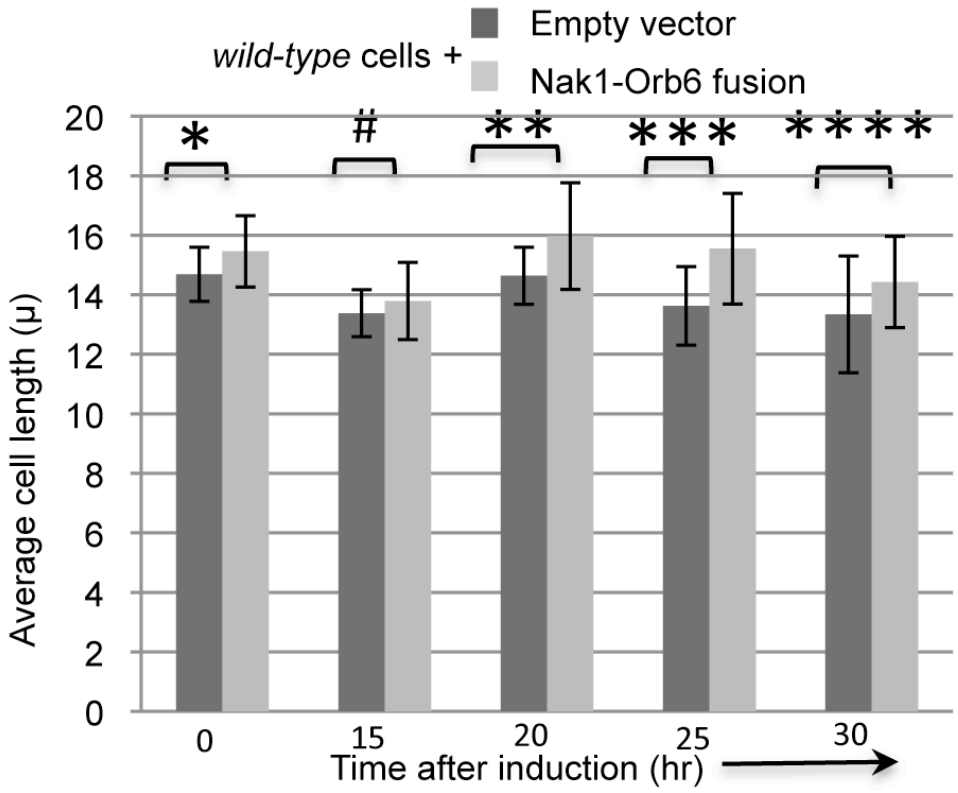


Figure 2-6: The Nak1-Orb6 fusion had mild effects in wild-type cells.

Figure 2-7. MOR inhibition becomes essential when cytokinesis is perturbed. (A) *sid2-250* cells expressing the vector alone or the Nak1-Orb6 fusion transgene were grown in medium lacking thiamine for 14 hours at 25°C to induce expression of the fusion protein before spotting 10-fold serial dilutions on minimal media plates lacking thiamine at the indicated temperatures. (B) Wild-type cells carrying the indicated plasmids were grown as in (A) and then 10-fold serial dilutions spotted on minimal media plates with or without 6µM Lat B and tested for growth at 25°C. (C) Wild-type cells carrying integrated copies of Cdc7-mcherry were transformed with the indicated plasmids, grown as in (A) for 19 hours before being treated with low dose (4µM) of Lat B. Samples were collected every 2 hours for 8 hours following drug addition (0 hr). Cells were fixed, stained with DAPI, and scored for number of nuclei, n (upper panel) and Cdc7 localization at the SPB (lower panel). At least 100 cells were analyzed for nuclei count and at least 50 for scoring Cdc7-mcherry localization. Error bars denote SD for three separate experiments. (D) Representative images of the Cdc7-mcherry cells expressing vector alone or the Nak1-Orb6 fusion at the 7hour time point are shown. (E) *cdc3-124 sid2-13Myc* cells expressing either the empty vector control or the fusion protein were grown in media lacking thiamine for 19 hours at 25°C then shifted to 36°C . Samples were collected for DAPI stain and nuclei count every hour for 8 hours following shift to 36°C. Error bars denote SD for three separate experiments. (F) Cells with the indicated genotypes were grown as in (E) and shifted to 36°C for three hours. Cell extracts were prepared

and Sid2-13Myc was immuno-precipitated with anti-Myc (Santa Cruz) antibody. The immunoprecipitates were split, with one portion used for western blotting using Myc antibodies (Santa-Cruz) (IP), and the other portion used for in vitro kinase assays using myelin basic protein, MBP (Sigma) as an artificial substrate as described previously and in Material and Methods (Leonhard and Nurse, 2005; Sparks et al., 1999). The kinase activity (KA) was measured using a Phosphor imager (Molecular Dynamics), quantified using the ImageQuant software, and normalized to the amount of Sid2 (IP). The activity relative to cells with the control plasmid is shown. Error bars denote SD of the relative KA. The difference in Sid2 kinase activity between cells expressing the vector and those expressing the fusion protein was not statistically significant based on t-test analysis from 3 different experiments, *p-value=0.1047.

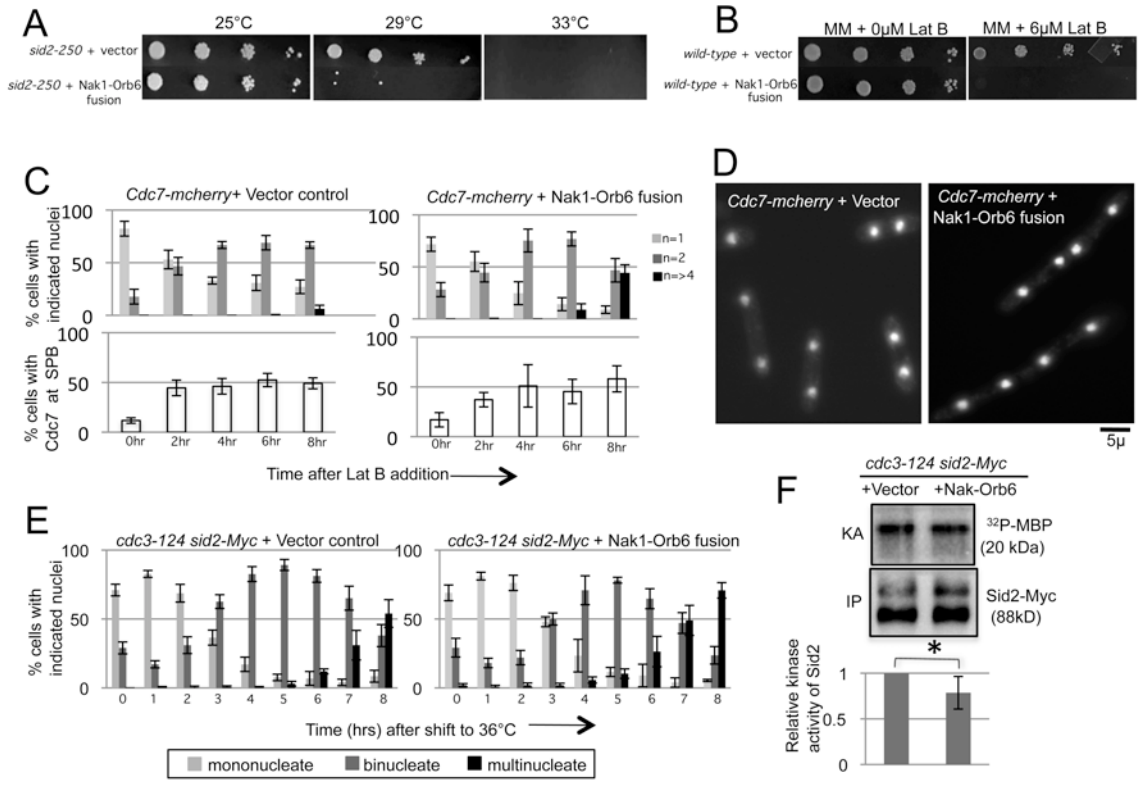


Figure 2-7: MOR inhibition becomes essential when cytokinesis is perturbed

Figure 2-8. MOR pathway mutant *orb6-25* does not promote persistent SIN signaling. The indicated strains were shifted to 36°C for 3 hours, methanol fixed and stained with DAPI and Calcofluor white (CW) for nuclei and septum staining respectively. Cdc7-GFP and Sid2-GFP, which localize to the SPB and division site respectively, during septum formation but not after septum completion in wild-type cells are used to monitor the status of the SIN signaling pathway. Arrowheads in montage images indicate complete septum while arrows mark the partial septum in the DAPI/CW panel and the Cdc7 and Sid2 signals in these cells in the GFP panel. The percentage of cells with complete septa that lost Cdc7 and Sid2 from the SPB and the cell division site respectively are indicated in the histogram plots for the wild-type and the *orb6-25* mutant backgrounds. The individual images in the montage presentations are separated by dashed dividing lines. The experiment was repeated once with similar results.

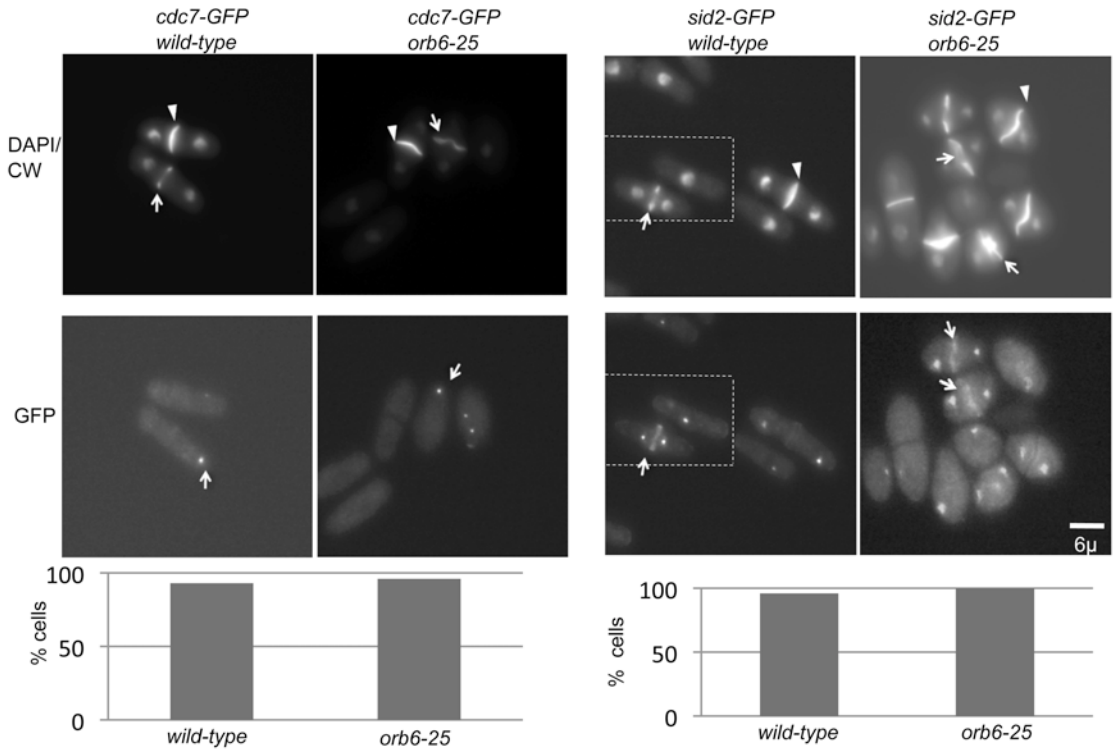


Figure 2-8:Orb6 inactivation does not promote persistent SIN signaling

Reduction in MOR activity allows weak SIN signaling to promote cytokinesis

If the MOR pathway interferes with the ability of the SIN to promote cytokinesis, then reduction in MOR pathway activity might be predicted to enhance cytokinesis in SIN mutants. Therefore we tested the phenotype of double mutants between the MOR mutant *orb6-25* and various SIN mutants. Interestingly the *orb6-25* temperature sensitive mutant was able to rescue the growth defect of some temperature sensitive SIN mutants at 29°C and 33°C, which are semi-permissive temperatures for *orb6-25* (Figure 2-9A). Although the *orb6-25* mutant dies at 36°C and was thus not able to rescue SIN mutants at this temperature, examination of double mutants between *orb6-25* and various SIN mutants at 36°C showed that the *orb6-25* mutation was able to partially rescue the septum formation defect in all SIN mutants tested (Figure 2-9B). We also examined this phenotype using *sid2-250, orb6-25,* and *sid2-250 orb6-25* cells that had been synchronized in G2 and then shifted to restrictive temperature (Figure 2-10). Consistent with results from asynchronous cells, the *sid2-250 orb6-25* cells showed a substantial increase in septation compared to the *sid2-250* single mutant cells, which totally failed in septation. Although MOR inactivation allowed septation in the *sid2-250 orb6-25* cells, absence of a completely functional SIN pathway could explain the reduced septation index in these cells relative to *orb6-25* alone. Inactivation of *orb6* was unable to promote septum formation in SIN double mutant cells with mutations in 2 different SIN

components (*sid2-250 sid1-239* and *sid2-250 cdc11-123*), which presumably completely ablates SIN signaling (Figure 2-9B), suggesting that inactivation of *orb6* does not bypass the requirement of the SIN in septum formation, but instead allows residual weak SIN signaling to promote cytokinesis.

To examine how loss of MOR activity rescued SIN mutants in more detail, we observed the dynamics of actomyosin ring assembly and septum formation in *orb6-25 sid2-250* double mutant cells. Previous studies have shown that SIN mutants form actomyosin rings that fall apart in anaphase and the cells do not form septa (Krapp and Simanis, 2008). Therefore, we examined actomyosin ring constriction and septum formation in the *sid2-250 orb6-25* double mutant cells expressing the GFP-tagged actomyosin ring component Rlc1 (Le Goff et al., 2000; Naqvi et al., 2000) at the restrictive temperature of 36°C using time-lapse microscopy. As expected, in wild-type cells, actomyosin rings formed then constricted (15/15 cells) (Figure 2-9C). By contrast, actomyosin rings formed in *sid2-250* single mutant cells, but failed to constrict and then disassembled in 16 out of 21 cells observed (Figure 2-9C). Unlike *sid2-250* mutant cells, but similar to wild-type cells, actomyosin rings formed and constricted in *sid2-250 orb6-25* double mutants (21/21 cells) (Figure 2-9C). Thus, loss of Orb6 activity allows SIN mutants to maintain actomyosin ring stability and complete cytokinesis. Thus loss of MOR activity allows weak SIN signaling to promote actomyosin ring constriction and septum formation.

Interestingly MOR inactivation also enhanced the ability of weak SIN signaling to promote ectopic septation. The Spg1-GFP allele has a weakly activated SIN phenotype, which causes occasional formation of interphase septa, or additional rounds of septum formation following normal cytokinesis (Garcia-Cortes and McCollum, 2009). When *spg1-GFP* was combined with *orb6-25*, or any other MOR mutant, the resulting double mutant cells showed an increased rate of ectopic septum formation (Figure 2-9D). Together these results show that reduction in MOR pathway activity enhances the ability of weak SIN signaling to promote cytokinesis.

Figure 2-9. The MOR pathway mutant *orb6-25* rescues growth and cell division defects of SIN mutants. (A) Cells of the indicated genotypes were grown at 25°C and their growth was tested at the indicated temperatures by spotting 10-fold serial dilutions on YE agar plates. (B) Cells of the indicated genotypes were shifted to 36°C for 4 hours, methanol fixed and stained with Calcofluor White (CW) to score for septum formation. Representative *cdc7-24* (montage) and *cdc7-24 orb6-25* cells show the septation status in respective cells and arrows mark the septum. Arrows mark the septum in the *cdc7-24 orb6-25* cells. The septation index (percentage of cells with septa) for all the genotypes indicated is shown in the histogram plot. At least 100 cells were scored for each strain. Error bars denote the SD for three separate experiments. (C) Time-lapse images of the indicated strains were acquired using a spinning disc confocal microscope (NikonTE 2000-E2). Cells were grown at 25°C, then placed on the microscope stage that was maintained at 36°C during the entire time of imaging. Arrowheads indicate completion of ring constriction in representative cells. (D) Cells of the indicated genotypes were grown as in (B) and the proportion of cells with ectopic septa (either septa present in mononucleate cells, or multiple septa) was scored. At least 100 cells were scored for each genotype. The experiment was done in triplicate and error bars denote the SD values. Arrows in the montage image point to the ectopic septa found in representative cells. Dashed dividing lines separate the individual images in the montage presentations, wherever applicable.

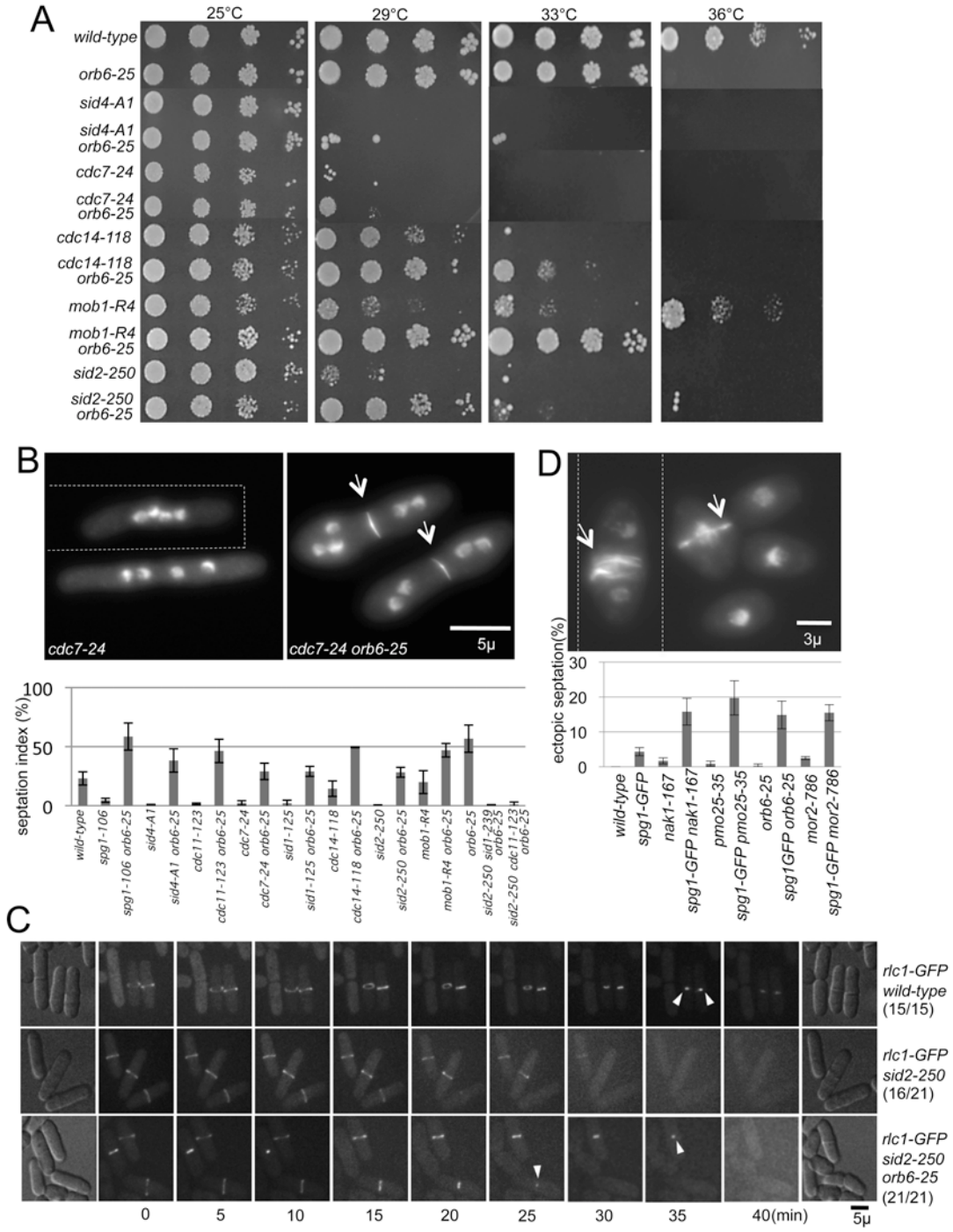


Figure 2-9: Orb6 inactivation rescues cytokinesis defects of SIN mutants

Figure 2-10. The septation index of cells of the indicated genotypes after G2 synchronization and release is shown. Cells of the indicated genotypes were synchronized using the lactose gradient method as described previously (Forsburg and Rhind, 2006). Following synchronization, cells were released into fresh media and shifted to 36°C. Samples were collected at the indicated times and scored for septation index. This experiment was repeated once with similar results.

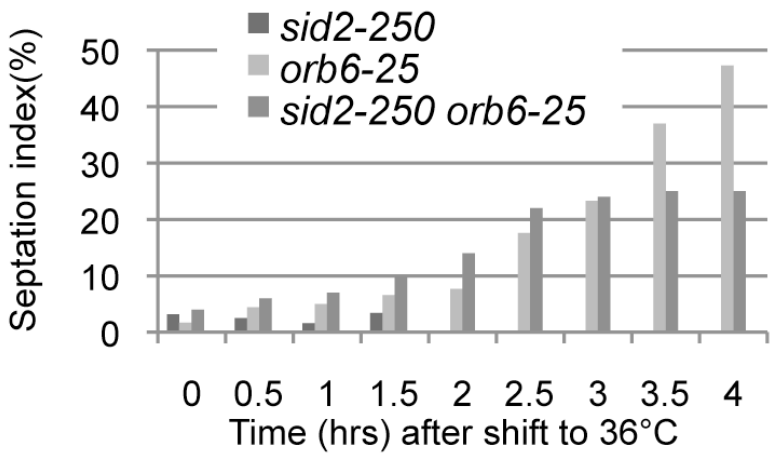


Figure 2-10: Septation rescue upon Orb6 inactivation in G2 synchronized cells

Discussion

The SIN antagonizes interphase polarity through inhibition of the MOR pathway

The SIN is required for actomyosin ring assembly and septum synthesis (Balasubramanian et al., 1998; Hachet and Simanis, 2008; Huang et al., 2008; Simanis, 2003). Previous studies have shown that when perturbation of the actomyosin ring causes a delay in cytokinesis, SIN activity is maintained during the delay and is required for cells to continue to promote actomyosin ring assembly, block polarized growth, and arrest cells with two G2 phase nuclei in order to allow cells to complete cell division and maintain normal ploidy (Liu et al., 2000; Mishra et al., 2004; Trautmann et al., 2001). However, it was unclear how the effects of the SIN on cell polarity and nuclear division are mediated. Although part of the SIN inhibition of interphase polarity might be through competition for shared components, we demonstrated that SIN signaling clearly interferes with interphase polarity through inhibition of the MOR pathway. Interestingly, the ability of the SIN to inhibit the G2/M transition might also be a consequence of inhibition of the MOR pathway, since MOR pathway mutants, like cells with activated SIN, display a Wee1-dependent block in G2/M progression (Hirata et al., 2002; Kanai et al., 2005). Consistent with this idea, we found that blocking complete inhibition of the MOR by the SIN using the Nak1-Orb6 fusion could partially bypass the SIN-dependent block in nuclear division. The failure of the Nak1-Orb6 fusion to completely bypass the SIN inhibition of

polarized growth and nuclear division could be because the SIN is still capable of partially inhibiting the MOR when the Nak1-Orb6 fusion is expressed.

Cross talk between the SIN and MOR pathways

The molecular details governing regulation of MOR/Orb6 activity by the SIN are likely to be complex. Orb6 kinase activity normally peaks at about the time when cells initiate bipolar growth in early G2 phase, and then decreases during mitosis (Kanai et al., 2005). We suspect that the decrease of Orb6 activity during mitosis may be biphasic. The initial reduction in Orb6 kinase activity at mitotic entry appears to be independent of the SIN, since the SIN is not active in early mitosis (Guertin et al., 2000), and because Orb6 activity still dropped at mitotic entry in the SIN mutant *cdc7-24* (This study and (Kanai et al., 2005). The initial drop in Orb6 activity may be triggered directly or indirectly by Cdk1 activation and mitotic entry, and then when Cdk1 activity drops in anaphase, the SIN becomes active and maintains inhibition of the MOR pathway until cytokinesis is complete. Intriguingly, some of the upstream SIN components are required for the increase in Orb6 activity that occurs in the following G2 phase when cells initiate growth at the new end (Kanai et al., 2005). The mechanistic details of how the SIN both activates and inhibits the MOR are as yet unclear. One explanation for these results could be that the SIN causes both inhibitory and activating modifications in the MOR pathway, with the inhibitory modifications being dominant. Removal of the inhibitory modifications following cytokinesis would then allow the MOR to

become active in interphase. To address this issue, future studies will need to focus on identification of SIN-dependent changes in the levels or post-translational modifications of MOR components.

