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Understanding Regulation of the Cytoskeleton during Cell Cycle Transitions through Examination of Crosstalk between Homologous Fission Yeast Pathways, Septation Initiation Network and Morphogenesis ORB6 Network: A Dissertation

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UNDERSTANDING REGULATION OF THE CYTOSKELETON DURING CELL CYCLE TRANSITIONS THROUGH EXAMINATION OF CROSSTALK BETWEEN HOMOLOGOUS FISSION YEAST PATHWAYS, SEPTATION INITIATION NETWORK AND MORPHOGENESIS ORB6 NETWORK.

A Dissertation Presented

By

Sneha Gupta

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I dedicate this thesis to Ma & Papa

for their love and support

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Abstract

The fission yeast *Schizosaccharomyces pombe* has become a powerful model system for studying cytokinesis, a process of cytoplasmic division by which one cell divides into two identical daughter cells. Like mammalian cells, *S. pombe* divides through the use of an actomyosin contractile ring, which is composed of a set of highly conserved cytoskeletal proteins. Cytokinesis in *S. pombe* is primarily regulated by the SIN pathway, which is activated in late mitosis and is required for actomyosin contractile ring and septum assembly, and also plays a role in spindle checkpoint inactivation, and telophase nuclear positioning. The various functions of the SIN are carried out by the terminal kinase in the pathway called Sid2. The lack of information in the downstream targets of Sid2 has limited our understanding of the different functions of the SIN. We recently showed that, in addition to its other functions, the SIN promotes cytokinesis through inhibition the MOR signaling pathway, which normally drives cell separation and initiation of polarized growth following completion of cytokinesis (Ray et al, 2010). The molecular details of this inhibition and the physiological significance of inhibiting MOR during cytokinesis was unclear. The results presented in Chapter II describe our approach to identify Sid2 substrates, particularly focusing on Nak1 and Sog2 that function in the MOR signaling cascade. We identified and characterized Sid2 phosphorylation sites on the Nak1 and Sog2 proteins. Chapter III explores how post translational modification of MOR proteins by Sid2 regulates polarized growth during cytokinesis. This includes delineating the effect

of Sid2 mediated phosphorylation of Nak1 and Sog2 on protein-protein interactions in the MOR pathway as well as on the regulation of their localization during late mitosis. Finally, results in Chapter IV demonstrate that failure to inhibit MOR signaling is lethal because cells initiate septum degradation/cell separation before completing cytokinesis thereby emphasizing the importance of cross-regulation between the two pathways to prevent initiation of the interphase polarity program during cytokinesis.

Table of Contents

List of Tables

List of Figures

List of Abbreviations or Nomenclature

3-AT	3-Aminotriazole
AD	Activation Domain
AM	Actomyosin
ATP	Adenosine triphosphate
BD	Binding Domain
CAR	Contractile Actomyosin Ring
CDK	Cyclin Dependent Kinase
CLP1	Cdc14-like-phosphatase 1
CW	Calcoflour White
DAPI	4',6-Diamidino-2-Phenylindole
DIC	Differential interference contrast
EMM	Edinburgh Minimal Medium
FRY	Furry
GAP	GTPase Activating Protein
GCK	Germinal Center Kinase
GEF	Guanine Exchange Factor
GFP	Green Fluorescent Protein
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
IP	Immunoprecipitation
KA	Kinase Assay
LatA	Latrunculin A
LATS1/2	Large Tumor Suppressor 1/2
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MAPK	Mitogen Activated Protein Kinase
MBP	Myelin Basic Protein
MEN	Mitosis Exit Network
MOR	Morphogenesis Orb6 Network
NDR	Nuclear Dbf2-related
NETO	New end take off
PAGE	Polyacrylamide gel electrophoresis
PAK	P21 activated kinase
PCH	Pombe Cdc15 Homology
PH	Pleckstrin homology

PMSF	Phenylmethylsulfonyl fluoride
RAM	Regulation of Ace2 and morphogenesis
RFP	Red fluorescent protein
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SIN	Septation Initiation Network
SIP	SIN Inhibitory Phosphatase
SPB	Spindle Pole Body (fission yeast centrosome)
TAP	Tandem affinity purification
WB	Western Blot
WT	Wild type
YE	Yeast extract
YFP	Yellow fluorescent protein