However, even without knowing the specific details of how the SIN affects the MOR pathway, our present data indicate that the SIN blocks signaling through the MOR pathway by inhibiting activation of the most downstream component of the pathway, the NDR family kinase Orb6. Our results showed that the SIN does not affect the kinase activity of the Orb6-activating kinase Nak1, suggesting that the SIN interferes with the ability of Nak1 to activate Orb6. Previous reports have suggested that the protein Mor2, which is required for Orb6 kinase activity, functions as a scaffold to bring together Orb6 with its activator, the GC-family kinase Nak1 (Kanai et al., 2005). Consistent with this idea, we found that fusion of Nak1 to Orb6 could rescue the *mor2-786* mutant. Interestingly, the SIN inhibition of polarized growth was also bypassed by expression of the Nak1-Orb6 fusion construct, suggesting that the SIN could inhibit Mor2 function, or the ability of Nak1 or Orb6 to associate with Mor2. A more detailed understanding of the molecular mechanism by which Orb6 is activated by Nak1 will be essential to uncover how the SIN interferes with the activation of Orb6 and the MOR pathway.

The MOR inhibits SIN-mediated actomyosin ring constriction and septum formation

So what is the purpose of the antagonistic relationship between the SIN and MOR pathways? Because each signaling pathway directs the actin cytoskeleton towards distinct processes, the mutual antagonism between them would keep each pathway from interfering with the functions of the other. The antagonism between the two pathways could be due to each pathway inhibiting signaling through the other, or by interfering with downstream functions such as actin organization. From our experiments, the SIN clearly inhibits signaling through the MOR pathway, although additional interference could come from competition over actin cytoskeletal components. In contrast we found no evidence that the MOR pathway inhibited SIN signaling, but instead, the MOR appears to interfere with cytokinesis downstream of the SIN, most likely through competition for shared components. The antagonism between the two pathways may both enhance the efficiency of each pathway by removing a competitor, and ensure that cytoskeletal rearrangements occur at the correct point in the cell cycle. For example, the ability of the MOR to interfere with SIN functions might be important to keep weak or leaky SIN signaling from triggering multiple rounds of cytokinesis. Consistent with this, we observe that inactivation of Orb6 allows weak interphase SIN signaling caused by the *spg1-GFP* allele to trigger ectopic septum formation. Overall our study identifies an antagonistic interaction between the two NDR pathways, the SIN and MOR, which is crucial for the ability

to maintain the quite different cytoskeletal arrangements present during cytokinesis and interphase (Figure 2-11).

Animal cells also undergo redistribution of cytoskeletal elements as they transition between interphase and mitosis. Interestingly, animals also have two pathways containing NDR kinases analogous to the SIN and MOR pathways called the Hippo/Lats and Ndr1/2 pathways respectively. Although the functions of these pathways in animal cells appear complex, they have been reported to function in mitotic exit/cytokinesis and polarized growth/morphogenesis as in fission yeast (Bothos et al., 2005; Chan et al., 2005; Guo et al., 2007; Hergovich et al., 2005; Hergovich et al., 2008; Preisinger et al., 2004; ten Klooster et al., 2009). Additionally, evidence for cross talk between the homologous NDR pathways has been observed both in *Drosophila melanogaster* (Emoto et al., 2006) and in mammalian cells (Vichalkovski et al., 2008). Therefore it is tempting to speculate that the cytoskeletal rearrangements that take place between anaphase/telophase and interphase might be regulated similarly in animal cells. Besides their role in polarized growth and mitotic exit, the animal homologs of the SIN and MOR pathway components are also involved in processes regulating cell proliferation and cell death. The homologs of the SIN kinase Sid2 in mammals, called Lats1/2, function as tumor suppressors (Hao et al., 2008; Hergovich et al., 2006; Seidel et al., 2007; Takahashi et al., 2005; Zhang et al., 2008). The role of Ndr1/2 in human diseases such as cancer is just beginning to be revealed (Hergovich et al., 2006) (Hergovich et al., 2008). Thus, a detailed

understanding of crosstalk between NDR pathways will likely have important implications for our understanding of how cells regulate both growth and proliferation as well as the cytoskeletal rearrangements that occur during the transitions between mitosis and interphase.

Figure 2-11. The SIN and the MOR pathways are involved in an antagonistic interaction. The SIN inhibits signaling through the MOR pathway to inhibit bipolar cell elongation during mitosis and the MOR inhibits the cytokinetic functions of the SIN.

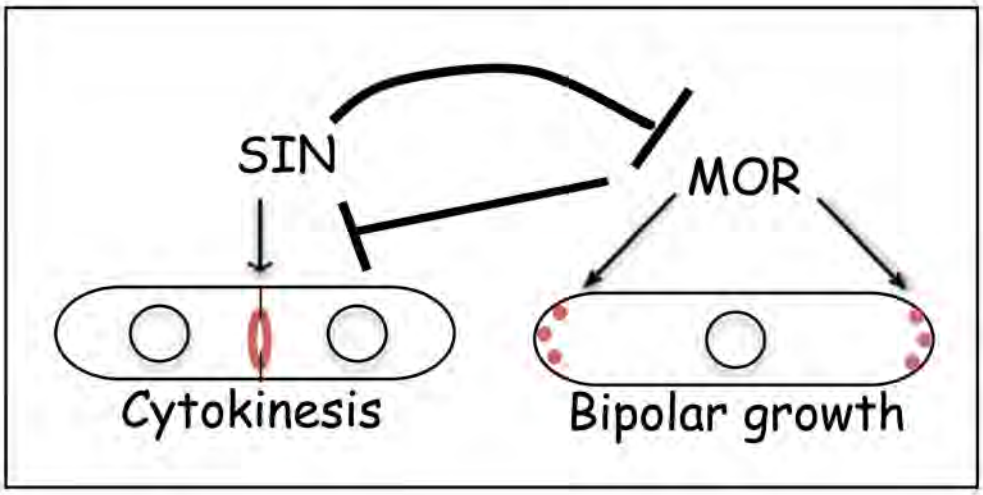


Figure 2-11: Antagonistic interaction between the SIN and MOR pathways

CHAPTER III

Exploring the SIN-MOR cross-talk at the spindle pole bodies

Summary

The MOR pathway is essential for initiation and maintenance of polarized growth in the fission yeast life cycle. It has been shown that, consistent with their role in bipolar cell elongation all the MOR components are localized at the cell ends in interphase. However, two of the upstream components, Nak1 and Pmo25, are localized to the SPB during mitosis. Further, the SPB localization of Pmo25 was shown to be dependent on the upstream SIN components Sid1 and Cdc7 (Kanai et al., 2005; Leonhard and Nurse, 2005; Mendoza et al., 2005). However, little was known about the regulation of Nak1 localization during mitosis. We examined the roles of SIN signaling in the mitosis specific localization of Pmo25 and Nak1. Our results showed that Nak1 and Pmo25 had distinct localization patterns during mitosis which were partially dependent on the SIN. However the significance of the differential localization pattern of the upstream MOR components remains unclear. We further found that constitutive association of Pmo25 with the SPB structural protein Ppc89 led to ectopic activation of the SIN. We expect these results will be important for future studies to tease out the mechanism by which

Pmo25 gets recruited to the SPB and to understand the significance of its anaphase specific localization.

Results and Discussion

SIN signaling causes spatial separation of Nak1 and Pmo25 during cytokinesis

To further examine the mitosis specific localization of upstream MOR components, we decided to follow the localization of Nak1 and Pmo25 through cell cycle using time-lapse movies. Nak1 was recruited to the SPB in early mitosis, consistent with a previous report (Leonhard and Nurse, 2005) but it disappeared in anaphase B around the time when the SIN gets activated (Guertin et al., 2000; Krapp et al., 2004) (Figure 3-1A,D). Interestingly, Pmo25 was recruited to the SPB in anaphase around the time when Nak1 disappeared (Figure 3-1A compare top and middle panels). Earlier studies have reported that Pmo25 recruitment to the SPB requires the upstream SIN components Cdc7 and Sid1 (Kanai et al., 2005; Mendoza et al., 2005). Thus to test whether SIN activation in anaphase cause simultaneous recruitment of Pmo25 and displacement of Nak1 from the SPBs, we followed Nak1 localization in different SIN mutants. Time-lapse analysis in SIN mutants was difficult because at the restrictive temperature for the SIN mutants (36°C) the Nak1-3GFP signal was faint and faded rapidly. Therefore, we analyzed Nak1-3GFP in *sid2-250* mutants

at 25°C, a temperature where the Sid2 mutant protein was known to have reduced activity (Chen et al., 2008; Mishra et al., 2005). Our time-lapse analysis of Nak1-3GFP in *sid2-250* cells showed that Nak1 persisted longer at the SPBs in anaphase B (Figure 3-1A). Similar results were obtained when we examined fixed *spg1-106*, *cdc7-24*, *sid1-125*, and *sid2-250* cells after incubation at the restrictive temperature of 36°C. The percentage of anaphase B cells (as judged by DAPI staining) that had the Nak1 signal at the SPBs and the average separation of the chromatin in these cells and wild-type was quantified (Figure 3-1C,D). We found that Nak1 was present at the SPBs in a considerably higher percent of anaphase B cells in SIN mutants compared to wild-type cells (Figure 3-1C). In addition, the SIN mutant cells maintained Nak1 at the SPBs longer in anaphase B compared to wild-type cells as judged by the average distance between the chromosomes in anaphase B cells with Nak1 at the SPBs (Figure 3-1D). Nak1 was typically observed at both SPBs in anaphase B wild-type and SIN mutants, with the exception of *sid1-125* cells, where Nak1 was often observed at a single SPB. Nak1 was not observed at the SPB in the SIN scaffold protein mutants *cdc11-119* and *sid4-A1* (data not shown). Together these and previous data (Kanai et al., 2005) suggest that although the SIN scaffold proteins Sid4 and Cdc11 are required for the SPB recruitment of Nak1 in early mitosis, SIN activation in anaphase displaces Nak1 from the SPB and causes SPB recruitment of Pmo25. Furthermore, consistent with previous reports (Kanai et al., 2005), ectopic activation of the SIN caused inappropriate recruitment of

Pmo25 to the SPB in interphase (Figure 3-1E). However, ectopic activation of the SIN failed to recruit Nak1 to the interphase SPB (Figure 3-1E). Thus SIN activation appears to cause simultaneous removal of Nak1 and recruitment of Pmo25 to the SPB. The net result is that the two upstream activators of the MOR are kept apart. Since Pmo25 is necessary for Nak1 activation, these results could provide a mechanism for how the SIN inhibits the MOR pathway.

Figure 3-1. The SIN inhibits co-localization of Nak1 and Pmo25. (A) Time-lapse images of the indicated strains were collected during mitosis using a spinning disc confocal microscope (Zeiss Axiovert 200) at 25°C. Ten stacks of images were captured for each time point, with a step size of 0.4μm. All the 22 Pmo25-4GFP and all 17 Nak1-3GFP wild-type cells observed showed similar pattern of Pmo25 and Nak1 localization respectively during mitosis, and 11 out of 11 Nak1-3GFP *sid2-250* cells observed maintained Nak1 at the SPBs in late anaphase B. (B) SIN mutants maintain Nak1 at the anaphase B SPBs longer than the wild-type cells. Wild-type and *sid2-250* cells expressing Nak1-3GFP were grown at 25°C, shifted to the semi-permissive temperature of 33°C for 2 hrs, methanol fixed and stained with DAPI. A montage of representative cells at different stages is shown. The Nak1-3GFP signal at the SPB is indicated by arrow-heads. (C) The indicated strains expressing Nak1-3GFP were treated as in (B) and the proportion of the anaphase B cells showing Nak1 at the SPB(s) was determined. Anaphase B cells were scored by DAPI staining (n> 50 in each case). (D) A comparison of the average nuclear separation in anaphase B cells showing Nak1 at the SPBs in the wild-type and the SIN mutant backgrounds. The average nuclear separation values were compared between wild-type and *spg1-106* (single asterisk,p=0.0003), *cdc7-24* (double asterisk, p<0.0001), *sid1- 125* (triple asterisk, p<0.0001) and *sid2-250* (quadruple asterisk, p<0.0001) by a *t*-test. At

least 50 anaphase B cells were examined in each case. Nuclear separation was measured as the distance between the distal edge of each DNA mass. (E) Ectopic SIN activation recruits Pmo25 but not Nak1 to the interphase SPB. Cells of the indicated genotypes were shifted to the semi-permissive temperature of 33°C following which GFP fluorescence was captured. Arrowheads denote SPB-localized Pmo25 in interphase cells.

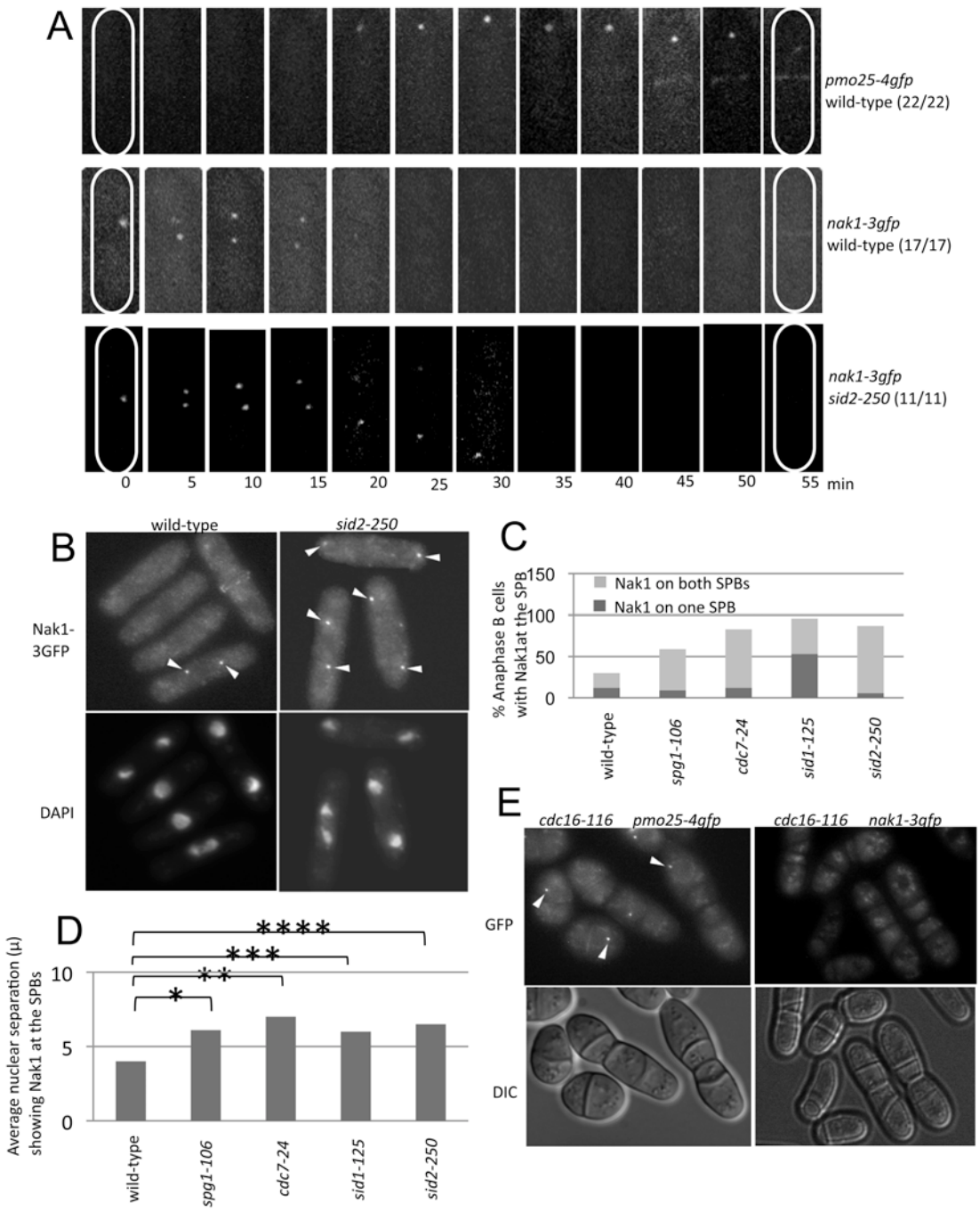


Figure 3-1: The SIN inhibits co-localization of Nak1 and Pmo25

To test this possibility and also explore the significance of the spatial separation between Nak1 and Pmo25 in mitosis, we made a fusion between the two MOR components and expressed the fusion protein under an inducible promoter so that we could regulate the level of its expression. Figure 3-2A shows a schematic of the fusion. The fusion was functional since it rescued the respective single mutants *nak1-167* and *pmo25-35* at the restrictive temperatures. In addition, at low levels of expression the fusion localized at the cell ends in interphase, to the SPB in metaphase and then to only one SPB in anaphase. Finally it was at the cell division site during cytokinesis.Thus the fusion between Pmo25 and Nak1 maintained the localization of both the individual proteins during the cell cycle (based on visual inspection).

We then tested if expression of the fusion could bypass the SIN inhibition of bipolar cell elongation. As mentioned in Chapter II, *cdc3-124 cdc16-116* cells have a constitutively active SIN at the restrictive conditions due to the *cdc16* mutation, but did not form ectopic septa due to the *cdc3* (profilin) mutation. To test if the fusion between Pmo25 and Nak1 could bypass the block in cell elongation in cells with activated SIN signaling, we measured the cell length of *cdc3-124 cdc16-116* cells at restrictive conditions upon expression of the fusion and compared that to those expressing the vector alone. We did not observe a significant increase in cell length upon expression of the fusion from the medium strength promoter (pREP41) (Figure 3-2B). However, expression of the fusion from a stronger strength promoter (pREP1) showed a statistically significant

amelioration of the cell elongtion defect by the fusion in the *cdc3-124 cdc16-116* cells (Figure 3-2C). To test if the separation of Nak1 from Pmo25 was important to maintain SIN signaling in anaphase, we tested if overexpression of the fusion could inhibit SIN functions and cause cytokinesis defects. Wild-type cells expressing the fusion had a longer morphology than those with vector alone but presence of the fusion did not cause any cytokinesis defects (based on eye estimation). One possibility is that the SIN inhibition is not efficiently bypassed by the fusion protein and the normal SIN activity in these cells allowed them to continue with cytokinesis and cell division. We then tested if the fusion was deleterious in cells with slightly compromised SIN activity. Using the SIN mutant *sid2-250* at the semi-permissive temperature of 30°C we observed a considerable increase in the percentage of cell lysis and cell division defects over prolonged expression of the fusion (Figure 3-2F). Thus overexpression of the fusion causes cytokinesis defects when cytokiness is partially compromised. To further test this hypothesis we used an alternative way to compromise the cell division machinery. Low doses of the actin-depolymerizing drug, Latrunculin B (Lat B) causes a cell division delay in wild-type cells (Trautmann and McCollum, 2005). During the delay the SIN remains active causing an arrest in polarized growth until cytokinesis is complete (Mishra et al., 2004). Consistent with the previous report, Lat B treated cells expressing vector alone arrested cell elongation following drug addition Figure 3-2D. However in sharp contrast, this arrest in cell elongation was bypassed in similarly treated cells upon expression

of the fusion. However in our experiments, expression of the individial genes by themselves also had an effect on cell length, suggesting that the effect of the fusion was only mildly stronger than that of the individual genes (Figure 3-2E). In conclusion, these results suggested that fusion between Pmo25 and Nak1 can partially bypass the SIN mediated growth arrest and inhibit other SIN functions particularly when cytokinesis is compromised.

However, the ability of the Pmo25-Nak1 fusion to bypass SIN inhibition was not as strong as that observed with the Nak1-Orb6 fusion (mentioned in Chapter II). One reason for this difference could reflect the degree of activation of the MOR pathway, which is much higher when the downtream kinase Orb6 is in direct association with its upstream activator Nak1. Hence there is only a partial rescue of the growth inhibition with the Pmo25-Nak1 fusion. Alternatively, the SIN targets could be further downstream of Pmo25 and Nak1, or, as suggested by our results in Chapter II, it could be interfering with the other regulator of Orb6 activity, which is the scaffold protein Mor2. However, we cannot exclude the possibility that the transgene expression in cells failed to phenocopy the exact in vivo nature of their interaction and hence did not have a substantial effect on cell cycle. In future, it might be useful to add modifications such as addition of a linker region between Pmo25 and Nak1 and test if the fusion has a stronger effect in inhibiting SIN functions towards cytokinesis.

Figure 3-2. The Pmo25-Nak1 fusion partially rescued SIN inhibition of cell growth but showed strong inhibition of SIN functions in SIN compromised background. (A) Schematic representation of the Pmo25-Nak1 fusion construct expressed from the thiamine repressible promoter, nmt1. (B) *cdc3-124 cdc16-116* cells carrying either the PREP41 vector alone or the pREP41 vector with the Pmo25-Nak1 fusion (fusion) were induced for 19 hours in media lacking thiamine and shifted to 36°C for indicated times (hrs) before collecting and fixing cells for cell length measurements (μ). (C) *cdc3-124 cdc16-116* cells expressing the indicated plasmids were induced for 19 hours as in (B) above and shifted to 36°C for 6 hours before collecting and fixing cells for cell length measurements. The plasmids used were the pRep1 vector (vector), or the pRep1 vector carrying Pmo25, Nak1, or the Pmo25-Nak1 (fusion). Cells were then stained with DAPI and scored for cell length measurements. Error bars in the cell length plot denote SD of the length measurements obtained from at least 30 different cells. Average cell length was compared between cells with vector control and those with the different transgenes as indicated in the figure at each time-point. Statistically significant increase in cell length (based on p-value calculation by t-test analysis) was observed in cells expressing the Pmo25 construct (*= 0.0196 significant), the Nak1 construct (**=0.0067 very significant) and the Pmo25-Nak1 fusion (*** =< 0.0001 extremely significant). (D) wild-type cells expressing the vector alone or the Pmo25-Nak1 fusion were grown for 19 hours before addition of 4μM Lat B. Cells were collected every 2 hours and cell length measurements taken. Error

bars denote the SD of the length measurements obtained from at least 30 different cells in each case. Average cell length comparison between cells with vector alone and those with the fusion showed statistically significant increase in cell length upon expression of the fusion (*; ** = p< 0.0001;extremely significant). (E) wild-type cells expressing the different transgenes as indicated were treated as in (D) above and cell length measurements taken after 6 hours following drug addition. Average cell length measurements were compared between cells with the vector and those with the different transgenes as shown in the figure at start (t=0) and end (t=6hrs) of experiment. Statistically significant increase in cell length (based on p-value calculation by t-test analysis) was observed in cells expressing the Pmo25 construct (*= 0.0256 significant), the Nak1 construct (**= 0.0049 very significant) and the Pmo25-Nak1 fusion (*** =< 0.0001 extremely significant). Pmo25 expressing cells had a statistically significant longer length than that of the vector expressing control at the start of the experiment (#=0.0005 extremely significant). (F) *sid2-250* cells expressing either the vector alone or the different transgenes as shown in the figure were grown at the semi-permissive temperature of 30°C for indicated time-periods (hrs). Cells were scored for cell division defects like cell lysis, multinucleate and collapsed nuclei phenotype and the percentage of cells with the respective phenotype were plotted.

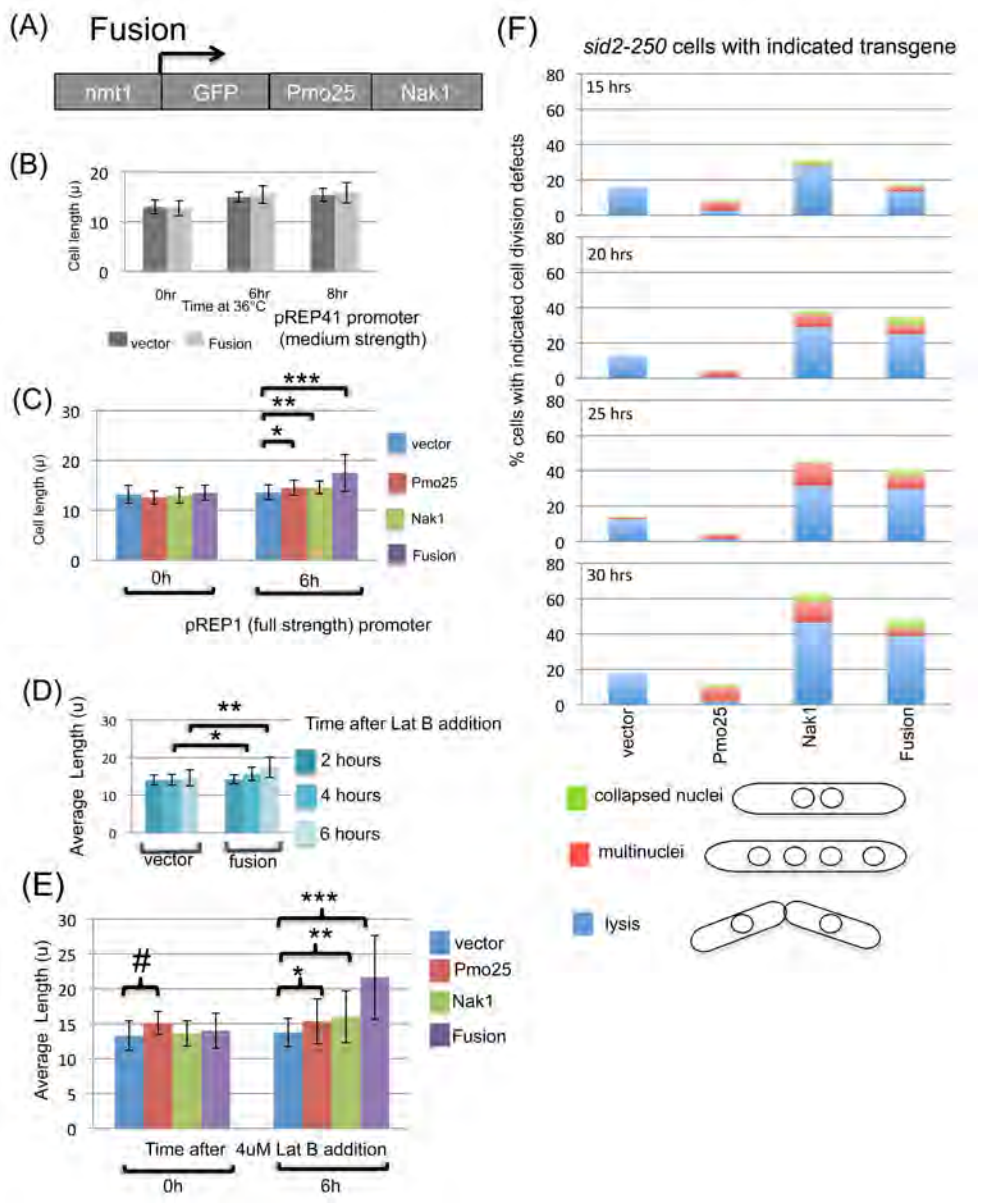


Figure 3-2: Effect of Pmo25-Nak1 fusion in normal and SIN compromized cells

SPB targeting of Pmo25 causes ectopic activation of the SIN

Besides removal of Nak1 from the SPB in anaphase, it remains mysterious why the two upstream SIN components, Sid1 and Cdc7, rescruits only the upstream MOR regulator Pmo25 to the SPB (Kanai et al., 2005; Mendoza et al., 2005)(Figure 3-1). In interphase, Pmo25 is in the cytoplasm and cell tips and is essential for MOR signaling through the kinases Nak1 and Orb6. Further, it has been shown to associate with Nak1 by immunoprecipitation experiments (Kanai et al., 2005). We hypothesized that the SIN mediated Pmo25 recruitment to the SPB could lead to its sequestration at the SPB. This could block MOR signaling by disrupting association of Pmo25 with its targets. FRAP (Fluorescence recovery after photobleaching) of Pmo25 at the SPB suggeted that the protein is highly dynamic at the SPB in normal as well as upon constitutive SIN activation (Balasubramaniam and Mishra, unpublished data). However, it remained possible that the population of Pmo25 at the SPB failed to associate with its downstream targets and hence failed to trigger MOR signaling. To test this possibility, we fused Pmo25 to the C-terminal portion of the SPB protein Ppc89, Figure 3-3A. Ppc89 has been shown to be essential for SIN activity and associates with Sid4 to act as a scaffold for the recruitment of the SIN components at the SPB (Rosenberg et al., 2006). Our results showed that Pmo25 at the SPB inhibited cell elongation resulting in round cells similar to MOR mutants. Further, the fusion did not rescue Pmo25 mutants and appeared

to be dominant negative for MOR since its expression in SIN mutants inhibited cell elongation.

Surprisingly, Pmo25 targeting to the SPB also caused ectopic septa formation as is observed when the SIN is constitutively activated (Figure 3-3B). Septation in these cells was associated with premature recruitment of both Sid1 and Cdc7 to the SPB (Figure 3-3C). Upon closer observation we found that Sid1 recruitment was stronger than Cdc7. Consistent with that, the ectopic septation phenotype was dependent on both Sid1 and Sid2, Figure 3-3D, but independent of the upstream GTPase Spg1 and Cdc7. Normally, Spg1 GTPase at the mitotic SPB recruits Cdc7,which then recruits Sid1 to the SPB in early anaphase that results in SIN activation. Our results suggest that Pmo25 at the SPB can bypass the requirement of the upstream SIN regulators for Sid1 recruitment and SIN activation. In the future, these results will be useful to identify specific domains within Pmo25 that are responsible for Sid1 recruitment and SIN activation at the SPB. Such studies will also help to understand the mechanism of Pmo25 recruitment to the SPB. Pmo25 is not required for normal SIN activation since MOR mutants can undergo cytokinesis. However, it remains to be tested if Pmo25 at the SPB causes recruitment and activation of other MOR components like Nak1 at the SPB .

Figure 3-3. Pmo25 targeting to the SPB causes ectopic SIN activation and septation. (A) Schematic of the Pmo25-Ppc89 (C-t) fusion used in our experiments. (B) Wild-type cells expressing the fusion produced ectopic septation in interphase cells upon 19 hours of induction at 25°C, shown by a schematic. Arrow denotes the septum. (C) Sid1-GFP and Cdc7-GFP recruitment was tested in cells with ectopic septa (arrows) after expression of the fusion as in (B) above. (D) Different SIN mutants, *spg1-106*, *cdc7-24*, *sid1-125* and *sid2-250* expressing the indicated transgenes or the vector (pREP41) alone were sifted to 36°C for 4 hours after initial induction at 25°C for 19hours. Cells were scored for ectopic or normal septa formation as shown and percentage cells plotted.

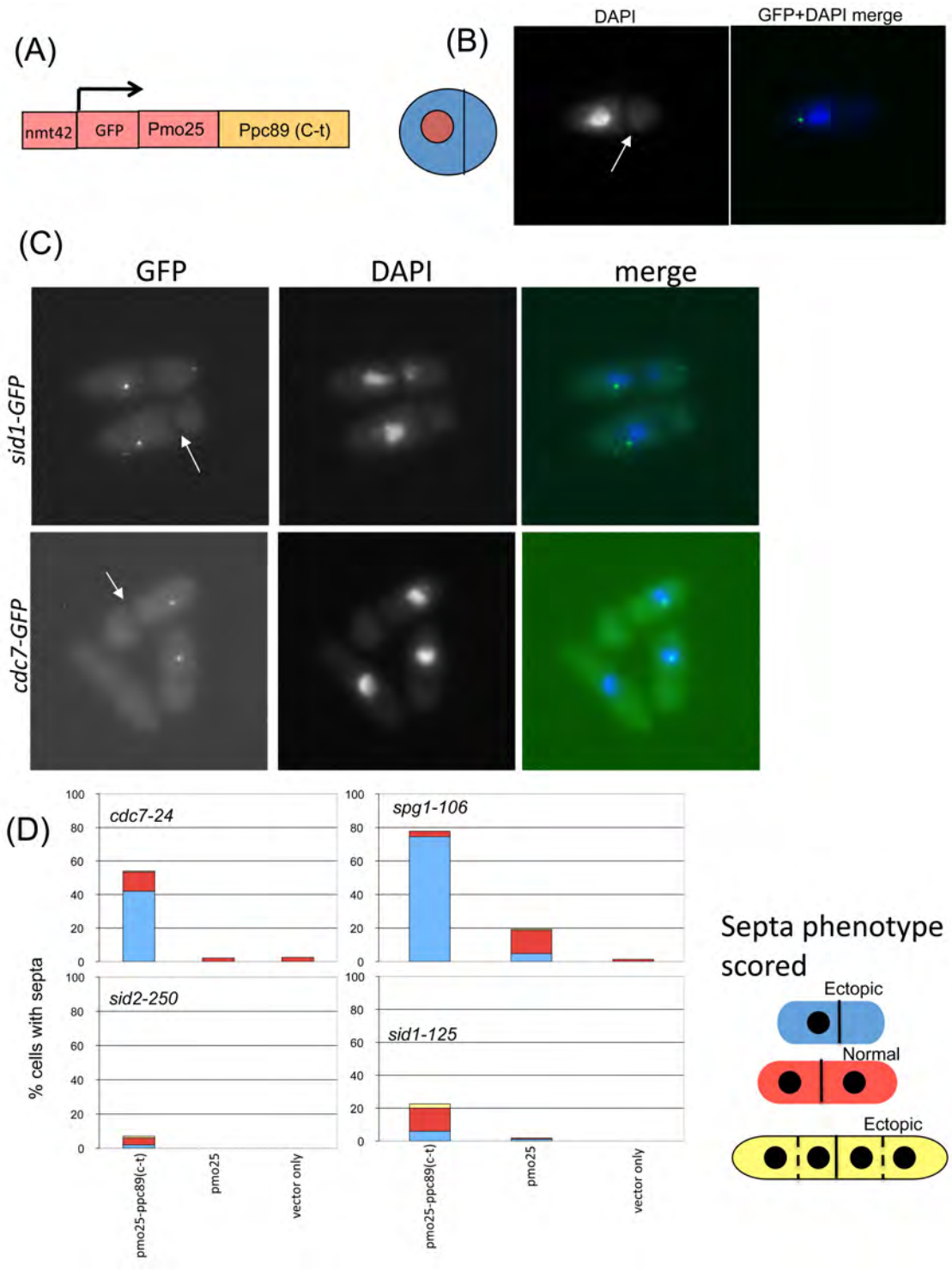


Figure 3-3: Pmo25 targeting to the SPB activates the SIN

Nak1 at the SPB is a putative inhibitor of ectopic septation

Several questions remain regarding the mitosis specific SPB localization of Nak1. It is intriguing that Nak1 is recruited to the SPB in early mitosis only to be removed from it in anaphase by active SIN. Mitotic onset has been associated with a drop in Orb6 kinase activity(Kanai et al., 2005), which is also consistent with the observation that bipolar growth stops at that time in cell cycle. In interphase, when MOR signaling promotes bipolar growth, the MOR components are localized at the cells ends or cytosol(Hirata et al., 2002; Kanai et al., 2005; Mendoza et al., 2005). Thus relocalization of Nak1 from cell tips to the SPB might be a mechanism by which cells inhibit MOR signaling in early mitosis. To this end, it will be interesting to test if failure to recruit Nak1 to SPB bypasses the cell growth arrest in mitosis. This can be achieved by constitutively binding Nak1 to the plasma membrane. Such experiments will also be useful to test if Nak1 association with SPB has other roles during early mitosis.

Intriguingly, in a serendipitous manner, I had observed that Nak1 mutants (*nak1-167*) formed ectopic septa in cells with condensed (metaphase) chromosomes (Figure 3-4). To test this further, we arrested cells in metaphase by overexpressing the *mad2*+ gene that has been previously reported to arrest cells with short spindles and inhbit initiation of cytokinesis(Guertin et al., 2000; He et al., 1997) . In my hands 30% wild-type cells arrested with metaphase chromosomes as judged by DAPI staining upon *mad2* overexpression at 25°C.

We found that inactivation of Nak1 triggered ectopic septation in metaphase arrested cells (Table 3-1). Since Nak1 is essential for Orb6 activity and MOR signaling, we tested if inactivation of Orb6 had a similar effect. Interestingly, we did not observe a substantial rise in ectopic septation in the metaphase arrested cells upon Orb6 inactivation. The small percentage of ectopic septation in the wild-type background could result due to cells leaking out of the arrest after prolonged *mad2* expression. Altogether our data suggest that Nak1 localization at the metaphase SPB might have a specific role in coordinating cytokinesis onset with nuclear division. Further work on this might reveal novel functions of the MOR components in regulating SIN activation.

Table 3-1. Ectopic septation in metaphase arrested cells upon Nak1 inactivation.

Strains	25°C (19 hours)	36°C (3 hours)
(wild-type)sid1-GFP sid4-RFP + *mad2*	4.3%	7.1%
Nak1-167 sid1-GFP sid4-RFP + *mad2*	2%	28%
Orb6-125 + *mad2*	6.5%	4%

Metaphase arrest was achieved by Mad2 over-expression. The *mad2* gene was expressed under the full strength thiamine inducible promoter pREP3X. Following induction for 19hrs at permissive temperature, cells were shifted to 36°C for 3 hrs before fixation and calcofluor staining to score for ectopic septa in metaphase arrested cells.

Figure 3-4. Nak1 inactivation triggers precocious septation.

Cell of the indicated genotypes were grown to log phase at permissive temperature of 25°C and shifted to 36°C for 3 hours. Cells were then collected and DAPI stained. The septum appears as dark line in these cells and is indicated by an arrow.

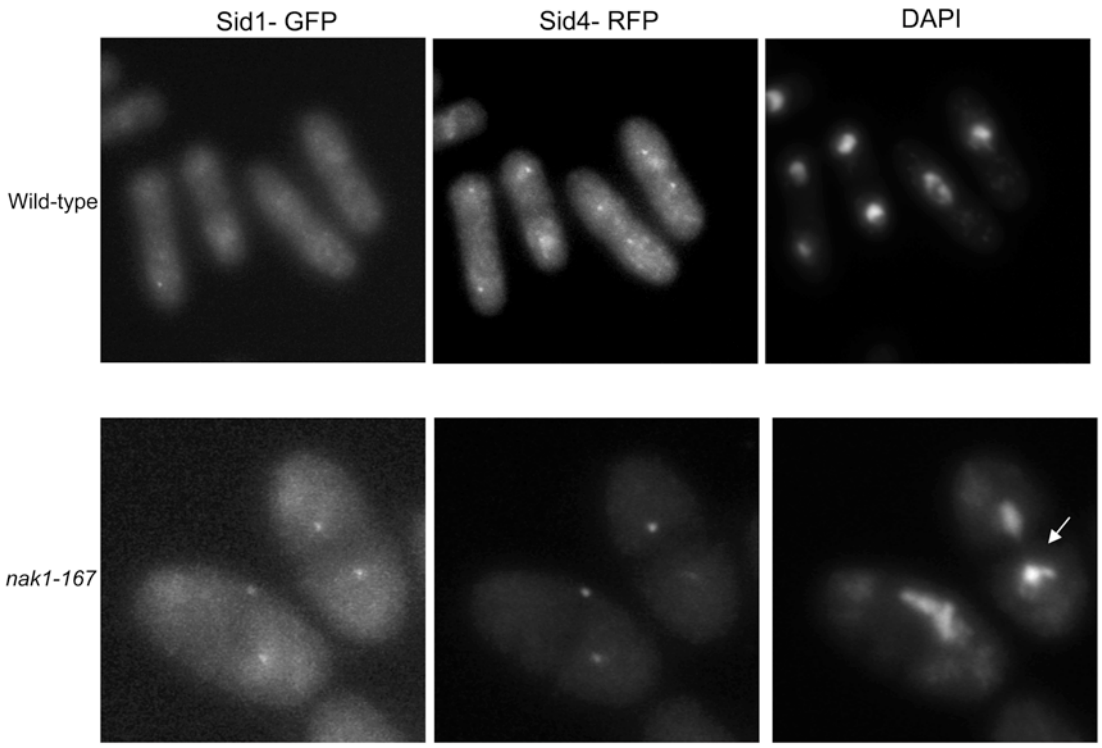


Figure 3-4:Nak1 inactivation triggers precocious septation

Although our observations presented here are preliminary, they gave rise to important questions regarding the regulation of polarized growth and cytokinesis. It has been known that fission yeast cells maintain a constant size during mitosis but it remains unclear how mitotic onset stops bipolar cell growth. Our observation that Nak1 is displaced from its cortical cell end localization to the SPB in early mitosis around the time when cells undergo a dip in Orb6 kinase activity and stop bipolar cell elongation (Kanai et al., 2005) opens up the possibility that Nak1 recruitment is triggered by activation of Cdk1 and is essential for the onset of the “constant volume” phase (Nurse et al., 1976). Encouragingly, mass spectrometry analysis of Nak1 in mitotic cells has identified phosphorylation of multiple putative CDK sites (Gould,McCollum and Chen,unpublished data). Future experiments will help to clarify the mechanism by which Nak1 is recruited to the SPB in early mitosis and its significance during the cell cycle. Furthermore, future work with the Pmo25-Ppc89 fusion will also help to understand mechanisms and significance of the SIN mediated recruitment of Pmo25 to the SPB.

CHAPTER IV

The nucleolar Net1/Cfi1-related protein Dnt1 antagonizes the septation initiation network in fission yeast

My contributions to this chapter: Figures 4-1B, 4-2, 4-3A, 4-4 and 4-7.

Summary

The SIN and MEN signaling pathways regulate cytokinesis and mitotic exit in the yeasts *Schizosaccharomyces pombe*, and *Saccharomyces cerevisiae* respectively. One function of these pathways is to keep the Cdc14-family phosphatase, called Clp1 in *S. pombe*, from being sequestered and inhibited in the nucleolus. In *S. pombe*, the SIN and Clp1 act as part of a cytokinesis checkpoint that allows cells to cope with cytokinesis defects. The SIN promotes checkpoint function by 1) keeping Clp1 out of the nucleolus, 2) maintaining the cytokinetic apparatus, and 3) halting the cell cycle until cytokinesis is completed. In a screen for suppressors of the SIN mutant cytokinesis checkpoint defect, we identified a novel nucleolar protein called Dnt1 and other nucleolar proteins including Rrn5 and Nuc1, which are known to be required for rDNA transcription. Dnt1 shows sequence homology to Net1/Cfi1, which encodes the nucleolar inhibitor of Cdc14 in budding yeast. Like Net1/Cfi1, Dnt1 is required for rDNA silencing, mini-chromosome maintenance, and both Dnt1 and Net1/Cfi1 negatively regulate the homologous SIN and MEN pathways. Unlike Net1/Cfi1, which regulates the MEN through the Cdc14 phosphatase, Dnt1 can inhibit SIN signaling independent of Clp1, suggesting a novel connection between the nucleolus and the SIN pathway.

Introduction

As in higher eukaryotes, cytokinesis in the fission yeast *Schizosaccharomyces pombe* occurs through the use of an actomyosin contractile ring. A spindle pole body (SPB)-based regulatory network, called septation initiation network or SIN, is required to initiate cytokinesis in late anaphase (reviewed in (Guertin et al., 2002a; Simanis, 2003)). A similar signaling cascade exists in budding yeast called the mitotic exit network (MEN). The MEN is essential for mitotic exit and plays an important but nonessential role in cytokinesis (reviewed in (Simanis, 2003; Stegmeier and Amon, 2004)). Both pathways function together with the Cdc14 phosphatase, which is called Clp1 in *S. pombe*. Cdc14 phosphatases reverse phosphorylation by cyclin dependent kinase (Cdk) (Kaiser et al., 2002; Visintin et al., 1998; Wolfe and Gould, 2004; Wolfe et al., 2006). The activities of Cdc14 and Clp1 are controlled at least in part by regulated sequestration and release from the nucleolus (Cueille et al., 2001; Shou et al., 1999; Trautmann et al., 2001; Visintin et al., 1999). In budding yeast, Cdc14 is kept sequestered and inactive in the nucleolus throughout most of the cell cycle by binding to the Net1/Cfi1 protein in the nucleolus (Shou et al., 1999; Visintin et al., 1999). In early anaphase Cdc14 is released from the nucleolus by the combined action of a set of proteins termed the FEAR network and Cdk1 phosphorylation of Net1 (Azzam et al., 2004; Stegmeier et al., 2002). The MEN then acts to maintain the release of Cdc14 from the nucleolus, which is sufficient to trigger mitotic exit. The essential function of the MEN is to keep Cdc14 out of the nucleolus so it can

promote mitotic exit. In fact, disruption of nucleolar localization of Cdc14 by mutation in the nucleolar inhibitor Net1/Cfi1 can rescue deletions of MEN pathway components (Shou et al., 2001; Shou et al., 1999).

Regulation of Clp1 in fission yeast has similarities and as well as important differences from Cdc14 regulation in budding yeast (Cueille et al., 2001; Trautmann et al., 2001). As with budding yeast Cdc14, Clp1 localizes to the nucleolus throughout interphase. However unlike budding yeast, but similar to mammalian Cdc14B (Cho et al., 2005), Clp1 is released from the nucleolus upon mitotic entry. Then in anaphase, much like the MEN in budding yeast, the SIN acts to keep Clp1 out of the nucleolus until cytokinesis is complete. The mechanism governing release of Clp1 from the nucleolus in early mitosis is not known but does not require homologs of the budding yeast FEAR pathway components (Chen et al., 2006a). How Clp1 is inhibited in the nucleolus is not known. An *S. pombe* homolog of Net1/Cfi1 has not been identified.

In fission yeast, Clp1 and the SIN each promote the others activity as part of a positive feedback loop that stays active until completion of cytokinesis (Cueille et al., 2001; Trautmann et al., 2001). Clp1 keeps the SIN active, and the SIN keeps Clp1 out of the nucleolus. This positive feedback loop functions as part of a surveillance mechanism, termed the cytokinesis checkpoint, that halts further cell cycle progression until cytokinesis is complete (Liu et al., 2000) (Mishra et al., 2004). Under normal growth conditions, the checkpoint is not essential for viability. However when cytokinesis is slowed, for example by

perturbation of the actomyosin ring, the checkpoint becomes essential for viability (Mishra et al., 2004). The checkpoint blocks further rounds of nuclear division, and maintains the cytokinetic apparatus, so the cells can eventually divide and retain normal ploidy. Cells with weakened SIN signaling, or a deletion of Clp1, are defective for the checkpoint and are unable to deal with defects in cytokinesis and become multinucleate and die when cytokinesis is delayed.

Here we describe a genetic screen for suppressors of the cytokinesis checkpoint defect in a weakened SIN mutant. As described below, our screen identified a protein similar to Net1/Cfi1 as well as several other nucleolar proteins. Unlike Net1/Cfi1, which regulates mitotic exit and MEN signaling through the Cdc14 phosphatase, Dnt1 affects the SIN independent of Net1, suggesting a new and unexpected link between the nucleolus and the SIN signaling pathway.

Results

A genetic screen for suppressors of the cytokinesis checkpoint defect of SIN mutants

To further understand the mechanisms controlling the SIN and the cytokinesis checkpoint in fission yeast, we carried out a genetic screen for suppressors of the cytokinesis checkpoint defects in SIN mutants. This screen took advantage of the fact that temperature-sensitive SIN mutants are viable at semi-permissive temperatures but have reduced SIN function and a defective cytokinesis checkpoint (Mishra et al., 2004). Due to lack of the checkpoint, these mutations become lethal when the actomyosin ring is slightly perturbed. As a result, these cells fail cytokinesis and die as multinucleate cells. This can be observed when examining the phenotype of single and double mutants between *cdc14-118* (Note that *S. pombe cdc14* encodes a subunit of the Sid1p kinase and is not a homolog of budding yeast Cdc14) and the type II myosin mutant *myo2-E1*. The temperature sensitive SIN mutant *cdc14-118* is viable at 30°C, but largely defective for the cytokinesis checkpoint (Mishra et al., 2004). The temperature sensitive type II myosin mutant *myo2-E1* is also viable at 30°C but is slow in completing cytokinesis, and its viability at this temperature depends on the cytokinesis checkpoint (Mishra et al., 2004). However, the *cdc14-118 myo2-E1* double mutant strain is dead at 30°C because of the combined delay in cytokinesis and defective cytokinesis checkpoint. As shown previously, these cells either fail to make septa or make incomplete aberrant septa and become

multinucleate (Mishra et al., 2004). The *cdc14-118 myo2-E1* strain was screened for spontaneous suppressing mutants at 30°C. From this screen, we identified many spontaneous suppressors, and picked different size colonies, which were back-crossed to wild-type. The weaker suppressors only poorly suppressed one of the single mutants, and the stronger suppressors suppressed both *cdc14-118* and *myo2-E1* (Figure 4-1A, and data not shown). The best suppressors we identified fell into 3 complementation groups: group I (6 members: *14*, *16*, *2-12*, *2-13*, *3-8* and *4-3*), group II (2 members: *6* and *3-3*) and group III (1 member: *4-12* called *sdc4* for suppressor of defective checkpoint). The first two groups are later referred to as *dnt1* and *rrn5* respectively (for reasons see Materials and Methods). Out-crossing revealed that although the *dnt1* mutant cells showed no obvious growth defect, the 2 alleles of *rrn5* were temperature sensitive on their own, and the *sdc4-12* strain grew slowly at all temperatures (Figure 4-1A).

Examination of the *cdc14-118 myo2-E1* cells carrying the different suppressor mutations showed that they all had similar morphology and had recovered the ability to form complete septa at 30°C (Figure 4-2, and data not shown). To characterize the phenotype more closely, we examined *cdc14-118 myo2-E1* and *cdc14-118 myo2-E1 dnt1* cells after shift from 25°C to 30°C for 4 hours. In the *cdc14-118 myo2-E1* cells the number of mononucleate cells decrease and the cells become bi and tetranucleate presumably due to cytokinesis defects caused by their inability to make proper septa (Figure 4-2). In contrast, the *cdc14-118 myo2-E1 dnt1* cells seem to have at least partially

restored cytokinesis checkpoint function, as these cells show a delay as binucleate cells but eventually can divide, since they maintain a mononucleate population and do not accumulate tetranucleate cells (Figure 4-2A). These cells also recover the ability to make proper septa (Figure 4-2B).

Figure 4-1. Suppressors of *cdc14-118 myo2-E1* double mutant. (A) Selected suppressor single mutants and triple mutants with *cdc14-118 myo2-E1* were grown in YE at 25°C and then serial dilutions were spotted on YE plates. Plates were incubated at different temperatures as indicated for 3-5 days before photography. (B) Nuclear phenotype of the suppressors. Wild type and suppressor mutants were first grown in YE at 25°C and then shifted to 30°C for 4 hours. Cells were fixed and stained with DAPI. Example nuclei from interphase cells are shown. Note the crescent shaped nuclear DNA in wild type and *dnt1*Δ cells and ring shaped nuclear DNA phenotype in *rrn5-S6*, *sdc4-12* and *nuc1-632* mutants. The percent of cells with the ring shaped nuclear DNA phenotype is indicated (n>100).

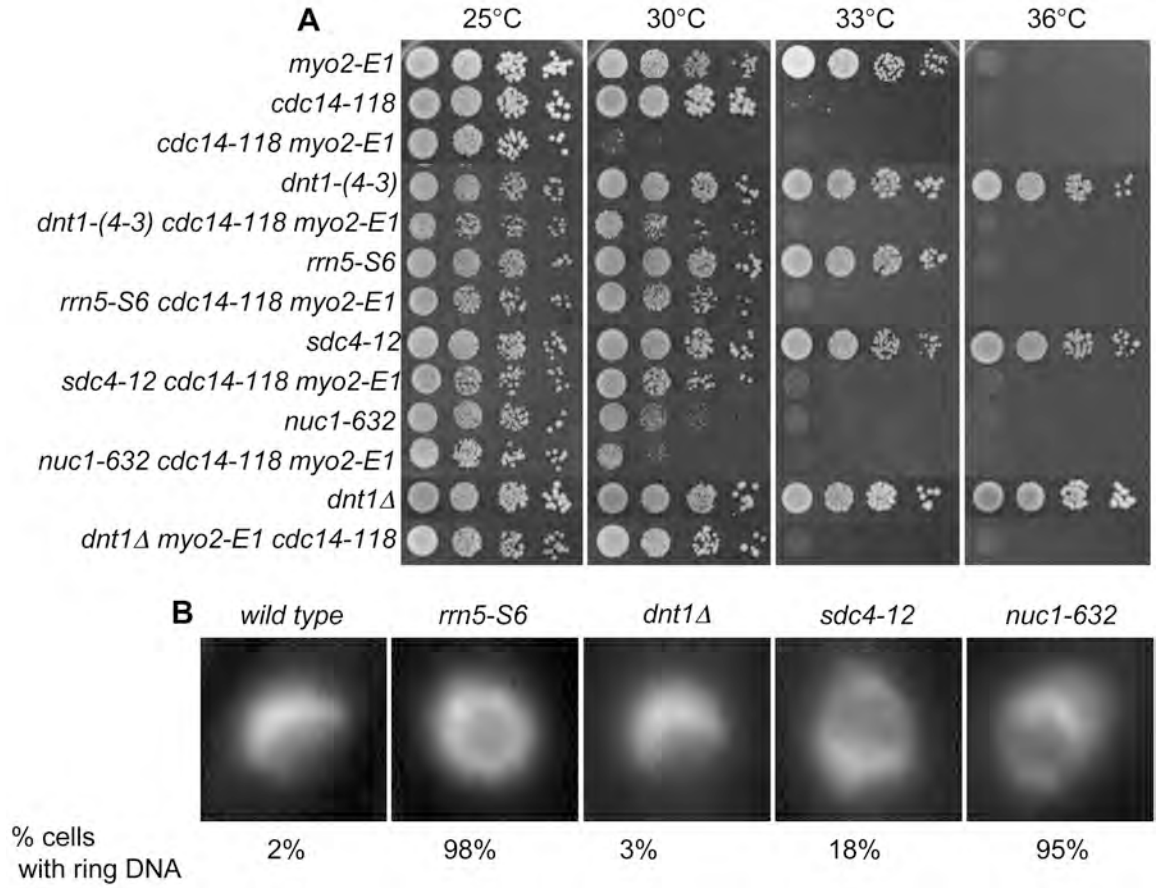


Figure 4-1: Suppressors of the cytokinesis defect had nuclear ring phenotype

Figure 4-2. *dnt1*Δ promotes proper completion of cytokinesis in the *cdc14-118 myo2-E1* cells. Asynchronous cells of the indicated genotypes were grown at 25ºC to log phase cell density and portions shifted to 30ºC for 4 hours. Cells were methanol fixed and stained simultaneously with DAPI and calcofluor to score for (A) the number of nuclei per cell, and (B) septum formation respectively. Cells were scored for the presence of normal, aberrant, or lack of septa between nuclei. At least 100 cells were examined for each strain and mean values (n=3) were plotted.

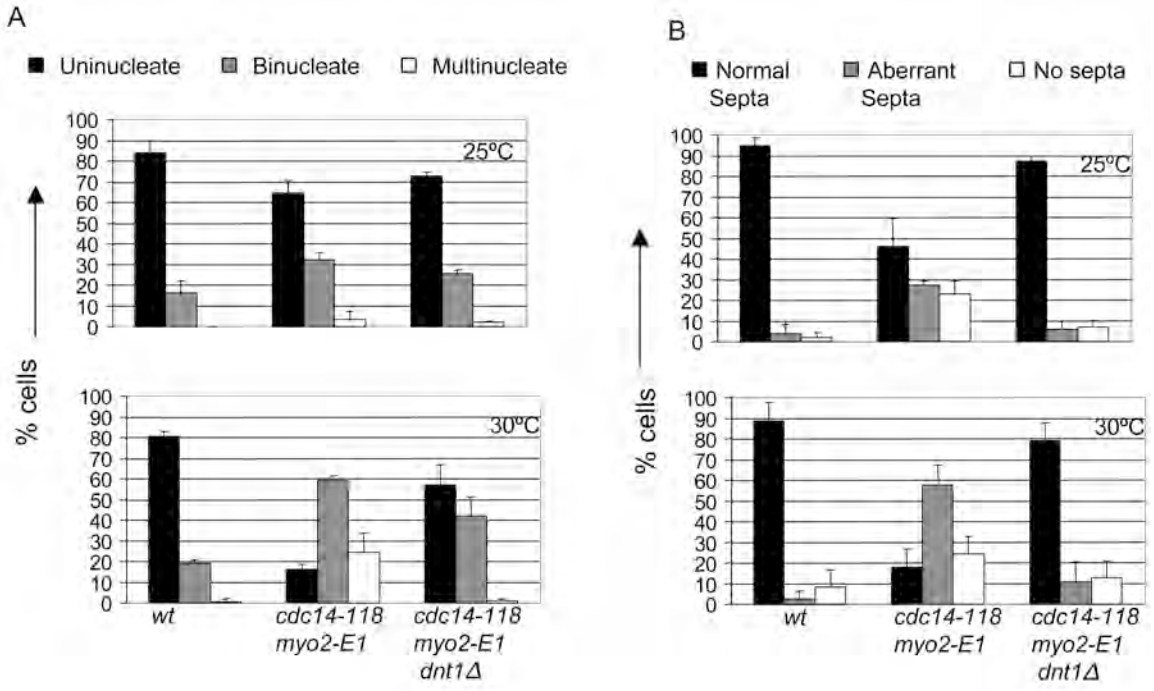


Figure 4-2: Loss of Dnt1 promotes cytokinesis

Suppressors encode nucleolar proteins

The group II suppressor gene *sup6* was cloned by complementation of its temperature sensitive phenotype and determined to be the *rrn5*$^{+}$ gene (see Materials and Methods for Chapter IV). The *rrn5*$^{+}$ gene encodes an upstream activating factor (UAF) for RNA polymerase I involved in transcription of ribosomal RNA (rRNA) (Liu et al., 2002). Examination of the phenotype of the *rrn5-S6* mutant at restrictive temperature revealed an unusual nuclear architecture. In wild-type cells, the nuclear DNA forms a crescent shape on one side of the nucleus, with the nucleolus occupying the other portion of the nucleolus. However, at its restrictive temperature of 36°C the *rrn5-S6* cells often had a ring shaped nuclear DNA pattern (Fig. 4-1B). The only other mutants reported to date with this phenotype are the topoisomerase I and II double mutants, and the temperature sensitive mutant *nuc1-632,* which carries mutation in the largest subunit of RNA polymerase I (Hirano et al., 1986b; Hirano et al., 1989). Interestingly, the *sdc4-12* mutant also displays the ring shaped nuclear DNA phenotype (Figure 4-1B), which, together with genetic analysis described below, suggests that it might also affect RNA polymerase I transcription. Both point mutations and null mutations in the *dnt1* did not cause the ring shaped chromatin phenotype. To determine the localization of Rrn5, we expressed plasmid-borne and GFP tagged Rrn5 in wild-type cells. Not surprisingly, similar to Nuc1, Rrn5 showed nucleolar localization as judged by colocalization with the non-DAPI staining region of the nucleus (Fig. 4-3A).

To investigate whether general perturbation of the RNA Pol I transcription complex can suppress *cdc14-118 myo2-E1* cells, we tested whether the *nuc1-632* could also rescue this double mutant at 30°C. Although the *nuc1-632* cells grew very poorly on their own at 30°C, the *nuc1-632* mutation could weakly rescue *cdc14-118 myo2-E1* cells at this temperature (Fig. 4-1A).

The group I suppressors turned out to be in the *dnt1* gene, which had been identified in our lab as part of an unrelated proteomics screen using mass spectrometry (see Materials and Methods, and Jin and McCollum unpublished observations). *dnt1*Δ deletion mutants were viable, and grew at rates similar to wild-type cells. The *dnt1*Δ mutation also rescued the growth defect of *cdc14-118 myo2-E1* cells at 30°C (Figure 4-1A). Interestingly, Dnt1 is also a nucleolar protein, as Dnt1-GFP is localized in the nucleolus as two or more punctate dots throughout the cell cycle (Figure 4-3B, D). Dnt1-GFP signals can also be observed faintly in the rest of the nucleoplasm. In late anaphase, Dnt1 localizes to the ends of the mitotic spindle (Figure 4-3C).

Figure 4-3. Nucleolar localization of suppressor proteins. The indicated strains were grown at 30°C. (A) Cells transformed with pREP42-*rrn5*$^{+}$-GFP were first grown in EMM with thiamine (repressed) and then induced for 18 hours in EMM without thiamine. Cells were fixed and DAPI stained. Note that the GFP signal varies due to variation in the plasmid copy number between cells . (B) Cells carrying integrated *dnt1-GFP* were fixed and DAPI stained (B). (C) Cells carrying integrated *dnt1-GFP* and mCherry-tubulin expressed from a plasmid were imaged live by fluorescence microscopy. A montage of 2 late anaphase cells is shown. (D) Cells expressing both integrated *dnt1-13myc* and *gar2-GFP* were fixed and subjected to immunofluorescence with antibodies against Myc and GFP. Dnt1-13myc foci were concentrated in the nucleolus as marked by Gar2-GFP. (E) Cells carrying integrated *nuc1-GFP* were fixed and DAPI stained.

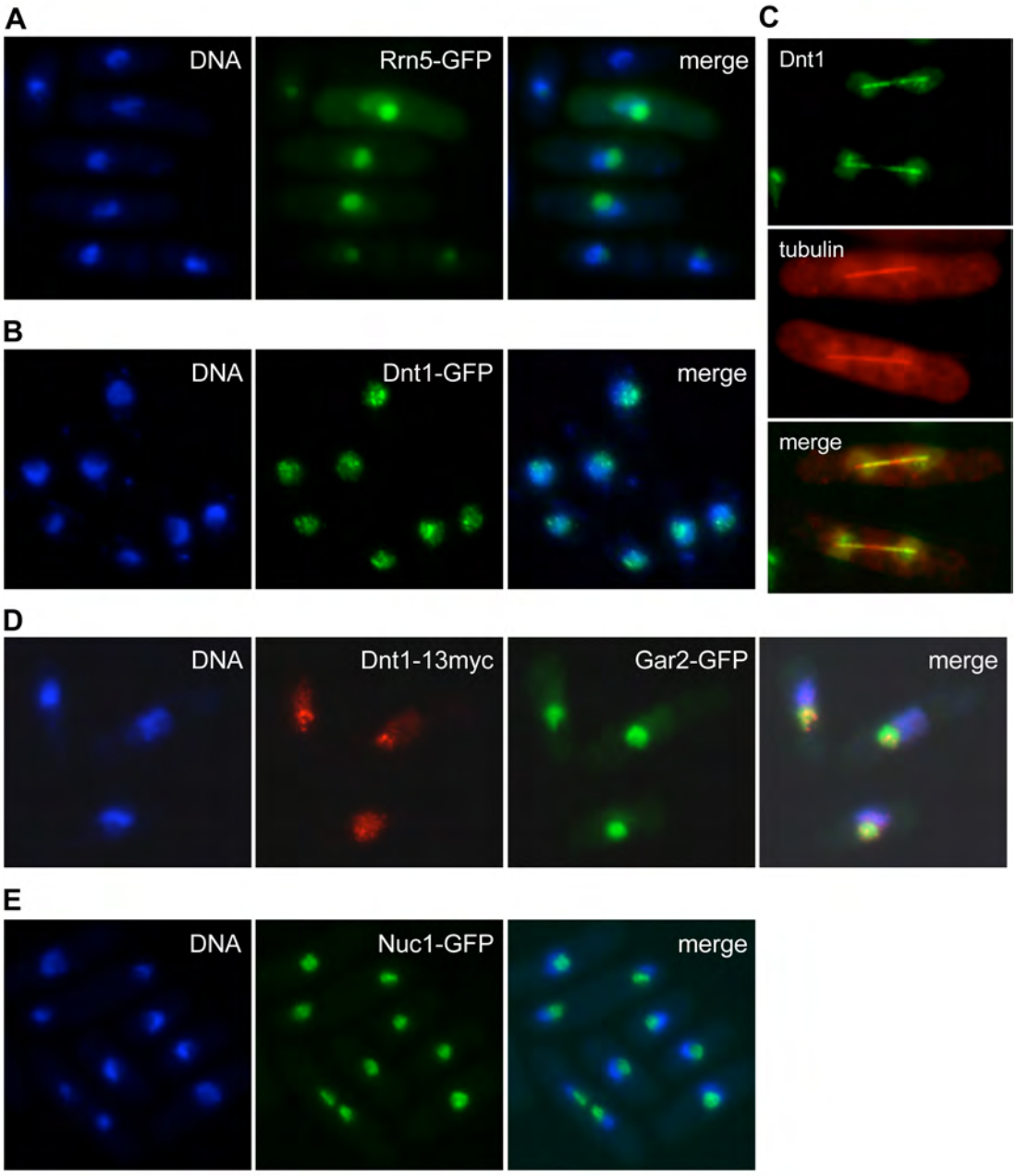


Figure 4-3: Suppressors were localized in the nucleolus

Functional interdependence of Dnt1 and suppressors involved in rDNA transcription

Three of our suppressors, *rrn5-S6, nuc1-632* and *sdc4-12*, showed a characteristic ring-shaped DNA phenotype, and they all grew slowly even at permissive temperature probably due to reduced rDNA transcription. Although *dnt1*Δ mutants did not have a reduced growth rate or defects in nucleolar positioning, genetic analysis suggested that it may also have a role in RNA Pol I transcription. Double mutant analysis revealed that all combinations of double mutants between *nuc1-632, rrn5-S6, sdc4-12* and *dnt1*Δ resulted in either synthetic lethality, or very sick and slow growing cells (Table 4-1). This data suggests that Dnt1 and Sdc4 might function in rDNA transcription like Rrn5 and Nuc1.

Table 4-1. Negative genetic interactions between suppressors *

	rrn5-S6	*sdc4-12*	*nuc1-632*
dnt1Δ	sick	sick	lethal
rrn5-S6		lethal	sick
sdc4-12			sick

* all the crosses were done at 25°C.

Further evidence also suggested an interaction between RNA Pol I and Dnt1. First, localization of Dnt1 to the nucleolus was disrupted in *rrn5-S6*, *sdc4-12* and *nuc1-632* cells (Figure 4- 4A). At 30°C, the temperature where all these mutations can suppress the growth defects of *cdc14-118 myo2-E1*, Dnt1 is not enriched in the nucleolus in these mutants, instead it was found in the nucleoplasm surrounding the nucleolus. This effect was not due to a global disruption of the nucleolus, since the nucleolar protein Gar2 still localized normally to the displaced nucleolus in these mutants at 30°C (Figure 4-5). This disrupted localization of Dnt1 was observed in *rrn5-S6* and *nuc1-632* mutants even at 25°C, the permissive temperature for these mutants (data not shown). We also found that Dnt1 is required for maintaining the exclusive nucleolar localization of Nuc1, as examination of Nuc1-GFP localization in *dnt1*Δ cells showed signals not only in nucleolus, but also in nucleoplasm (Figure 4-4B). This localization pattern is distinct from wild type cells, in which Nuc1-GFP is almost exclusively found in nucleolus (Figure 4-4B). We noticed that *Nuc1-GFP dnt1*Δ cells grew very poorly, although strains carrying either individual allele grew well, this might be because GFP tagged Nuc1 is not completely functional, thus *nuc1-GFP dnt1*Δ cells demonstrate a negative genetic interaction (data not shown). Consistent with the genetic interactions we observed between all suppressors, we also found that nucleolar Nuc1-GFP localization is slightly disrupted in *rrn5-S6* and *sdc4-12* mutants. Like in *dnt1*Δ cells, strong Nuc1-GFP signals were

found at the periphery of the nucleolus and weaker signals in the nucleoplasm (Figure 4-4B).

Figure 4-4. Nucleolar localization of Dnt1 and Nuc1 is disrupted in suppressor mutants. Wild-type and mutant cells with the indicated genotypes were first grown at 25°C and then shifted to 30°C for 4 hours before being visualized by fluorescence microscopy. Arrows indicate the nuclei enlarged in insets.

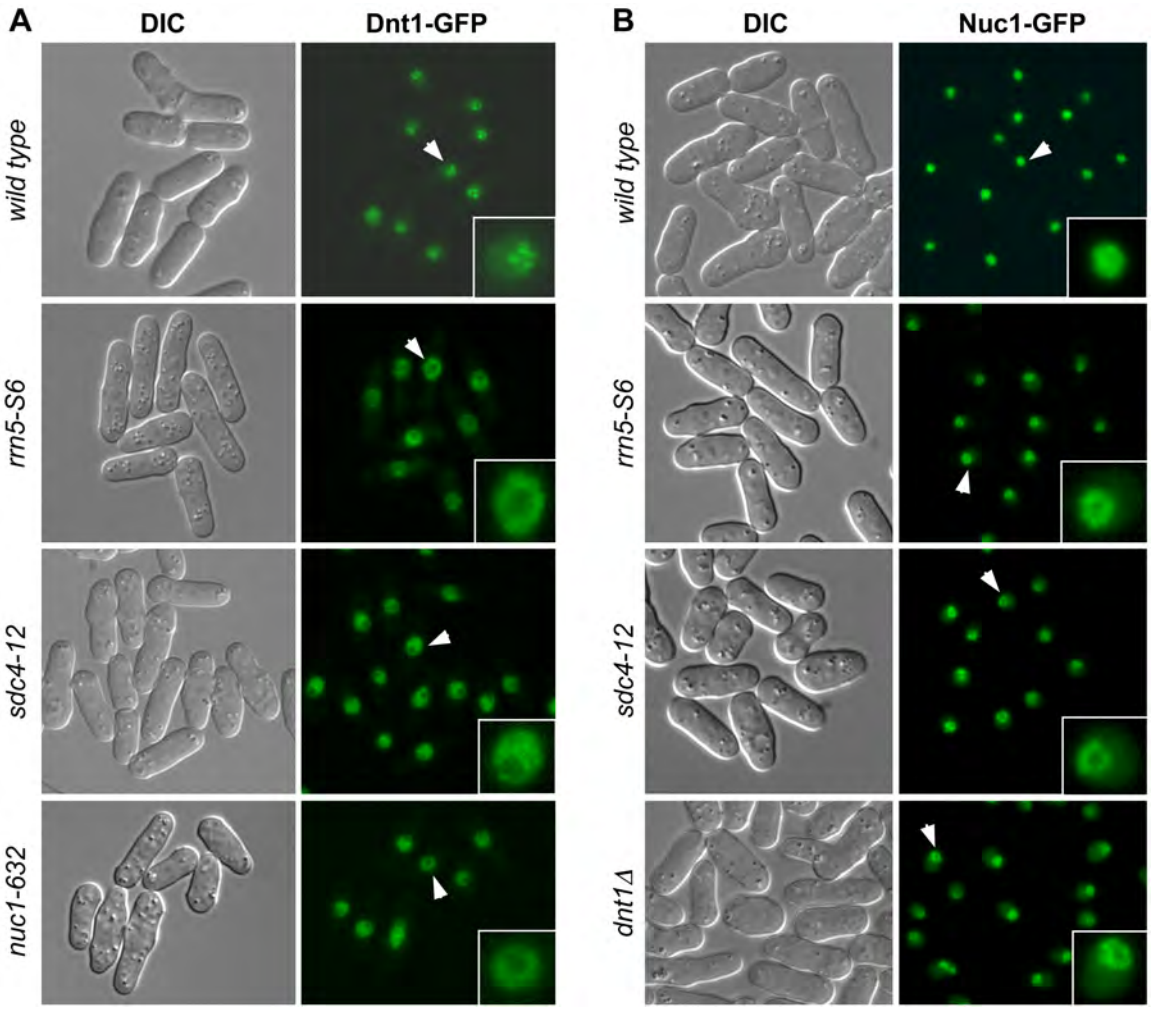


Figure 4-4:Nucleolar localization of Dnt1 and Nuc1 in suppressor mutants.

Figure 4-5. Suppressor mutations do not cause premature release of Gar2-GFP from nucleolus. Wild-type and mutant cells with indicated genotypes were first grown at 25°C and then shifted to 30°C for 4 hours before being imaged by DIC or fluorescence microscopy for GFP signal.

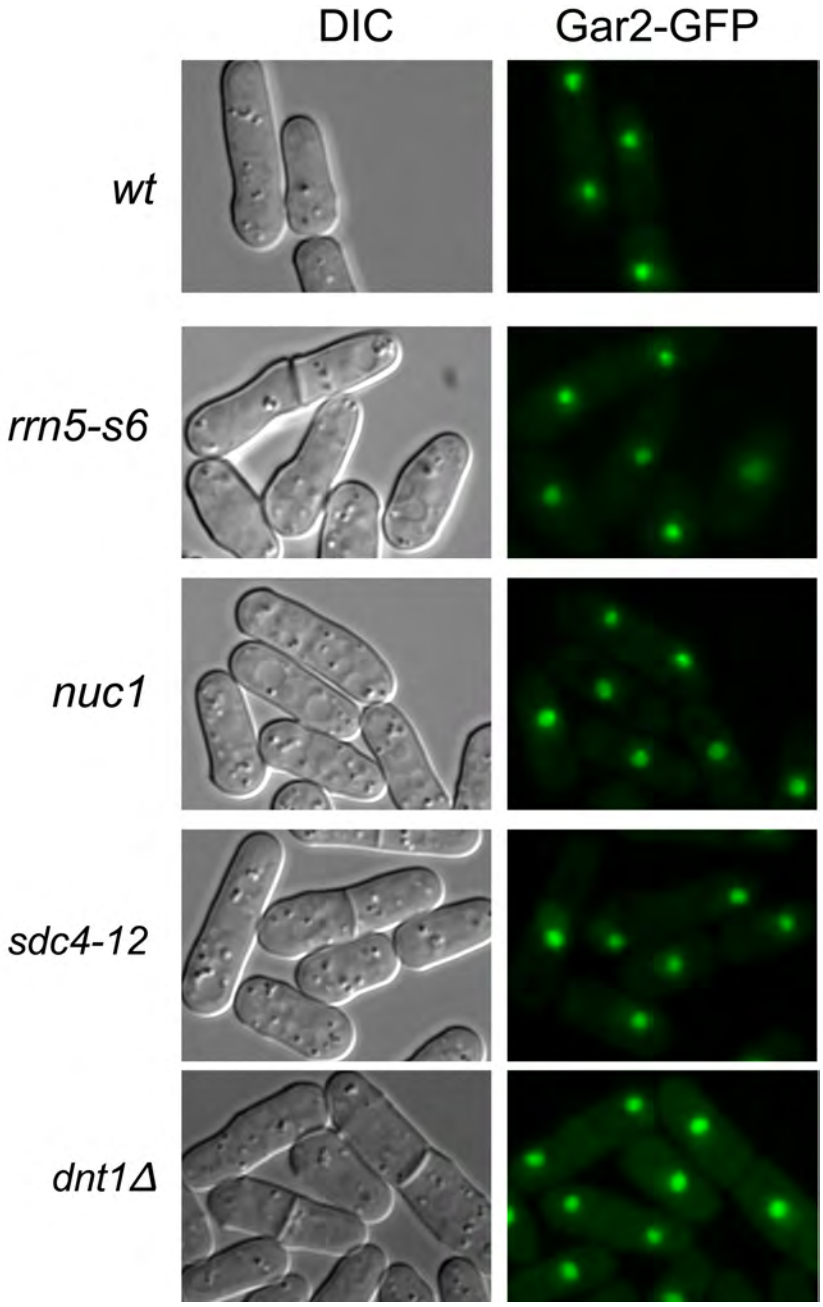


Figure4-5: Suppressor mutations do not disrupt general nucleolar architecture

S. pombe Dnt1: a homolog of budding yeast Net1/Cfi1?

So, why might these mutations in nucleolar proteins suppress *cdc14-118 myo2-E1*? A similar screen in *S. cerevisiae* identified Net1/Cfi1, the nucleolar inhibitor of the Cdc14 phosphatase (Shou et al., 1999). Our basic database searches had not revealed any obvious homologs of Net1/Cfi1 in *S. pombe*. However in the course of this study, more sophisticated database searching (with advice from Dr. Aaron Neiman at SUNY Stoneybrook) identified a candidate *S. pombe* protein called SPBC25D12.02c, which corresponds to Dnt1. Among Net1/Cfi1 homologs in related budding yeast, only the N-terminal 180 amino acids are conserved. This region is also conserved in a second nucleolar protein in *S. cerevisiae* called Tof2 (Huang et al., 2006) (Figure 4-6A). Psi-Blast searches with this region identified SPBC25D12.02c as the best homolog in *S. pombe* of the *S. cerevisiae* NET1/CFI1 and TOF2 genes (Figure 4-6A).

The similarity of Dnt1 to Net1/Cfi1 is intriguing because both Dnt1 and Net1/Cfi1 were identified in similar screens for suppressors of the SIN and MEN pathways respectively. As described above, we found that *dnt1*Δ rescues not just the *cdc14-118 myo2-E1* double mutant, but it also rescues either single mutant (Table 4-2). Further study showed that *dnt1*Δ can weakly suppress other SIN mutants (Table 4-2), consistent with Dnt1 being an inhibitor of the SIN. To examine functional conservation between Net1/Cfi1 and Dnt1, we tested whether Net1/Cfi1 could reverse the effects of *dnt1*Δ on the SIN. We had found that *dnt1Δ* partially rescues the growth defect of the *cdc14-118* mutant, allowing it to grow at

33°C (see Table 4-2). We expressed Net1/Cfi1 from plasmids (pREP41X -*NET1*-GFP, pREP3X-*NET1*-GFP) under the control of inducible *nmt1* promoter in *dnt1Δ cdc14-118* cells, and observed that these cells are dead at 33°C while cells with control plasmid grow well, showing that Net1/Cfi1 expression reversed the rescue phenotype of *dnt1Δ* at this temperature (Figure 4-6B). It is possible that this simply reflects toxicity associated with expression of *NET1/CFI1* in *S. pombe*. However Net1/Cfi1 expressed from either strong (pREP3X) or intermediate (pREP41X) *nmt1* promoters gives a similar reversion phenotype, and also the reversion of suppression occurred even in the presence of thiamine when only low levels of Net1/Cfi1 are expressed. Furthermore, Net1/Cfi1 expression did not inhibit growth at lower temperatures showing that at these expression levels it was not acting as a general growth inhibitor.

Other similarities between Dnt1 and Net1/Cfi1 include the fact that both proteins localize to the nucleolus (Figure 4-3), and like Net1/Cfi1 (Shou et al., 2001; Straight et al., 1999), we found that Dnt1 is required for rDNA silencing as judged by increased expression (i.e. derepression) of a reporter gene (*ura4*$^{+}$) integrated into the rDNA repeats (Thon and Verhein-Hansen, 2000) (Figure 4-6C). Although we have not tested directly whether other suppressors have a similar effect on rDNA silencing, it would not be surprising if they did because RNA Pol I transcription activity is required for rDNA silencing in *S. cerevisiae* (Shou et al., 2001).

Table 4-2. Summary of rescue of *SIN* mutants by *dnt1Δ*.

	25°C	30°C	33°C	36°C
sid4-A1	++	-	-	-
dnt1Δ sid4-A1	++	+	-	-
cdc11-123	++	++	++	-
dnt1Δ cdc11-123	++	++	++	-
spg1-106	++	-	-	-
dnt1Δ spg1-106	++	-	-	-
cdc7-24	++	++	++	+
dnt1Δ cdc7-24	++	++	++	+
sid1-125	++	-	-	-
dnt1Δ sid1-125	++	+	-	-
sid1-239	++	++	++	-
dnt1Δ sid1-239	++	++	++	+
cdc14-118	++	++	-	-
dnt1Δ cdc14-118	++	++	++	-
sid2-250	++	-	-	-
dnt1Δ sid2-250	++	+	+/-	-
mob1-1	++	+	-	-
dnt1Δ mob1-1	++	+	-	-

Growth was examined with serial dilution drop test at different temperatures.

++: good growth; +: weak growth; +/-: weak growth with variations in growth in different clones; -: no growth.

Figure 4-6. Relationship between *S. pombe* Dnt1 and *S. cerevisiae* Net1/Cfi1. (A) The sequence alignment between Dnt1, Net1, and Tof2 at the N-termini of the three proteins is shown. (B) Rescue of *cdc14-118* by *dnt1Δ* can be reversed by expression of budding yeast *NET1. dnt1Δ cdc14-118* cells transformed with either empty vectors (pREP41X-GFP or pREP3X-GFP) or the *NET1* expressing plasmid (pREP41X-*NET1*-GFP or pREP3X-*NET1*-GFP) were first grown at 25°C in EMM medium plus thiamine, then washed, diluted and spotted on EMM plates with (not shown) or without thiamine. Plates were incubated at different temperatures as indicated for 3 to 5 days. (C) Dnt1 is involved in rDNA silencing. Wild-type and *dnt1Δ* cells carrying *ura4*$^{+}$ gene inserted at the rDNA repeats (rDNA::*ura4*$^{+}$) and control wild-type cells carrying endogenous (*ura4*$^{+}$) or deleted *ura4*$^{+}$ gene (*ura4-D18*) were grown at 30°C and serially diluted and spotted onto the indicated plates. The plates were incubated at 30°C for 3 to 5 days before photography. (D) Dnt1 and Clp1 interact in the two-hybrid assay. Clp1 was fused with the DNA-binding domain of GAL4 (BD) and Dnt1 with the transcriptional activation domain of GAL4 (AD). Both fusion constructs were coexpressed and the growth on SD, -Leu, -Trp and SD, -Leu, -Trp, -His is shown. As controls, coexpressions of Clp1 and empty AD vector, and empty DB vector with Dnt1 (negative control) or an AD fusion with *S. pombe* Cdc25 (positive control) are shown. The two-hybrid interaction between Clp1 and Cdc25 has been shown previously (Wolfe and Gould, 2004).

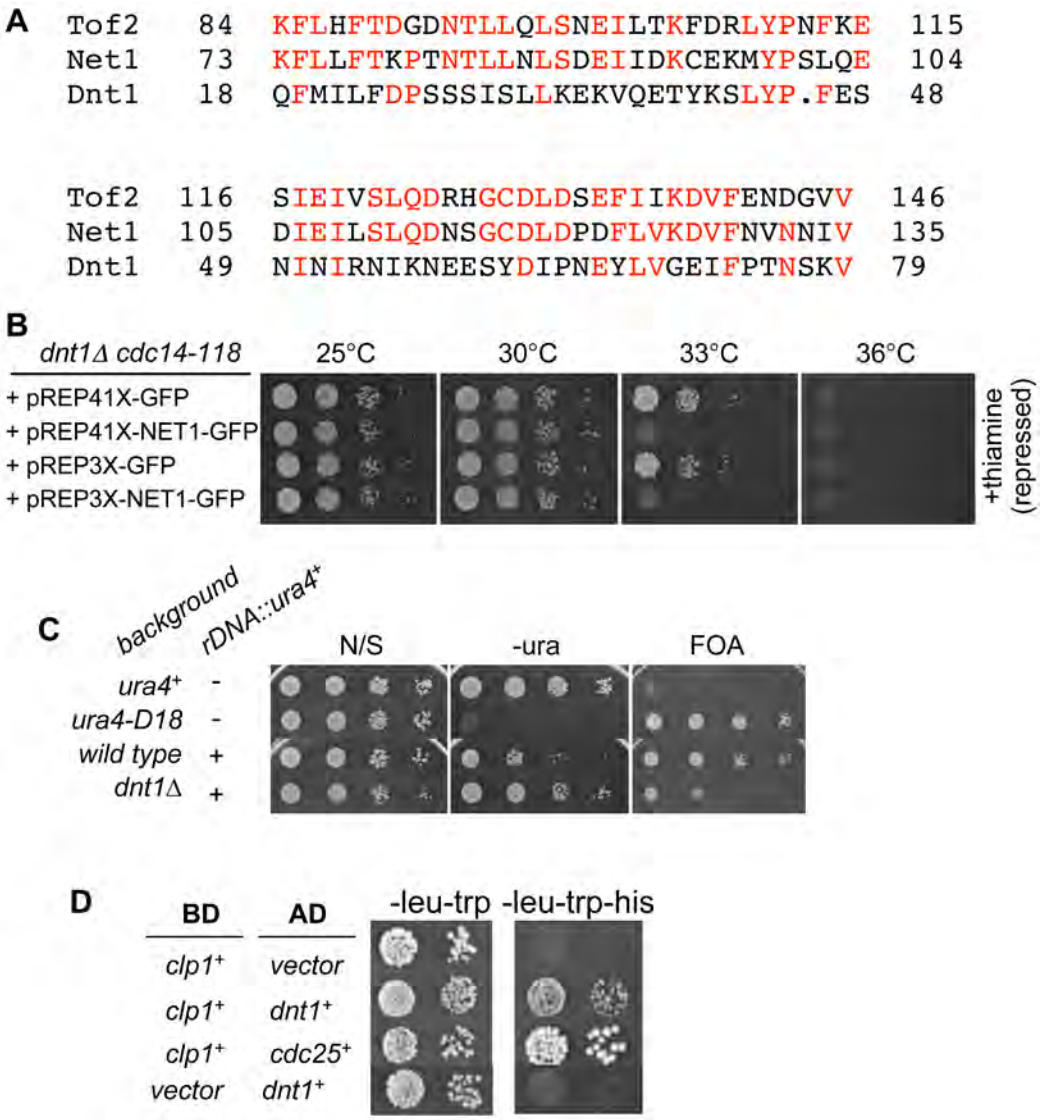


Figure 4-6: Relationship between Net1 and Dnt1

Additionally, as with Net1/Cfi1 (Shou and Deshaies, 2002), Dnt1 is also involved in maintenance of mini-chromosomes. *dnt1Δ* cells showed an almost 100-fold increase in mini-chromosome loss rate compared with wild type cells: with loss rate of 1.78×10^{-2} in *dnt1Δ* cells versus 2×10^{-4} in wild-type cells.

Although the genetic interactions we observe between *dnt1*Δ and mutants in the RNA polymerase I machinery suggest that Dnt1 may participate in Pol I transcription like Net1/Cfi1, we do not think that it plays a direct role, since unlike the *net1*Δ*/cfi1*Δ mutant, *dnt1*Δ cells do not have reduced growth rates compared to wild-type cells. Additionally, we did not observe cross complementation between Dnt1 and Net1/Cfi1 for their putative roles in Pol I transcription. Specifically, we found that Net1/Cfi1 could not rescue the synthetic growth defects we observed in *dnt1*Δ *rrn5-S6* strains (data not shown). We also tested whether Dnt1 could rescue the growth defects of *net1*Δ*/cfi1*Δ cells at high temperatures, which are thought to be due to defects in Pol I activity, since they can be rescued by overexpression by Pol I transcription factors (Shou et al., 2001). However, Dnt1 was not able to rescue the growth defects of *net1*Δ*/cfi1*Δ cells at high temperatures (data not shown). In summary, although we found some interesting similarities between Dnt1 and Net1/Cfi1, the proteins do not appear to be functionally interchangeable.

Does Dnt1 act by antagonizing Clp1?

We next tested whether Dnt1 and Net1/Cfi1 inhibit the SIN and MEN signaling pathways respectively through a common mechanism. It is known that Net1/Cfi1 inhibits MEN signaling by binding to the Cdc14 phosphatase, and both sequestering it in the nucleolus and inhibiting its phosphatase activity (Traverso et al., 2001). Although we were able to detect a modest interaction between Dnt1 and Clp1 in the yeast two-hybrid assay (Figure 4-6D), we have been unable to detect an interaction between endogenous or bacterially expressed Dnt1 and Clp1 by coimmunoprecipitation or *in vitro* binding assays (data not shown). In addition, bacterially expressed Dnt1 does not seem to inhibit Clp1 phosphatase activity in vitro (Ray and McCollum, unpublished data).

***dnt1*Δ and the other suppressors do not cause premature release of Clp1 from nucleolus**

Because *dnt1*$^{+}$ and the other suppressors we identified encode nucleolar proteins like Clp1, we thought that the suppressors might act by causing release of Clp1 from the nucleolus and allowing it to remain active and promote cytokinesis checkpoint signaling. To address whether the suppressors we isolated have effects on nucleolar localization of Clp1, we examined the localization of Clp1-GFP in *dnt1*Δ, *rrn5-S6*, *sdc4-12* and *nuc1-632* mutant strains (Figure 4-7A). All cells were first grown at 25°C, then shifted to 30°C for 4 hours, the temperature

at which they showed suppression of *cdc14-118 myo2-E1*. Except for *dnt1*Δ mutant, all the other mutants show a characteristic ring shaped DNA phenotype at permissive or restrictive temperature, with the nucleolus in the center of the nucleus. However, we did not find Clp1 to be released prematurely in interphase cells in any of the suppressor mutants, even at fully restrictive temperature (Figure 4-7A, and data not shown). We also compared Clp1-GFP localization in *cdc14-118 myo2-E1* and *cdc14-118 myo2-E1 dnt1*Δ cells to *myo2-E1* cells after shift to 30°C. By comparing the ratio of the mean intensity of nucleolar verses cytoplasmic fluorescence in telophase cells we found, as expected, that Clp1 remains out of the nucleolus in *myo2-E1* cells (Figure 4-7B) as normally occurs when cytokinesis is perturbed and the cytokinesis checkpoint is activated. However Clp1-GFP returned to the nucleolus prematurely in both *cdc14-118 myo2-E1* and *cdc14-118 myo2-E1 dnt1*Δ cells (Figure 4-7B). Together, this data indicates that the suppressors of the *cdc14-118 myo2-E1* double mutant do not rescue by simply keeping Clp1 out of nucleolus.

Figure4-7. Suppressors do not cause premature release of Clp1 from nucleolus. (A) Wild-type and mutant cells with indicated genotypes were first grown at 25°C and then shifted to 30°C or 36°C for 4 hours before being fixed and stained with DAPI. Arrows indicate the nuclei enlarged in insets. (B) Cells of the indicated genotypes were grown to log phase at 25ºC and then shifted to 30ºC for 4 hours. These were then methanol fixed and imaged for Clp1-GFP localization. The ratio of the mean average intensity of the Clp1-GFP signal in the nucleolus and cytoplasm is shown (n>50). Representative images are shown. Only cells that were in telophase, as judged by nuclear positioning, were analyzed.

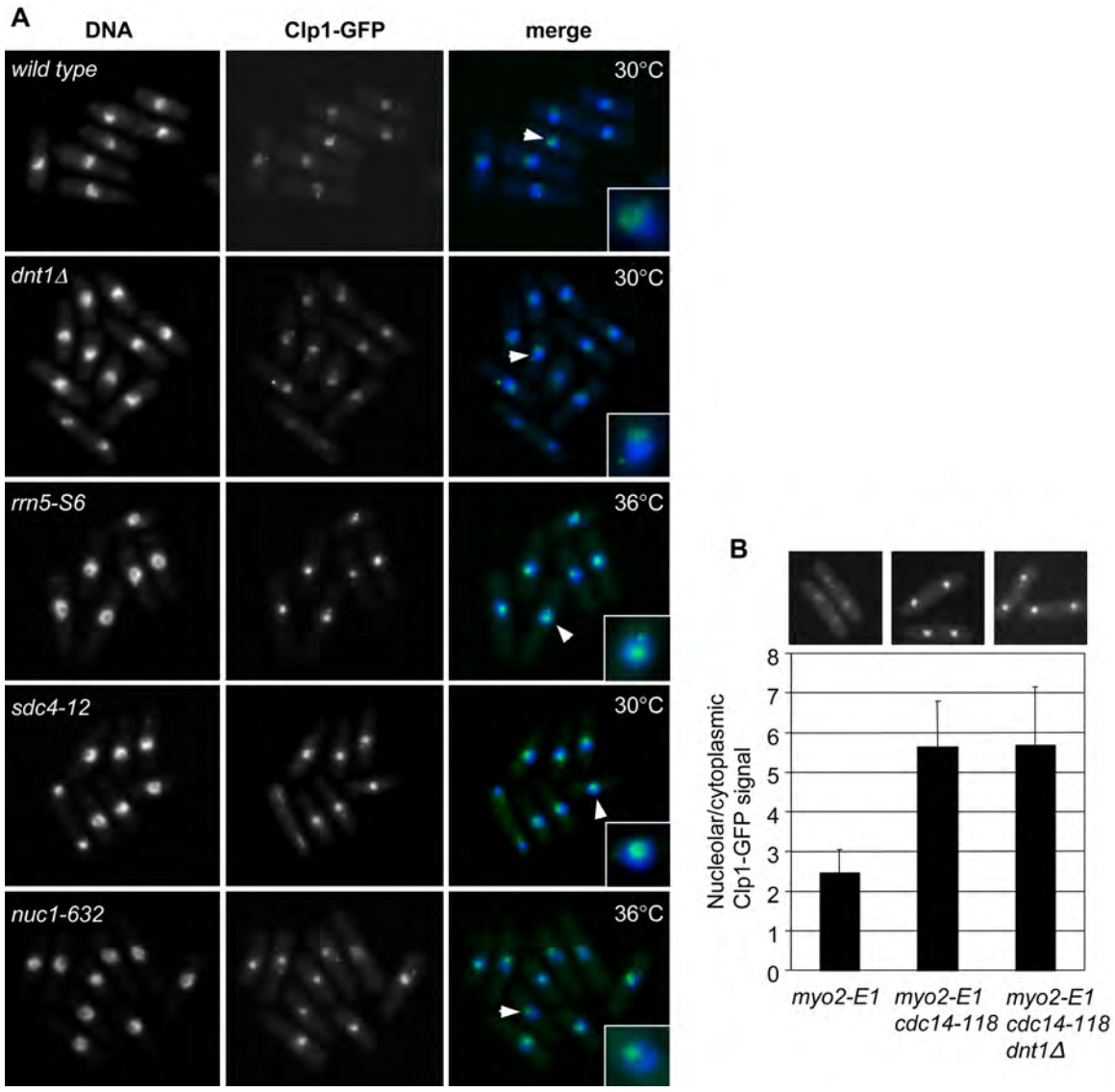


Figure 4-7: Suppressors do not cause premature release of Clp1 from nucleolus.

Dnt1 can regulate the SIN independent of Clp1

As described above, we found that *dnt1*Δ rescues not just the *cdc14-118 myo2-E1* double mutant, but it also rescues either single mutant as well as other SIN mutants. If the rescue of SIN mutants by *dnt1*Δ was through Clp1, then it should depend on *clp1*$^{+}$. To test this, we compared the phenotypes of different combinations of single, double, and triple mutants between *cdc14-118, clp1*Δ, and *dnt1*Δ (Figure 4-8). We found that *cdc14-118* cells can grow up to 30°C, but die at the restrictive temperature of 33°C. As expected, deletion of Clp1 and Dnt1 have opposite effects on the *cdc14-118* mutant, with *clp1*Δ reducing the restrictive temperature of *cdc14-118* cells to 30°C, and *dnt1*Δ raising the restrictive temperature of *cdc14-118* cells to 36°C. Interestingly deletion of *dnt1*$^{+}$ raises the restrictive temperature of the *cdc14-118 clp1*Δ double mutant from 30°C to 33°C, showing that Dnt1 can affect the SIN even in the absence of *clp1*$^{+}$. We also found that *dnt1*Δ could promote growth of the *myo2-E1* mutant in the absence of Clp1 (data not shown). Together these results clearly show that *dnt1*Δ can promote SIN function independent of Clp1, and the simplest interpretation of our results is that Clp1 and Dnt1 act on the SIN independently.

Figure 4-8. Dnt1 can regulate the SIN independent of Clp1. Wild-type and mutant cells with indicated genotypes were first grown in YE at 25°C and then serially diluted and spotted on plates of YE. Plates were incubated at different temperatures as indicated for 3 to 5 days before photography.

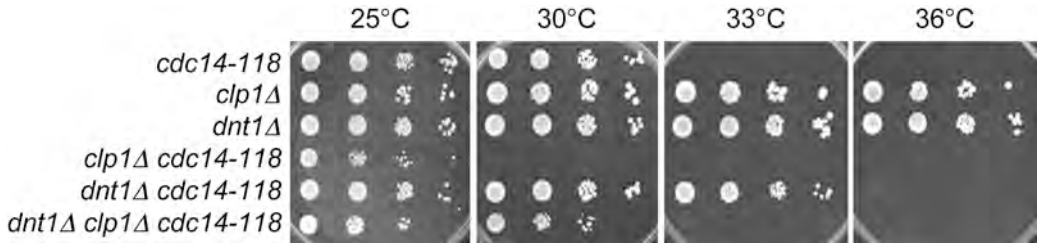


Figure 4-8: Dnt1 can regulate SIN functions independent of Clp1

Another piece of evidence supporting the idea that Dnt1 can regulate SIN signaling and cytokinesis checkpoint in the absence of Clp1 came from our analysis of checkpoint activation and SIN signaling in *dnt1*Δ and *clp1*Δ single and double mutant cells. These cells also expressed Cdc7-GFP whose localization to the SPB can serve as a marker for SIN activation (as reviewed in (McCollum and Gould, 2001)). Low-dosage of Latrunculin, a drug that prevents actin polymerization (Ayscough et al., 1997), has been shown to be able to activate the cytokinesis checkpoint in *S. pombe* by slowing down actomyosin ring contraction (Mishra et al., 2004). We compared the kinetics of nuclear cycle progression in synchronized wild-type, *dnt1*Δ*, clp1*Δ and *clp1*Δ *dnt1*Δ cells upon treatment with 4μM Latrunculin B in liquid cultures (Figure 4-9A). Wild-type and *dnt*Δ cells, which have an intact checkpoint, remained binucleate with the SIN activated and slowly form a septum (Figure 4-9A, B). In contrast, *clp1*Δ cells, which lack the checkpoint, are unable to maintain SIN signaling and become multi-nucleate and are unable to form complete septa (Figure 4-9A, B) (Mishra et al., 2004). Unlike *clp1*Δ single mutants, *clp1*Δ *dnt1*Δ cells maintain SIN signaling and remain blocked as binucleate cells for an extended period of time, though not as long as wild-type or *dnt1*Δ cells (Figure 4-9A). These data suggest that *dnt1*Δ allows cells to maintain SIN signaling and keep the cytokinesis checkpoint active even in the total absence of Clp1. Therefore, Dnt1 must be able to affect SIN signaling through an alternative pathway.

Figure 4-9. *dnt1Δ* has a positive effect on SIN signaling even in the absence of Clp1. Wild-type and mutant cells with the indicated genotypes (all carrying Cdc7-GFP) were first grown in YE at 30°C and then synchronized in early G2 by centrifugal elutriation and treated with 4μM latrunculin B. Cells were withdrawn in every 30 minutes for a period of 6 hours and fixed and stained with DAPI. (A) Left panels show the number of nuclei over time for each strain, and examples of DNA staining and Cdc7-GFP signal at the 4 hour time point for each strain are shown on the right. (B) Quantification of Cdc7-GFP signals are shown. At least 200 cells were counted for each time point.

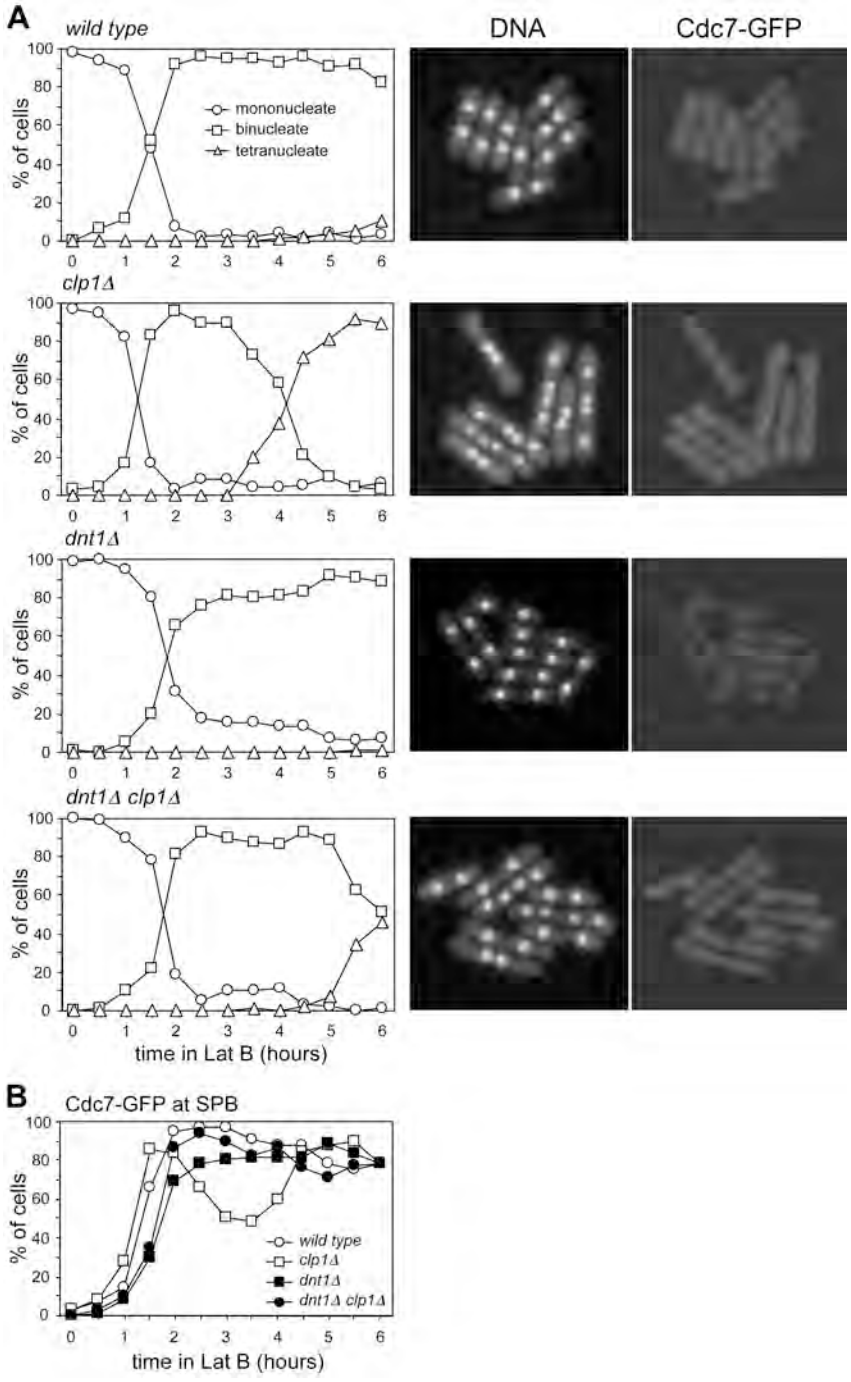


Figure 4-9: Dnt1 deletion promotes SIN signaling independent of Clp1

Discussion

Our screen for suppressors of the cytokinesis checkpoint of SIN mutants identified several nucleolar proteins, Dnt1, Rrn5, Nuc1, and one uncharacterized protein. Dnt1 seems to act as an inhibitor of SIN signaling and it is also involved in rDNA silencing. Dnt1 has sequence similarity to 2 nucleolar proteins from budding yeast called Net1/Cfi1 and Tof2 that are both involved in rDNA silencing. Interestingly, *S. cerevisiae* Net1/Cfi1 and a second nucleolar protein involved rDNA silencing called Fob1 also have roles in regulating the MEN pathway (Huang and Moazed, 2003; Shou et al., 2001; Stegmeier et al., 2004; Straight et al., 1999). It is not known whether Tof2 regulates MEN signaling. The effects of Net1/Cfi1 and Fob1 in mitotic exit control seem to be through the Cdc14 phosphatase. The key function of Net1 in controlling MEN in budding yeast is to bind to and inhibit the Cdc14 phosphatase in the nucleolus (Shou et al., 1999). However our data suggests that unlike Net1/Cfi1, Dnt1 can regulate the SIN independent of the Clp1 phosphatase. We only found very weak interaction between Dnt1 and Clp1 by two-hybrid analysis but not in coimmunoprecipitation or *in vitro* binding assays. The weak interaction might suggest that Dnt1 is a substrate of Clp1, consistent with our observations that Dnt1 is phosphorylated on Cdk1 sites in vivo (Jin and McCollum, unpublished observations). Furthermore the absence of Dnt1 does not lead to premature release of Clp1 into nucleoplasm or cytoplasm. Given the apparent differences between Net1/Cfi1

and Dnt1, it is surprising that Net1 could reverse the suppression of *cdc14-118* cells by *dnt1*Δ. However this could be explained if both proteins inhibit the SIN through different mechanisms. The simplest model to explain our data on the relationship between Dnt1 and Clp1 is that the 2 proteins independently regulate the SIN.

The mechanism by which the suppressors we identified promote SIN signaling is unclear. We do not think that the suppression is due to loss of rDNA silencing because deletion of Sir2, which is required for rDNA silencing (Shankaranarayana et al., 2003), does not rescue *cdc14-118 myo2-E1* mutants (by visual inspection of cell growth). In principle, perturbation of RNA Pol I activity could rescue *cdc14-118 myo2-E1* mutants through an indirect mechanism such as reduction of protein synthesis rates caused by Pol I inhibition, or changes in general transcription rates leading to increased levels of Cdc14 or Myo2 protein. We think this is unlikely, primarily because *dnt1*Δ cells grow at wild-type growth rates and thus are unlikely to be significantly impaired for Pol I activity, and secondly, graded reduction in overall protein synthesis rates using tetracycline did not promote rescue of *cdc14-118 myo2-E1* mutants, nor does deletion of *dnt1* have a significant effect on Cdc14, Sid1, or Myo2 protein levels (as observed through visual inspection). The other mutations in RNA Pol I factors might then rescue SIN signaling, because they all disrupt nucleolar localization of Dnt1, but the exact function of Dnt1 in SIN signaling remains to be determined.

What is the link between nucleolar proteins and SIN regulation? Definition of the nucleolus as the site of rDNA transcription and ribosome biogenesis is well established in both yeast and higher eukaryotes. Recent studies have expanded the functions of nucleolus to include roles in recruitment and exclusion of regulatory complexes (Garcia and Pillus, 1999; San-Segundo and Roeder, 1999; Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). The synthesis of ribosomes consumes a vast amount of the resources in rapidly growing cells, and the nucleolus is emerging as a key control point for the regulation of cell growth and division, both in yeast and human cells (reviewed in (Dez and Tollervey, 2004)). More generally, the high metabolic cost of ribosome synthesis may have selected for its close integration with cell growth and division. Together, these results suggest that the nucleolus may serve as a site to integrate signals governing cell growth and cell cycle progression, and this will serve as an exciting avenue for further research.

CHAPTER V

General Discussion

The fission yeast *S.pombe* has emerged as an excellent model to study regulation of polarized growth. These rod shaped cells grow mainly by elongation at the ends and upon acquiring an optimum size (~14 microns) undergo cytoplasmic cleavage at the cell middle. The actin cytoskeleton is essential for both cell elongation and division, hence the transition between interphase and mitosis is associated with remodeling of the actin cytoskeleton from cell ends to the cell middle. Two conserved signaling pathways, the MOR (Morphogenesis) pathway and the SIN (Septation Initiation Network), are essential to drive polarized growth and cytoplasmic division (cytokinesis) respectively. Several studies have focused on how the separate pathways regulate their respective functions but not much was known about the mechanisms that coordinate the cytoskeletal remodeling during the transition between cytokinesis and bipolar growth.

The SIN inhibits MOR signaling by blocking activation of the most downstream kinase Orb6

In Chapter II we show that the SIN and MOR pathways are involved in a cross-talk interaction to regulate the transition between cytokinesis and bipolar growth. Precedence for a role of the SIN in inhibiting bipolar growth came from observations that cells with inactive SIN signaling fail to maintain the medial cytokinetic ring apparatus but instead continue with bipolar elongation by repolarizing actin to the cell ends (Mishra et al., 2004). To test if SIN signaling could directly inhibit interphase bipolar growth, we tested the effect of ectopic SIN activation on interphase cell morphology and on MOR signaling. Our results showed strong inhibition of bipolar growth and mitotic progression similar to that observed upon inactivation of the MOR pathway (Hirata et al., 2002; Kanai et al., 2005) (Figure 2-1). Further analysis showed that the SIN directly inhibits MOR signaling by inhibiting the activity of the most downstream MOR component, the NDR kinase Orb6 (Figure 2-3). This data was apparently contradictory to earlier findings by Kanai et al (Kanai et al., 2005) that showed that SIN signaling was essential for activation of the MOR pathway in the next interphase following cell division. However, after following the effect of Orb6 under more physiological conditions in synchronous cell population we were able to reconcile the discrepancy (Figure 2-3B). We found that inactivation of the SIN results in moderate level of Orb6 activity that was able to maintain bipolar growth in these cells. However, upon activation of cytokinesis checkpoint when cells were

arrested with active SIN signaling there was a reduced level of Orb6 activity consistent with our observation in *cdc16-116* cells. Together, these data suggested that the SIN is not essential for Orb6 activation per se, but was important for the increase in its activity during the transition to bipolar growth following cytokinesis.

We then tested the mechanism by which the SIN inhibits Orb6 activity. Orb6 activation requires phosphorylation by the Ste20-like kinase Nak1. Besides Nak1, Mor2 is also involved in Orb6 activation since it associates with both Nak1 and Orb6 and serves as a scaffold to facilitate Nak1 mediated Orb6 activation (Kanai et al., 2005). We showed that constitutive association between Nak1 and Orb6, achieved by making a fusion of the two proteins, was able to bypass the SIN mediated block in cell growth and nuclear division (Figure 2-4C and 4D). This suggested that the SIN inhibits Orb6 activation by blocking its association with Nak1. However it remains to be tested if the SIN interferes with direct association between Orb6 and Nak1 (that have been shown to associate in yeast two-hybrid assays) or whether SIN has other targets in the MOR pathway that disrupt Orb6 activation. We also found that the fusion protein was able to bypass the growth defect of *mor2-786* cells, consistent with the proposed role of Mor2 as a linker between Nak1 and Orb6. In the future, it will be interesting to biochemically test if SIN activation inhibits the association of Mor2 with Nak1 and/or Orb6.

Independent experiments have suggested that the MOR pathway kinase Nak1 is a potential target of Sid2, the most downstream SIN kinase. Previous work by Chun-ti Chen in the lab has shown that phosphorylation by the SIN component Sid2 creates binding sites for the 14-3-3 protein Rad24 (Chen et al., 2008). Rad24 protein binding has been shown to produce varied effects including cytoplasmic retention of target proteins and inhibition of binding with other proteins (van Heusden and Steensma, 2006). Using Rad24-TAP as bait, mass spectrometry analysis was performed to identify Sid2 targets under different genetic backgrounds (unpublished work Chen and McCollum). Interestingly, such an approach showed that Nak1 was bound to Rad24-TAP in a SIN dependent manner. Future work will be required for validation of this result by checking if Rad24-TAP can directly associate with Nak1 and/or if Nak1 can be in-vitro phosphorylated by Sid2. Although based on mass spec data it is reasonable to speculate that the SIN disrupts association between Mor2 and Nak1, it will be interesting to test if the Mor2-Orb6 association is also affected. Previously immunoprecipitation and two-hybrid experiments have shown that Mor2 physically associates with Orb6 (Kanai et al., 2005), however the implication of this interaction is not clear. One possibility is that Mor2 binding serves to stabilize Orb6 association with Nak1 in interphase and SIN signaling disrupts this interaction in mitosis. This possibility will also suggest that the SIN uses multiple mechanisms to inhibit Orb6 activation.

MOR inhibition by the SIN is essential for successful cytokinesis

To test the significance of MOR inhibition by the SIN, we tested if expression of the Nak1-Orb6 fusion, which was able to bypass the SIN mediated cell elongation block, was also antagonistic to the SIN function of cytokinesis. Although in wild-type cells the fusion did not cause any cytokinetic defects, it was toxic in cells with weakened SIN signaling or with perturbed cytokinetic machinery (Figure 2-7). This was consistent with genetic interaction studies that showed that inactivation of the MOR rescued cytokinesis defects of different SIN mutants and allowed ectopic septum formation in cells with weakly activated SIN signaling (Figure 2-9). These results suggested that the MOR inhibition by SIN signaling was essential to maintain SIN functions, especially when the cytokinesis machinery was perturbed. The inability of the Nak1-Orb6 fusion to antagonize SIN functions in wild-type cells could suggest that the SIN has other targets in the MOR pathway and hence the MOR inhibition was not completely bypassed in our experiments.

To test the mechanism by which MOR signaling inhibited SIN functions when cytokinesis was compromised we followed localization and activity of the SIN upon expression of our fusion. The MOR pathway could either directly inhibit SIN signaling or could act downstream of the SIN to interfere with cytokinesis. We reasoned that if MOR inhibited signaling through the SIN then inactivation of the MOR would promote persistent SIN signaling. To test this possibility, we examined localization of two SIN pathway markers (Cdc7 and Sid2) in *orb6-25*

cells (mutation in the most downstream MOR component, Orb6). During cytokinesis Cdc7 is at the SPB and Sid2 at the cell division site. However, concomitant with completion of cytokinesis both Cdc7 and Sid2 are lost from these sites (Kanai et al., 2005; Sparks et al., 1999). We thus examined localization of both Cdc7 and Sid2 in *orb6-25* cells, however our data ruled out persistent SIN signaling following cytokinesis and septum formation upon MOR inactivation (Figure 2-8). We then tested if over-expression of the Nak1-Orb6 fusion could inhibit kinase activity of Sid2, the most downstream SIN component but found otherwise. Together these results suggest that the MOR does not inhibit SIN signaling per se but interferes with its downstream functions. Since MOR promotes growth at the cell ends, it is reasonable that it titrates away essential ring components from the cell middle thereby interfering with cytokinesis. In future it will be important to identify cytokinesis associated cytoskeletal elements that are targeted by the MOR to perturb cell division.

One potential candidate would be the essential ring component Cdc15 (Fankhauser et al., 1995; Wachtler et al., 2006). As mentioned earlier, Cdc15 is a phosphoprotein whose localization during the cell cycle is regulated by its phosphorylation status. The hyperphosphorylated state of Cdc15 maintains its localization to the cell ends in interphase but inhibits its mitosis specific localization at the cell middle (Clifford et al., 2008; Roberts-Galbraith et al., 2010). Cdc15 has Sid2 target sites (RXXS)(Chen et al., 2008) and it has been shown that dephosphorylation of the RXXS sites is essential for its role in

maintenance of stable contractile ring and cytokinesis (Roberts-Galbraith et al., 2010). This observation is paradoxical since Sid2 is a kinase but Cdc15, a prospective downstream effector required for ring assembly and cytokinesis, is dephosphorylated when SIN is activated. Since Orb6 and Sid2 belong to the same family of proteins it is conceivable that interphase Orb6 activity maintains Cdc15 phosphorylation and tip localization. However, based on our results (Chapter II) SIN signaling would inhibit Orb6 activity resulting in Cdc15 dephosphorylation, which would maintain its medial ring localization and cytokinesis specific functions. This would also explain the apparent paradox that despite seeming to function downstream of the SIN, Cdc15 needs to be dephosphorylated for its cytokinesis specific functions. This mode of regulation would make Cdc15 a common target of both the SIN and MOR pathways. Hence successful cytokinesis would require timely inactivation of the MOR by SIN signaling to allow complete dephosphorylation and activation of Cdc15. However, inability to inhibit the MOR would maintain the phosphorylated state of Cdc15 causing cytokinetic defects. In future, it will be interesting to test if Cdc15 is directly phosphorylated by Orb6 and if overexpression of the hyperphosphorylated form of Cdc15 rescues bipolar growth defect in *cdc3-116 cdc16-116* cells. Alternatively, we could use the hypophosphorylated form to look for rescue of SIN mutants. Further experiments with overexpression of other putative cytoskeletal targets, either through a candidate approach or using genomic library, in SIN mutants might help to identify other shared cytoskeletal

elements between the two pathways. Future work will help to elucidate the detailed mechanism of the antagonistic interaction between the SIN and MOR components and their functions.

SIN signaling regulates the differential SPB localization of the MOR components Nak1 and Pmo25

Both the MOR pathway component Pmo25 and its downstream target Nak1 have been observed at the SPB in anaphase (Kanai et al., 2005). We decided to look into the mitosis specific localization of Nak1 and Pmo25 in further detail using time-lapse movies and found that Pmo25 and Nak1 had very distinct localization patterns at the SPB in mitosis. We observed that Nak1 was localized at the SPB in early mitosis but was displaced from there in late anaphase around the time when the SIN becomes active (Figure 3-1). Consistent with previous studies, we found Pmo25 at the SPB in late anaphase and its recruitment was dependent on the upstream SIN kinases Sid1 and Cdc7. It is curious that despite the early presence of Cdc7 at the mitotic SPB, Pmo25 localization coincides with Sid1 recruitment in late anaphase. Since Sid1 localization at the active (new) SPB follows Cdc7, Pmo25 might be directly dependent on Sid1 and indirectly on Cdc7 for its SPB positioning. Furthermore, ectopic activation of the SIN in interphase was also associated with the premature recruitment of Pmo25 to the SPB in interphase cells. Thus SIN activation at any point in the cell cycle was

able to drive Pmo25 to the SPB (Kanai et al., 2005; Mendoza et al., 2005) (Figure 3-1, our study).

Several interesting questions remain unanswered about these observations. It is intriguing that ectopic SIN activation recruits only Pmo25 and no other MOR components to the SPB. Since SIN activation is associated with inhibition of Orb6 activity, one possibility is that SPB recruitment of Pmo25 disrupts its association with its target proteins in the MOR pathway and thereby blocks MOR signaling. To test if Pmo25 recruitment to the SPB could block MOR signaling we made a fusion between Pmo25 and Ppc89(C-t), a SPB structural protein that successfully targets the SIN scaffold protein Sid4 to the SPB (Rosenberg et al., 2006). Our results showed that SPB targeting of Pmo25 inhibited normal bipolar growth evident from their short, round morphology. This suggested that the MOR pathway is inhibited by the constitutive SPB localization of Pmo25. In future, Orb6 kinase assays will be useful to directly test if recruitment of Pmo25 at the SPB inhibits downstream signaling through the MOR.

However, to our surprise, constitutive targeting of Pmo25 to the SPB triggered ectopic septation in interphase suggesting that the SIN was prematurely activated in our experiments (Figure 3-3B). Intriguingly, SPB localized Pmo25 caused premature recruitment of the upstream SIN components Sid1 and Cdc7 (Figure 3-3C). Further analysis showed that the ectopic septation phenotype was independent of the upstream SIN regulators Spg1 and Cdc7, but

was still dependent on Sid1 and Sid2 kinases (Figure 3-3D). Sid1 recruitment to the SPB is highly cell cycle regulated and is essential for the recruitment of Pmo25 to the anaphase SPB during normal cell cycle (Guertin et al., 2000). However we found that targeting Pmo25 to the SPB also leads to ectopic Sid1 recruitment to the SPB and SIN activation. It is conceivable that due to an inherent affinity between Pmo25 and Sid1 either protein can direct the other to the SPB (Figure 5-1). These data suggested that Pmo25 targeting at the SPB might act as a scaffold to promote Sid1 recruitment and SIN signaling. In the future, it will be important to test if Pmo25 shows physical interaction with Sid1 or the SIN scaffold proteins Sid4 and Cdc11 to identify the mechanism by which it can prematurely activate the SIN. Complementary experiments would include identification of the specific SPB binding or SIN activating regions within Pmo25 to better understand its role at the SPB. Pmo25 shows high sequence similarity to the human MO25 (Kanai et al., 2005). Based on crystal structure analysis, MO25 has a helical-repeat structure that can interact with putative ligands via distinct surfaces on the protein (Milburn et al., 2004). It will be interesting to test if Pmo25 also uses separate binding regions for binding to its MOR pathway targets in the cytoplasm and the SIN pathway components at the SPB.

Another possibility is that Pmo25 recruitment to the SPB is required for activation of the MOR in the following interphase (Figure 2-3C and (Kanai et al., 2005). This is reasonable since both SPB recruitment of Pmo25 and MOR activation in the next interphase require the same subset of SIN components. In

any case, how the SIN can both inhibit and activate the same pathway will be important to address in future studies. It is conceivable that it uses different mechanisms to promote the different physiological consequences at two different stages of the cell cycle. It will be important to test the possibility that the SIN causes specific post-translational modifications to Pmo25 either at the SPB or at the ring that leads to enhanced MOR signaling in the next interphase.

Figure 5-1. Sid1 and Pmo25 recruit each other to the SPB. (A) Sid1 localization to the SPB is highly regulated during the cell cycle to allow SIN activation in anaphase. Sid1 recruitment at the SPB in early anaphase promotes Pmo25 recruitment there. (B) Ectopic association of Pmo25 by fusion with the SPB protein Ppc89 causes precocious recruitment of Sid1 and SIN activation.

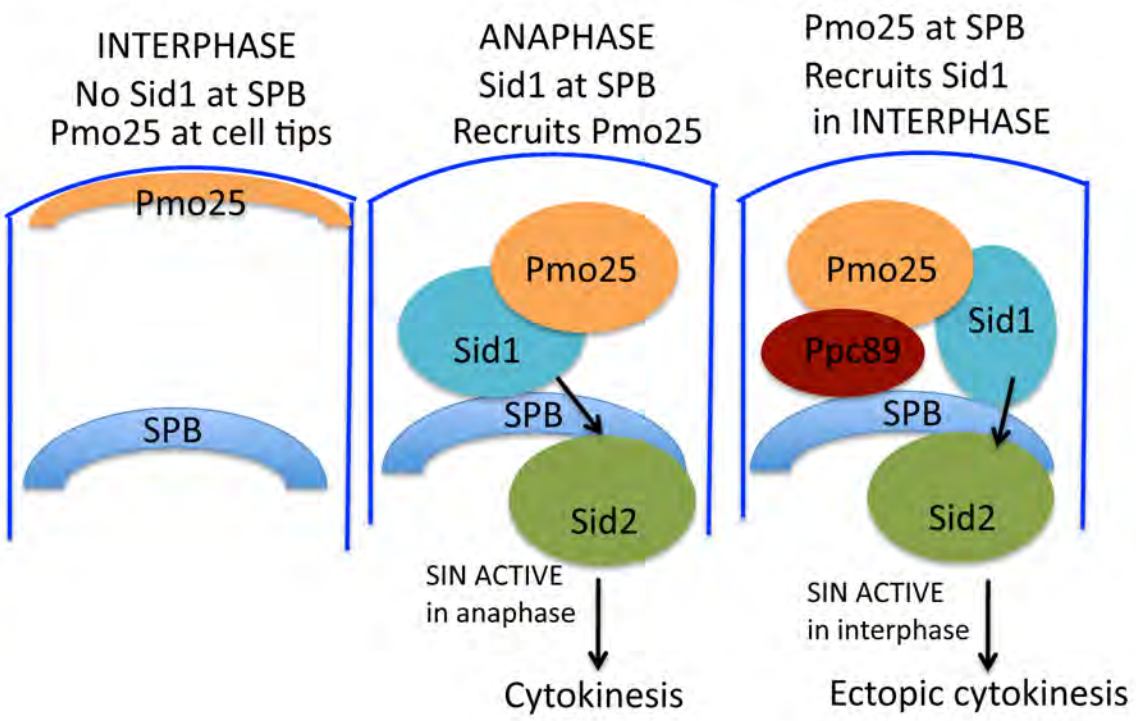


Figure 5-1: Sid1 and Pmo25 can recruit each other to the SPB

In contrast to the MOR component Pmo25, its downstream target Nak1 was localized to the SPB in early mitosis and was lost in early anaphase B around the time of SIN activation. Nak1 localization was independent of the activity of upstream SIN components Spg1 and Cdc7 that are normally at the SPB in early mitosis. It is possible that Nak1 recruitment is dependent on mitosis specific factors like Cdk1 and Plo1 (Archambault and Glover, 2009; Hagan, 2008). Interestingly, Nak1 has several putative Cdk1 phosphorylation sites and it will be worth testing if mutating those sites to either inactivating alanine mutations or phosphomimetic glutamate or aspartate mutations affects its recruitment to the SPB. Plo1 is localized at the SPB in early mitosis and remains there till early anaphase B (Mulvihill et al., 1999), which is very similar to Nak1 localization in mitosis. Future work using Plo1 mutants will help to clarify if Plo1 activation in mitosis also regulates Nak1 recruitment at the SPB.

We found that Nak1 recruitment was independent of all the SIN components at the SPB except the scaffold proteins Sid4 and Cdc11. Interestingly, disappearance of Nak1 from the SPB occurred around the time of SIN activation in late anaphase (Figure 3-1A). Subsequent analysis suggested that Nak1 disappearance was driven by SIN signaling (Figure 3-1C and 3-1D). Inactivation of all the SIN components except the scaffold proteins resulted in persistent Nak1 localization at the SPB till the end of spindle elongation. This suggested that SIN has a dual role in regulating MOR pathway components at the SPB; it recruits Pmo25 while displacing Nak1 from the same. One interesting

point to study is the mechanism by which SIN activation displaces Nak1 from the SPB. One possible mechanism of Nak1 displacement might involve Sid2 mediated phosphorylation of Nak1 resulting in Rad24 binding and subsequent displacement into the cytoplasm. This idea is based on previous work done by Chun-ti Chen (mentioned earlier) showing that Sid2 phosphorylation creates binding sites for 14-3-3 proteins and her discovery that Rad24-TAP pull down followed by mass spectrometry yielded Nak1 as a SIN target. It will be interesting to test if Nak1 localization to the SPB is retained in absence of Rad24. However, the other SIN kinase Sid1 would also be an important candidate to test.

Thus our data suggest that SIN signaling is essential for recruitment of Pmo25, and concomitant displacement of Nak1 from the SPB in early anaphase B. Since Nak1 kinase activity depends on Pmo25 and both these proteins physically associate (Kanai et al., 2005), it is possible that the SIN mediated displacement of Nak1 in anaphase is important to inhibit Nak1 activation and MOR signaling in anaphase by SPB localized Pmo25. This regulation could be important to maintain the inhibition of bipolar cell growth during cytokinesis. However expression of a fusion between Pmo25 and Nak1 showed only a partial bypass of the growth arrest in *cdc3-124 cdc16-116* cells that arrest with high SIN activity (Figure 3-2). One possibility is that the SIN maintains MOR inhibition by acting downstream of Pmo25-Nak1 interaction step and this was further supported by our Nak1-Orb6 fusion experiments in the same conditions. However, it is also possible that the fusion was not able to mimic the exact in vivo

nature of interaction between Pmo25 and Nak1 resulting in partial rescue of MOR inhibition in our experiments. Further work with the fusion will help to clarify the significance of the simultaneous recruitment and displacement of Pmo25 and Nak1 respectively at the anaphase SPB. It will be interesting to test the effect of constitutive SPB localization of the Pmo25- Nak1 fusion. Conversely, it is worth testing if cells have severe cell division defects when Nak1 and Pmo25 fail to localize at the SPB. Such experiments might also help to understand if Nak1 recruitment to the SPB in early mitosis is a mechanism to stop bipolar growth upon mitotic onset. Figure 5-2 presents a model for how SIN signaling regulates localization of the upstream MOR components at the SPB and maintains Orb6 inactivation.

Figure 5-2. Model: The SIN regulates SPB localization of the upstream MOR components and inhibits MOR pathway during mitosis. The MOR pathway components are localized at the cytoplasm and cell tips (green), consistent with their role in bipolar growth during interphase when the SIN in inactive. In early mitosis Nak1 (violet) is localized to the SPB by an unknown mechanism. In early anaphase, Sid1 recruits the upstream MOR component Pmo25 (blue) to the (SIN active) SPB. Simultaneous with the recruitment of Pmo25, Nak1 is displaced from the SPB. Pmo25 remains at the SPB until the end of cytokinesis, however, all the MOR components are localized as a ring on both sides of the septum at the time of septum formation.

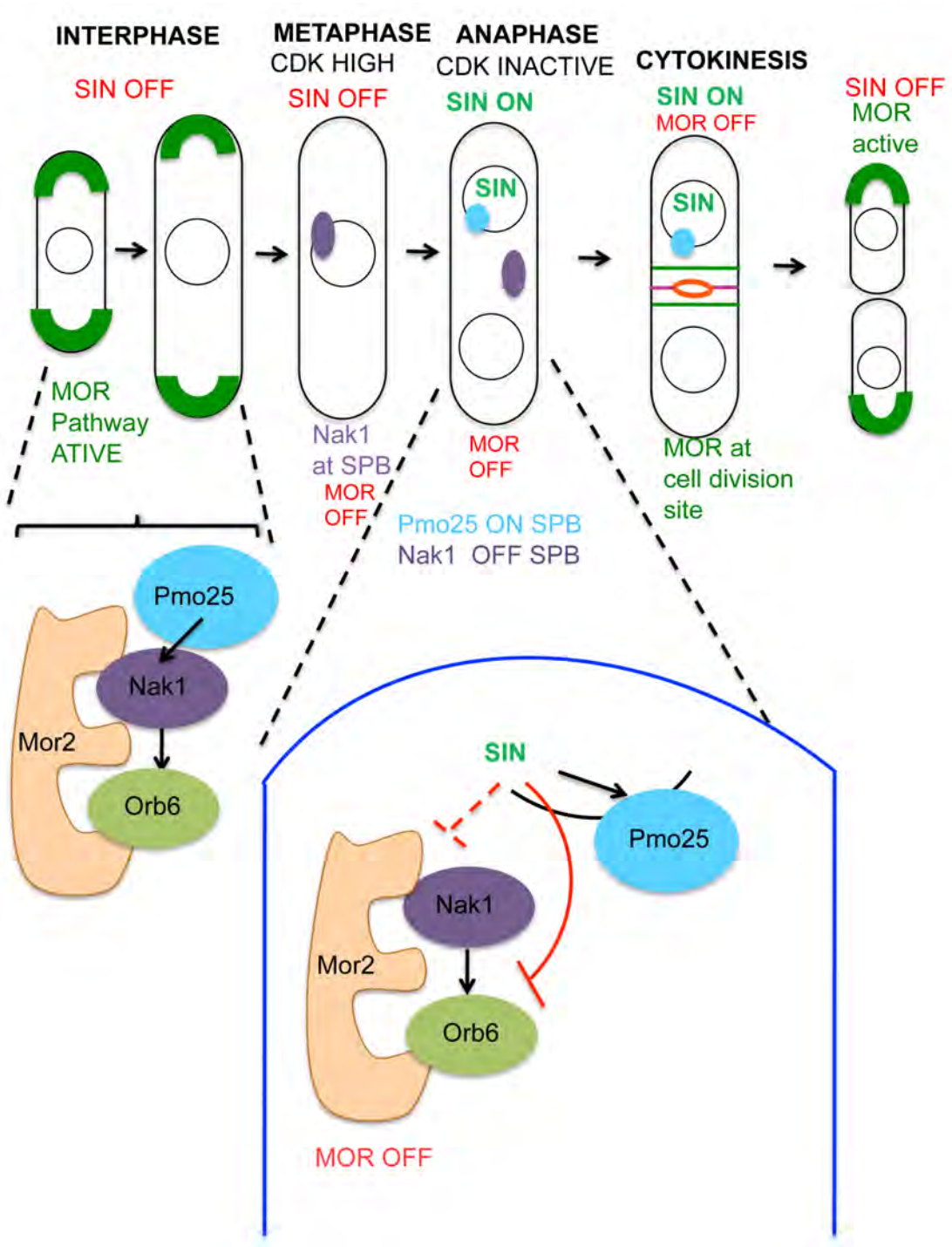


Figure 5-2: The SIN regulates localization and activity of the MOR in mitosis

The observation that *nak1-167* mutant (kinase inactive) produces ectopic septation in metaphase arrested cells (Figure 3-4 and Table 3-1) implicates Nak1 in inhibition of SIN signaling in early mitosis. Thus removal of Nak1 in late anaphase could be crucial to promote and maintain SIN activation and its functions. It will be interesting to check if the role of Nak1 at the SPB is dependent on its kinase activity alone or does it physically compete with other SPB localized SIN components like Sid1 and Sid2. In that respect it will be useful to check localization of the *nak1-167* allele. Failure of *nak1-167* to localize to the SPB would imply that Nak1 could physically compete with its homolog in the SIN pathway Sid1 to block its SPB localization in early mitosis.

Role of nucleolar regulators in SIN signaling

Activation of the SIN is closely correlated with inactivation of mitotic cyclin and loss of Cdk1 activity. In contrast SIN signaling appears to inhibit CDK activity (Trautmann et al., 2001). This reciprocal correlation between the SIN and Cdk1 activity makes SIN and its functions sensitive to the rising Cdk1 activity of the next cell cycle if cytokinesis is delayed due to pertubation of the cell division machinery. However, to get around this issue the SIN acts in close association with Clp1 phosphatase (*S.pombe* homolog of the *S.cerevisiae* Cdc14). The SIN acts positively on Clp1, which dephosphorylates Cdk1 sites and inhibits Cdk1 activity by inactivating the Cdk1 activator Cdc25 phosphatase (Wolfe et al., 2006). Clp1 is not essential for normal cytokinesis but becomes essential in the

event of a cytokinetic delay to inhibit Cdk1, allowing the SIN to remain active until cytokinesis is complete (Trautmann et al., 2001). Clp1 is a nulceolar protein in interphase but exits into the cytoplasm during mitosis where it is retained till the end of cytokinesis. The cytoplasmic localization of Clp1 is essential for maintenance of SIN signaling and cytokinesis since premature entry of Clp1 into nucleolus is associated with cytokinesis failure. Interestingly, the activity of Clp1 is in turn dependent on the SIN, which maintains its cytoplasmic localization during mitosis (Chen et al., 2008). Hence the SIN and Clp1 are involved in a posititve feedback interaction that is crucial for the maintenance of cytokinesis checkpoint in the event of a cytokinetic delay (Figure 5-3).

Figure 5-3. SIN-Clp1 interaction. Positive feedback interaction between the SIN and Clp1 maintains the cytokinesis checkpoint.

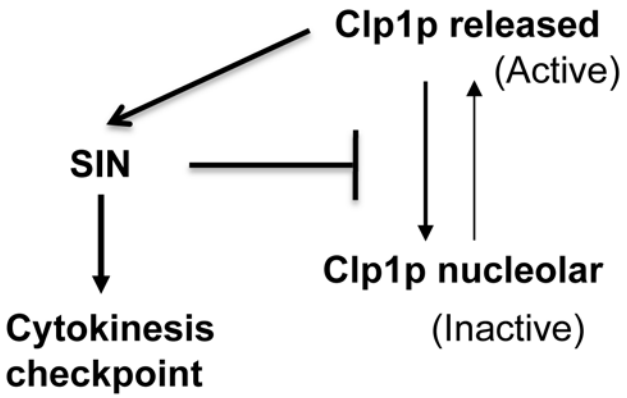


Figure 5-3: Positive feedback interaction between the SIN and Clp1

In an attempt to identify other regulators of the SIN and the cytokinesis checkpoint, we performed a screen to look for suppressors of the cytokinesis defect in weakened SIN mutants. It had been previously shown that the temperature sensitive SIN mutant *cdc14-118* was able to grow at semi-permissive temperature of 30°C but was defective in SIN signaling and the cytokinesis checkpoint (Mishra et al., 2004). Hence when combined with the ring mutation (*myo2-E1)*, which caused a delay in cytokinesis, *cdc14-118 myo2-E1* cells failed in cytokinesis and died as multinucleate cells at 30°C. We used the *cdc14-118 myo2-E1* cells to look for spontaneous suppressors of the cytokinesis checkpoint defect at 30°C. Our screen identified Dnt1, Rrn5, and one unidentified protein that we named Sdc1 (Suppressor of defective checkpoint). Astonishingly, Rrn5 was the nucleolus localized upstream activator of RNA Polymerase (Pol) 1 (Figure 4-3). Further, *rrn5-s6* and *sdc4-12* showed unique ring like nuclear staining characteristic of mutants defective in DNA topoisomerase I and II and the RNA polymerase I largest subunit mutant *nuc1-632* (Hirano et al., 1986a; Hirano et al., 1989). Based on the nuclear phenotype of the mutants, the *nuc1+* gene has been implicated in nucleolar organization and function. Interestingly, the *nuc1-632* mutant also rescued the cell division defect of *cdc14-118 myo2-E1* cells suggesting that general inactivation of rRNA transcription machinery can rescue the cytokinesis defects. Consistent with defective rRNA transcription in *rrn5-s6*, *nuc1-632* and *sdc4-12* cells, these mutants grew slowly at permissive temperature. Although

Dnt1 inactivation did not result in reduced growth or characteristic nuclear ring phenotype, Dnt1 was also localized in the nucleolus and showed strong negative genetic interaction with the other Pol1 defective suppressors (Figure 4-3,Table 4-1).

Interestingly, homology searches showed strong sequence conservation of the N-terminal 180 amino acids between Dnt1 and the *S.cerevisiae* Net1/Cfi and Tof2. Both Tof2 and Net1 are nucleolar and involved in rDNA silencing. Net1 was identified in a similar screen for suppresssors of the MEN (Mitotic Exit Network), which is the budding yeast homolog of the SIN. The main function of Net1 is to maintain the nucleolar localization of the budding yeast Clp1 homolog, Cdc14. In budding yeast Cdc14 phosphatase is nucleolar and inactive in interphase and is not released until early anaphase when it triggers cyclin destruction and mitotic exit (Stegmeier and Amon, 2004) . Since a nucleolar anchor for the fission yeast homolog Clp1 has not yet been identified, we tested if Dnt1 had similar functions as Net1. Unfortunately, our results showed that Dnt1 was not required for Clp1 nucleolar localization (Figure 4-7). Further, Dnt1 failed to recue *net1Δ* cells suggesting that Net1 and Dnt1 are not functionally interchangeable. Interestingly, we also found that *dnt1Δ* rescued the *cdc14-118 myo2-E1* cells independent of Clp1 (Figure 4-8 and 4-9). Altogether, these results ruled out the possibility that Dnt1 was the Net1 homolog in fission yeast and suggested that Net1 and Dnt1 use different mechanisms to regulate the MEN and SIN respectively.

The results in Chapter 4 revealed a novel role for nucleolar RNA Pol1 components in regulation of the SIN and cytokinesis. Since RNA Pol1 transcription activity is essential for rDNA silencing and at least one of the suppressors, Dnt1, when tested was found to be required for rDNA silencing (Figure 4-6C), we wondered if loss of rDNA silencing was responsible for the rescue of cytokinesis defect. However, our experiment with deletion of Sir2, which is required for rDNA silencing (Shankaranarayana et al., 2003) showed no rescue of the *cdc14-118 myo2-E1* suggesting that the RNA Pol1 components have a novel mechanism of rescuing cytokinesis defects in fission yeast cells. Besides, rRNA transcription is associated with ribosome biogenesis, which is essential for protein synthesis, thus we also tested the possibility that the rescue was due to general defects in protein synthesis rates or protein levels. However, that did not seem to be the case either. Future work will be important to understand how RNA Pol1 inhibits SIN signaling in fission yeast cells. It will be interesting to systematically test the effect of the individual suppressors on recruitment and activity of different SIN components. Why does the RNA Pol1 machinery inhibit SIN and its functions? Although our results seem perplexing it is conceivable that SIN inhibition is essential to ensure that cytokinesis is not initiated until the completion of rDNA segregation which has been reported to lag behind the majority of the genomic DNA during mitotic chromosome segregation in yeast cells (Granot and Snyder, 1991) . In budding yeast the main target of the MEN, Cdc14, inhibits Pol 1 transcription

in late anaphase to allow rDNA condensation and faithful genomic separation (Clemente-Blanco et al., 2009). However in fission yeast, the SIN target Clp1, is not essential for rDNA segregation (Chen et al., 2006b). Interestingly, budding yeast nucleolar protein Nop15 that is essential for pre-rRNA processing has been shown to be essential for regulation of cytokinesis (Oeffinger and Tollervey, 2003) suggesting that the nucleolus is an essential regulatory hub for cell growth and division (Dez and Tollervey, 2004). Our results in fission yeast suggest a negative interaction between rRNA transcription machinery and the cytokinesis apparatus. Thus it is possible that the connection between nucleolar regulators and cell division is different between the budding and fission yeast systems. Conversely, it will also be interesting to test if the SIN has a counter-inhibitory effect on Pol 1 transcription machinery. Future work will help to understand the molecular details of the regulation of cytokinesis by Pol1transcription machinery and its implications during the cell cycle.

Concluding Remarks

Rearrangement of the actin cytoskeleton is one of the essential factors that needs to be regulated during the transition between bipolar growth and cytokinesis and vice versa. In fission yeast, SIN signaling is essential regulator for stable maintenance of the contractile ring apparatus and septum formation at the end of mitosis. Following cytokinesis the MOR pathway becomes essential to

initiate polarized growth in the next cell cycle. Our data unravels a novel connection between these two conserved pathways and suggests that coordination between these pathways is essential for spatial and temporal regulation of the yeast cytoskeleton during the transition between essential cell cycle stages. In the process, we also unraveled clues to suggest that the upstream MOR components might have mitosis specific roles that are mediated by their SPB localization. Future work will be important to clarify our results and to understand the mechanisms that drive those processes. In an independent study, we also unraveled role of several nucleolar regulators in cytokinesis regulation. In future it will be worth exploring if Pol1 transcription machinery can directly affect SIN signaling or has independent mechanisms to regulate SIN functions during cell division.

Homologous NDR pathways exist in mammals; and while the homologous pathways in mammals are not identical to those in yeast, nevertheless, regulation and functions of some of the key components of these pathways are conserved between yeast and humans (Figure 5-4 shows a comparison of the different NDR pathways between fission yeast and humans). Given the complexity of the human system compared to single celled yeast cells, it will not be surprising if regulation of the SIN and MOR components in humans involved several other dimensions specific to cell type, cellular environment and developmental stage.

Figure 5-4. NDR kinase cascades are similar between fission yeast and humans**.** Although identical signaling cascades similar to the SIN and MOR have not been identified in humans, the basic activation mechanisms of the different NDR kinases and their regulators are conserved.

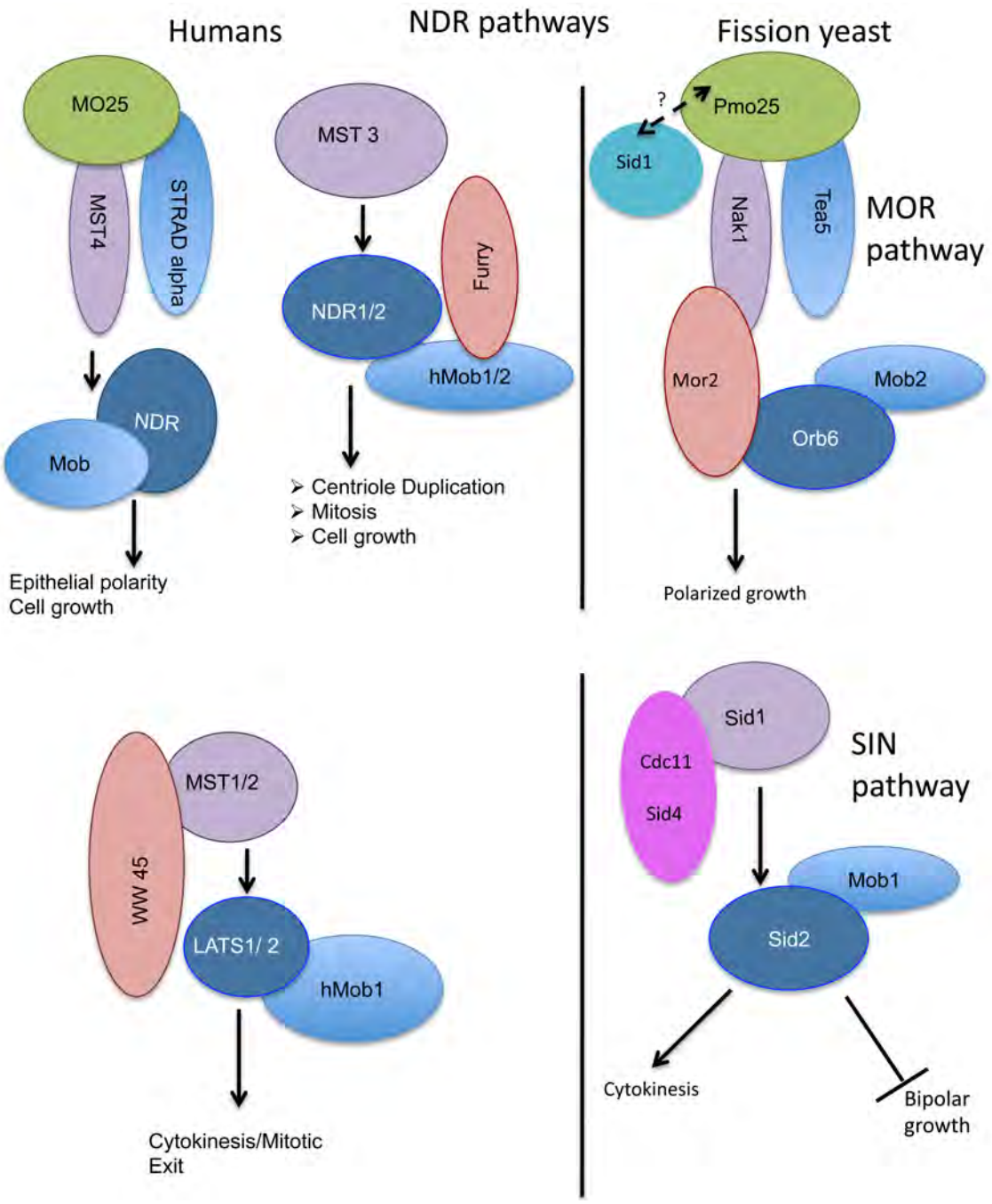


Figure 5-4: NDR kinase pathways are conserved between fission yeast and humans

Nevertheless, in the future it will be interesting to test if regulation of cell division and growth by mammalian NDR pathways involves similar cross-talk mechanisms as those identified by our studies.

The existence of cross-talk between the two separate NDR pathways is an example of how cells coordinate the timely and precise execution of distinct processes in a cell cycle dependent manner. Instances of similar cross-talk interactions among signaling cascades has also been found in the MAPK (mitogen-activated protein kinase) pathway, which is one of the most extensively studied signal transduction pathway. Cross-pathway interaction amongst the different MAPK pathways ensures efficiency of signaling through a particular cascade in response to the appropriate stimuli to produce the desired output (Schwartz and Madhani, 2004). Disruption of the normal cross-talk interaction can cause unregulated growth and proliferation, often associated with tumor formation (Zhang and Liu, 2002). Work in genetically tractable systems like the budding yeast, in which many of the major MAPK signaling components are highly conserved, have helped to dramatically increase our understanding of the molecular mechanisms that connect MAPK pathways and the cell cycle machinery (Schwartz and Madhani, 2004; Wilkinson and Millar, 2000). Along similar lines, further research towards the detailed understanding of the molecular mechanisms that connect NDR pathways and cell cycle machinery in fission yeast could help to advance our understanding of similar regulation by the

NDR pathway components in humans. We are just beginning to understand the biological roles of the NDR kinases during the animal cell cycle. Cell cycle regulation is essential for the normal proliferation and development of multi-cellular organisms; loss of control ultimately leads to cancer. Moreover, NDR kinases have been identified as tumor suppressors and oncogenes in different cancers (Hergovich et al., 2008). Thus, complete understanding of the NDR pathways will be important to not only understand how normal proliferating cells maintain the balance between growth and division but could also be useful for identification of drug targets for disease therapy.

Appendix A

Characterization of the *S.pombe* homolog of the budding yeast Sog2

The MOR pathway is similar to the RAM pathway in budding yeast in terms of the functional architecture. Some of the key MOR components Orb6, Mob2 and Mor2 are orthologous to the core RAM pathway components Cbk1,Mob2 and Tao3 respectively(Nelson et al., 2003). However, no Sog2 orthologs have been characterized in the fission yeast. Upon database search we found that the fission yeast ortholog of budding yeast Sog2 is a leucine-rich repeat protein Sog2 with the accession number NP_596483. We decided to further characterize the putative fission yeast Sog2.

Sog2 is essential: Knock-out of the Sog2 ORF inhibited spore germination and spores died with “orb” phenotype suggesting that Sog2 was an essential gene, which was required for polarized growth (Figure A-2).

Sog2 Localization: We then tested the localization of the protein by tagging the genomic locus of Sog2 with GFP. We did a time-lapse movie to follow Sog2 localization through cell cycle and found that the localization of Sog2 was very similar to that of Nak1 (Figure A-1). In interphase G2 cells, it was mostly in the cytoplasm and partially at the cortical cell ends. However, in mitosis it was localized to the SPB similar to Nak1. It remained at the SPB till early anaphase,

when it was lost from the SPB similar to Nak1. Interestingly, around the time of cytokinesis, Sog2 was localized at the cell middle near the division site like all the other MOR components (Figure A-3). These results suggested that Sog2 could be a part of MOR pathway.

Sog2 as a binding partner of Nak1: Since Sog2 localization was similar to that of Nak1 during the cell cycle we tested if there was any association between the two proteins by traditional co-immunoprocipitation experiment. As shown in Figure A-4, Nak1 and Sog2 showed physical association in our IP experiments. Since SIN activation caused inhibition of MOR signaling, we tested if SIN also had an effect on the association between Sog2 and Nak1. However, the asociation between Nak1 and Sog2 was intact in the *cdc16-116* background (Figure A-4). Further we also observed that similar to Nak1, removal of Sog2 from the SPB in anaphase depended on SIN signaling since in the SIN mtuants, Sog2 was maintained at the SPB longer than the wild-type background (data not shown). These results suggest that Sog2 could be the binding partner of Nak1. Further work would be facilitated by availability of a temperature-sensitive mutant of Sog2 that has been constructed by the lab of Dai Hirata. It will be interesting to identify how this new MOR component regulates cell growth, particular MOR signaling.

Figure A. Characterization of the fission yeast homolog of the budding yeast Sog2.

(1) Localization of Sog2 during mitosis. (2) Sog2 knock-out was lethal. (3) GFP tagged Sog2 localized to the cell division similar to other MOR pathway components at the time of cytokinesis. (4) Sog2 associates with Nak1 in normal and SIN hyper-activated background.

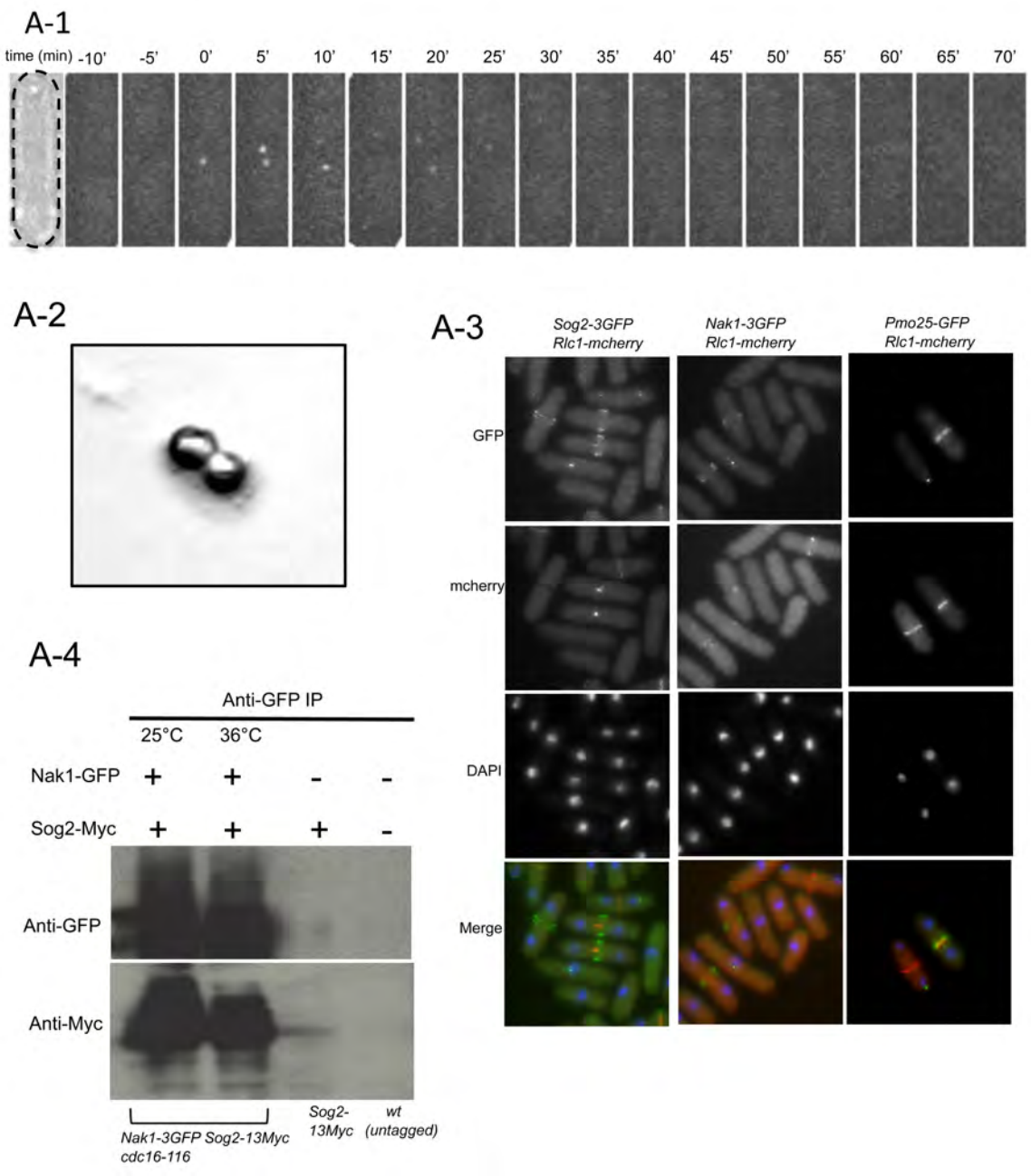


Figure A: Characterization of the fission yeast Sog2

Appendix B

MATERIALS AND METHODS

CHAPTER II

Yeast strains culture and flow cytometry conditions

Fission yeast media, growth conditions, and manipulations were carried out as described previously (Moreno et al., 1991). Except where noted, cells were grown in yeast extract (YE) medium. Flow cytometry analysis (FACS) were performed on isolated nuclei as described previously (Forsburg and Rhind, 2006) using the Becton-Dickinson FACScan flow cytometer**.**

Microscopy

GFP and fusion proteins were observed in cells that were grown in YE (unless otherwise mentioned) after fixation with -20°C methanol or in live cells for time-lapse studies. DNA and septum material were visualized by staining with 4'6',-diamidino-2 phenylindole (DAPI-Sigma Aldrich) and Calcofluor White (CW-Sigma Aldrich) respectively. Cell staining with DAPI and Alexafluor-488-conjugated phalloidin were performed as described previously (Balasubramanian et al., 1997). Images were acquired at room temperature using a Nikon Eclipse E600 microscope equipped with a Hamamatsu Orca-ER cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and IPlab

Spectrum software (Scanalytics, Fairfax, VA). Z-series of images were captured with a 100X oil (numerical aperture (N.A.) =1.3) objective lens and 3D stacked view was obtained using the IP Lab software. For time-lapse studies, exponentially growing cells were concentrated and 1.8µl of cell suspension (in YE) was placed on a microscope slide between a 2% YE-agar pad and a cover slip, which was sealed using VALAP. The cells were maintained at 36°C in a 20/20 Technologies micro-incubator and images were acquired on a Nikon TE 2000-E2 inverted microscope equipped with a Solamere Technology Group CSU10B spinning disk confocal system controlled by MetaMorph software. Time-lapse Z-series of images was captured with a Nikon 60x Plan Apo oil objective (NA =1.4) using a Rolera MGi EMCCD camera and processed using Metamorph software to get the maximum projection of the z-stack images.

Plasmid construction

The fusion construct between the Nak1 and Orb6 genes was expressed in the pREP41-GFP vector system(Craven et al., 1998). The fusion products were sequenced to ensure there were no frame shift mutations. The kinase dead allele of Nak1 was made by mutating the conserved Lysine (K39) site in the proposed ATP binding region of the Nak1 kinase to Arginine (R) using the Stratagene Quickchange site-directed mutagenesis kit. The resulting constructs were sequenced to confirm the changes.

Immunoprecipitation and Immunodetection

Preparation of cell extracts, immunoprecipitation, immunodetection, and kinase assays were performed as previously described (Huang et al., 2003; Kanai et al., 2005; Sparks et al., 1999). Briefly, exponentially growing 2 X 10^8 cells were collected by centrifugation and cell pellets were frozen in liquid nitrogen. All subsequent steps were carried out on ice or at 4°C. Cells were thawed in ice after addition of NP-40 Buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 6 mM Na_2HPO_4 , 4 mM NaH_2 PO_4) supplemented with protease inhibitor cocktail for fungal and yeast extract (Sigma P8215). After centrifugation for 3 minutes at 3000 rpm (Beckman), 1.5 ml of glass beads (425-600µm G8772 Sigma) were added to the cells and vortexed vigorously for 1 minute. Another 1ml of NP-40 buffer was added and the lysates were cleared by centrifugation at 8X1000 rpm for 10 minutes in a microfuge and the supernatant was collected. The cell lysates were subject to a pre-clearing step by incubation with 20µl of Protein G-Agarose or 15µl Dyna1 beads (as appropriate, see below) by rocking at 4°C for 45 minutes followed by centrifugation to collect pre-cleared lysates. Relative protein amounts in the pre-cleared lysates were estimated using BCA Protein Assay Kit (Pierce Biotechnology).

For immunoprecipitations of Myc-tagged Sid2, 0.2 µg anti-Myc monoclonal IgG (9E10, Santa cruz) was added to the NP-40 pre-cleared lysates (described above) and incubated on a rocker for 1 hr at 4°C. Immune complexes were

prepared by adding 30 µl of a 1:1 slurry of Protein G-Agarose beads (Sigma), incubating for 1 hour followed by centrifugation in a microfuge for 1 min. Beads were washed three times with 1 ml NP-40 buffer. For immunoprecipitation of GFP-tagged Nak1 and Myc-tagged Mob2, pre-cleared cell lysates in NP-40 buffer (as above) were added to anti-GFP monoclonal IgG (A1120, Invitrogen) or anti-Myc monoclonal IgG bound magnetic Protein G Dynabeads (DYNAL,Invitrogen) and incubated at 4°C on a roller for at least 2 hours, following which beads were washed thrice in NP-40 buffer as before.

Pre-binding the Protein G Dynabeads to anti-GFP monoclonal antibody: Appropriate volume of dynabeads was washed once with Citrate phosphate buffer (pH=5) and re-suspended in equal volume of the buffer. Required amount of antibody was added to the bead suspension in separate tubes and incubated on a roller at 4°C for at least 3 hours. Before the addition of the precleared lysate, beads are washed once with 100 µl of the citrate phosphate buffer.

For detection of the epitope tagged proteins, cell lysates and immunoprecipitates were separated by SDS-PAGE (7%) and transferred to Immobilon-P nylon (Millipore) using a wet transfer apparatus (Biorad). Blots were probed with anti-Myc IgG (Santa Cruz) or anti-GFP IgG (Santa Cruz) at a 1:500 dilution, and developed using HRP chemiluminescent detection system (West Pico, Thermoscientific).

In vitro Kinase assays

Kinase assays were performed as described previously (Huang et al., 2003; Sparks et al., 1999; Leonhard et al., 2005). Briefly, immune complex bead preparations were washed twice in 1ml kinase assay buffer (10mM Tris-HCl, pH 7.4, 10mM $MgCl_2$, 0.1mM sodium vanadate, 1mM DTT for Sid2 kinase assays; and 50mM Tris-HCl, pH 7.4, 100mM NaCl, 10mM $MgCl_2$, 1mM $MnCl_2$, 1mM DTT for Nak1 kinase assays) supplemented with a yeast protease inhibitor cocktail (Sigma). Washed immunoprecipitates were incubated at 30°C for 30 mins in 20 µl respective kinase buffer containing 10 µg myelin basic protein (M1891, Sigma) for Sid2 kinase assays, or 5 µg alpha-casein (C-8032, Sigma) for Nak1 kinase assays, 5µCi of [P^{32}] γATP (Perkin Elmer) and 50 µM unlabelled ATP per reaction. Reactions were stopped with the addition of 20 µl of 2X SDS sample buffer and subject to SDS-PAGE. The gels were dried and quantified using a phosphor imager.

CHAPTER III

Yeast strains culture and flow cytometry conditions

Fission yeast media, growth conditions, and manipulations were carried out as described previously (Moreno et al., 1991). Except where noted, cells were grown in yeast extract (YE) medium.

Plasmid construction

The fusion construct between Pmo25 and Nak1 and that between Pmo25 and Ppc89 (C-t) genes was expressed in the pREP41/42-GFP vector system(Craven et al., 1998). For both the fusions, Pmo25 was PCR amplified from genomic DNA with Nde1 and BamH1 sites in the primers. Nak1 was PCR amplified from the genomic DNA fragment (pBluescript containing the entire Nak1 gene in the HindIII insert) with the BamH1 and Sma1 sites in the primers. Ppc89 (C-t) = Ppc89 (280-783 amino acid) was also PCR amplified from the stock vector with the BamH1 and Sma1 sites in the primers. Following restriction digestion of the inserts, they were inserted into appropriately cut vectors through a three-piece ligation. The fusion products were sequenced to ensure there were no frame shift mutations.

Microscopy

GFP and fusion proteins were observed in cells that were grown in YE (unless otherwise mentioned) after fixation with -20°C methanol or in live cells for time-lapse studies. DNA was visualized by staining with 4'6', -diamidino-2 phenylindole (DAPI-Sigma Aldrich). Images were acquired at room temperature using a Nikon Eclipse E600 microscope equipped with a Hamamatsu Orca-ER

cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and IPlab Spectrum software (Scanalytics, Fairfax, VA). Z-series of images were captured with a 100X oil (numerical aperture (N.A.) =1.3) objective lens and 3D stacked view was obtained using the IP Lab software. For time-lapse studies, exponentially growing cells were concentrated and 1.8µl of cell suspension (in YE) was placed on a microscope slide between a 2% YE-agar pad and a cover slip, which was sealed using VALAP. Time-lapse images were taken at room-temperature using a Zeiss Axiovert 200 microscope with an argon ion laser system (Mellers Griot), images were captured with an IEEE 1394 digital CCD camera C4742-80-12AG (Hamamatsu) and UltraVIEW^{TM} RS confocal imaging system software (Perkin Elmer).

CHAPTER IV

Yeast media, strains and genetic manipulations: The fission yeast strains used in this study are listed in Table 1. Genetic crosses and general yeast techniques were performed as previously described (Moreno et al., 1991). *S. pombe* strains were grown in rich medium (yeast extract [YE]) or Edinburgh minimal medium (EMM) with appropriate supplements (Moreno et al., 1991). EMM with 5 µg/ml of thiamine was used to repress expression from the *nmt1* promoter. YE containing 100 mg of G418 (Sigma) per liter was used for selecting

Kan^r cells. For latrunculin B treatment in cytokinesis checkpoint experiments, a final concentration of 2-5µM was used. YE containing 5 µg/ml TSA (trichostatin A) (Sigma) was used to test the sensitivity of mutant cells. For serial dilution drop tests for growth, 3 serial 10-fold dilutions were made and 5µl of each was spotted on plates with the starting cell number of 10^4. Cells were pre-grown in liquid YE or EMM at 25°C and then spotted onto YE or EMM plates at the indicated temperatures and incubated for 3 to 5 days before photography. In rDNA silencing assays, N/S refers to EMM with glutamate, containing adenine, leucine, and uracil at 75 mg/liter each. 5-Fluroorotic acid (5-FOA) plates contain N/S supplemented with 2 g of 5-FOA (Toronto Research Chemicals) per liter. Ura$^-$ is N/S medium without uracil. The minichromosome loss assay was performed as previously described (Allshire et al., 1995). The rate of minichromosome loss was determined by the frequency of half-sectored colonies. *S. cerevisiae* strain PJ69-4A was used as the host strain in two-hybrid analyses (James et al., 1996) and was transformed using the LiAc/PEG procedure (Gietz et al., 1995). Leu$^+$ and Trp$^+$ transformants were selected and scored for positive interactions by spotting onto synthetic dextrose plates lacking histidine. Synchronous populations of *S. pombe* cells at early G2 were generated by centrifugal elutriation using a Beckmann JE 5.0 rotor.

Isolation of suppressors of cytokinesis checkpoint and cloning of *rrn5*$^{+}$

The *cdc14-118 myo2-E1* strain was grown at 25°C and then plated at 30°C to select for random suppressors. In total, 68 colonies were picked and crossed to wild-type to determine if they represented single mutations, and whether they had phenotypes on their own. Most suppressors only weakly suppressed either the *cdc14-118* or *myo2-E1* single mutants and were not analyzed further. However, 9 mutants could suppress *cdc14-118* and *myo2-E1* single and double mutants. These mutants fell into 3 complementation groups: group I (6 members: *14*, *16*, *2-12*, *2-13*, *3-8* and *4-3*), group II (2 members: *6* and *3-3*) and group III (1 member: *sdc4-12*). In the course of mapping group I mutations, we also crossed them to *dnt1Δ*, because, although the *dnt1Δ* strain was generated as part of a separate study, we suspected *dnt1* might be identified in our screen and we knew that *dnt1Δ* could rescue *cdc14-118 myo2-E1*. Group I mutations were all tightly linked to *dnt1*$^{+}$, with no double mutants with *dnt1Δ::ura4*$^{+}$ isolated out of more than 20 complete tetrads dissected. This suggested that group I mutations might be alleles of *dnt*$^{+}$. Subsequently, we amplified the *dnt1*$^{+}$ gene from the group I mutants by PCR and sequenced the PCR products confirming that 3 of the group I mutants carried mutations in the ORF of the *dnt1*$^{+}$ gene: in suppressor *14*, it had a 17 nucleotides insertion between nucleotides 230 and 231 in the coding region, which introduced a premature stop codon TAA; in *sup16*, one nucleotide is deleted at base 1503 causing a frameshift and a stop codon 20 amino acids before the C-terminal end of the protein; in suppressor *4-*

3, nucleotide 492 is deleted, causing a premature stop codon at nucleotide 500. For the other 3 group I mutants (*2-12*, *2-13*, *3-8*) obtained from our screen, sequencing of the ORF of the *dnt1*$^{+}$ gene did not reveal any mutations, suggesting that they may carry mutations outside the coding region, that affect gene expression or RNA stability. Suppressor number *6* was cloned by complementation of its temperature sensitive phenotype using an *S. pombe* genomic library (Clontech). Several plasmids were identified, and one gene called *rrn5*$^{+}$ was determined to be responsible for the rescuing activity. Therefore, we renamed suppressor *6* as *rrn5-S6.*

Epitope Tagging, Gene Deletion, and Cloning

Carboxy-terminal GFP and 13-*myc* epitope tagging of Dnt1 was done by PCR-based gene targeting (Bahler et al., 1998b). To construct the *dnt1* deletion strains, the entire *dnt1*-coding region was replaced with the *ura4*$^{+}$ gene or *kanR* cassette by homologous recombination.

For complementation experiment of budding yeast *NET1/CFI1*, *NET1/CFI1* gene was amplified by PCR from wild type budding yeast genomic DNA. The full-length *NET1/CFI1* open reading frame was first cloned into the entry vector of the Gateway System (Invitrogene), then it was cloned into destination vectors based on pREP41X and pREP3X, which were designed for expression in *S. pombe* (Choi and McCollum, unpublished data).

Microscopy:

GFP-fusion proteins were observed in cells after fixation with cold methanol or in live cells. DNA was visualized with 4', 6-diamidino-2-phenylindole (DAPI, Sigma) at 2µg/ml. Immunofluorescence microscopy of Gar2-GFP and Dnt1-13*myc* was done as described previously (Balasubramanian et al., 1997). Briefly, cells expressing Gar2- GFP and Dnt1-13*myc* fusions were fixed with methanol and digested with lysing enzymes (Sigma), followed by indirect immunofluorescence with polyclonal rabbit anti-GFP (Molecular Probes) and monoclonal mouse anti-myc (a gift from Dr. Kathy Gould). Secondary anti-mouse Texas red and Alexa 594-IgG (Molecular Probes) were used. Photomicrographs were obtained using a Nikon Eclipse E600 fluorescence microscope coupled to a cooled CCD camera (Hamamatsu, ORCA-ER) and image processing and analysis was carried out using IPLab Spectrum software (Signal Analytics Corporation, Vienna, VA).

APPENDIX A

Tagging and knock-out primers: Tagging and knock-out of the Sog2 genomic locus were performed using PCR based homologous recombination as described previously (Bahler et al., 1998b).

Sog2 genomic locus tagged at the C-terminal end using the following primers:

Forward: CAATTCCGGCGT CAGCCTATAG TACTAATAAA ATCGATTACT TTACTGACGC GGATGGGAAC GTGGAAGGTT TACAGCGC CGG ATC CCC GGG TTA ATT AA

Reverse: ACCATCATCCTTTAC AATTTGTCCT TGGTTTTGTT ATTTGAATAA GAAATAAATT AATCAAATTC AATCGTGCAT CATTT GAATTCGAGCTCGTTTAAAC

Following Primers were used for the Sog2 genomic locus deletion using ura4 as the selectable marker.

Forward:

TATTACTTCTTAGAGTTCCTTTTTTTTTCAACAAAACCTTCGGATTTACGTCTG AGGTGATCGATCTTTTCATGCT - CGCCAGGGTTTTCCCAGTCACGAC

Reverse:

TAAATTTATAGTCTTTGTAGGCTTTTGAGTTATCGAGTTACCAATTATTCCTCA TCAATCTTCATTAAAAAATTCT - AGCGGATAACAATTTCACACAGGA

Sog2 KO clone was tested using the forward primer: GCGAACTAGC TTGGCAGTAA AT and the reverse primer was used as described previously(Bahler et al., 1998b).

Microscopy:

GFP-fusion proteins were observed in cells after fixation with cold methanol or in live cells. DNA was visualized with 4', 6-diamidino-2-phenylindole (DAPI, Sigma)

at 2µg/ml. Images were acquired at room temperature using a Nikon Eclipse E600 microscope equipped with a Hamamatsu Orca-ER cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and IPlab Spectrum software (Scanalytics, Fairfax, VA). Z-series of images were captured with a 100X oil (numerical aperture (N.A.) =1.3) objective lens and 3D stacked view was obtained using the IP Lab software.

For time-lapse studies, exponentially growing cells were concentrated and 1.8µl of cell suspension (in YE) was placed on a microscope slide between a 2% YE-agar pad and a cover slip, which was sealed using VALAP. Time-lapse images were taken at room-temperature using a Zeiss Axiovert 200 microscope with an argon ion laser system (Mellers Griot), images were captured with an IEEE 1394 digital CCD camera C4742-80-12AG (Hamamatsu) and UltraVIEW^{TM} RS confocal imaging system software (Perkin Elmer).

Immunoprecipitation and Immunodetection

Preparation of cell extracts, immunoprecipitation, immunodetection, and kinase assays were performed as previously described (Huang et al., 2003; Kanai et al., 2005; Sparks et al., 1999). Briefly, exponentially growing 2 X 10^8 cells were collected by centrifugation and cell pellets were frozen in liquid nitrogen. All subsequent steps were carried out on ice or at 4°C. Cells were thawed in ice after addition of NP-40 Buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 6 mM Na_2HPO_4 , 4 mM NaH_2 PO_4) supplemented with protease inhibitor cocktail for

fungal and yeast extract (Sigma P8215). After centrifugation for 3 minutes at 3000 rpm (Beckman), 1.5 ml of glass beads (425-600µm G8772 Sigma) were added to the cells and vortexed vigorously for 1 minute. Another 1ml of NP-40 buffer was added and the lysates were cleared by centrifugation at 8X1000 rpm for 10 minutes in a microfuge and the supernatant was collected. The cell lysates were subject to a pre-clearing step by incubation with 15µl Dyna1 (invitrogen) beads by rocking at 4°C for 45 minutes followed by centrifugation to collect pre-cleared lysates. Relative protein amounts in the pre-cleared lysates were estimated using BCA Protein Assay Kit (Pierce Biotechnology).

For immunoprecipitation of GFP-tagged Nak1, pre-cleared cell lysates in NP-40 buffer (as above) were added to anti-GFP monoclonal IgG (A1120, Invitrogen) bound magnetic Protein G Dynabeads (DYNAL,Invitrogen) and incubated at 4°C on a roller for at least 2 hours, following which beads were washed thrice in NP-40 buffer as before.

For detection of the epitope tagged proteins, cell lysates and immunoprecipitates were separated by SDS-PAGE (7%) and transferred to Immobilon-P nylon (Millipore) using a wet transfer apparatus (Biorad). Blots were probed with anti-Myc IgG (Santa Cruz) or anti-GFP IgG (Santa Cruz) at a 1:500 dilution, and developed using HRP chemiluminescent detection system (West Pico,Thermoscientific).

Appendix C

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