Preface

Copyright Information

Results presented in Chapters II, III and IV have appeared in publications listed below:

1) **Gupta S**, McCollum D. Crosstalk between NDR kinase pathways co-ordinates cytokinesis with cell separation in *S. pombe.* (*Manuscript in preparation*)

2) **Gupta S***, Cipak L*,Rajovic I, Anrather D, Jin QW, Ammerer G, Dannel McCollum and Juraj Gregan. (**Co-first authors*) Cross-talk between casein kinase II and Ste20-related kinase Nak1. Cell Cycle 2013

3) **Gupta S**, Mana-Capelli S, McLean JR, Chen CT, Ray S, Gould KL, and McCollum D. Identification of SIN pathway targets reveals mechanisms of crosstalk between NDR kinase pathways. Curr. Biol. 2013.

4) Ray S, Kume K, **Gupta S**, Ge W, Balasubramanian M, Hirata D, McCollum D. The mitosis-to interphase transition is coordinated by cross talk between the SIN and MOR pathways in Schizosaccharomyces pombe. J. Cell Biol. 2010; 190:793–805.

5) **Gupta S**, McCollum D. Crosstalk between NDR kinase pathways coordinates cell cycle dependent actin rearrangements. Cell Div. 2011; 6:19.

List of Third Party Copyrighted Material

Declaration of figure/table contributions by co-authors on publications listed in the previous section:

Chapter II

Table 2.1 was contributed by Sebastian Mana Capelli and Chun-Ti Chen

Tables 2.2 and 2.3 were contributed by Janel R McLean from Kathleen Gould's lab at Vanderbilt University School of Medicine

Figure 2.1 was contributed by Sebastian Mana Capelli

Figure 2.2A was contributed by Samriddha Ray

Figure 2.3 C was contributed by Sebastian Mana Capelli

Chapter III

Figure 3.8A was contributed by Samriddha Ray

Appendix A

Figure A.1 was contributed in part (Pmo25 localization) by Samriddha Ray

CHAPTER I

General Introduction

All proliferating cells alternate between two primary cell cycle stages, namely growth and division. Transition between the two stages is accompanied by dynamic rearrangements of the cytoskeleton, which is composed of actin, tubulin and associated accessory or regulatory proteins (Figure 1.1). For instance, motile interphase cells maintain the actin cytoskeleton near the cell periphery where it undergoes rapid reorganization to determine cell shape and movement based on extracellular stimuli such as growth factors. Such reorganization results in formation of distinct cytoskeletal structures such as lamellipodia, filopodia, focal adhesions and stress fibers (Figure 1.2) (Heng and Koh, 2010). However, during cell division, the cells reassemble the actin cytoskeleton at the division plane in the cell middle where it forms the contractile actomyosin ring (CAR) (Figure 1.2). Constriction of the ring, which is attached to the plasma membrane, creates a cleavage furrow that partitions the cell into two daughters (Eggert et al., 2006; Green et al., 2012; Glotzer, 2005). Execution of such drastic reorganization of the cytoskeleton in coordination with cell cycle progression necessitates a tight regulatory interaction between cytoskeleton signaling networks overseeing interphase and mitotic events. In fission yeast, Nuclear Dbf2-related (NDR) kinase signaling cascades called Morphogenesis Orb6 Network (MOR) and Septation Initiation Network (SIN) regulate growth and division respectively. NDR kinase pathways are conserved in higher eukaryotes where they retain similar

functions but have also acquired new ones (Gupta and McCollum, 2011; Johnson et al., 2012). How these signaling networks interact to coordinate cytoskeletal redistribution with cell cycle transitions is not well understood. A large part of my research focuses on delineating mechanisms of crosstalk between the two networks and understanding its impact on interphase growth and mitotic cell division in the fission yeast experimental system.

Figure 1.1 - Regulation of cytoskeletal remodeling is essential for cell cycle transitions.

This figure is a representation of actin localization (indicated in blue) in animal and fission yeast cells as they switch between interphase and mitosis.

Figure 1.1 - Regulation of cytoskeletal remodeling is essential for cell cycle transitions

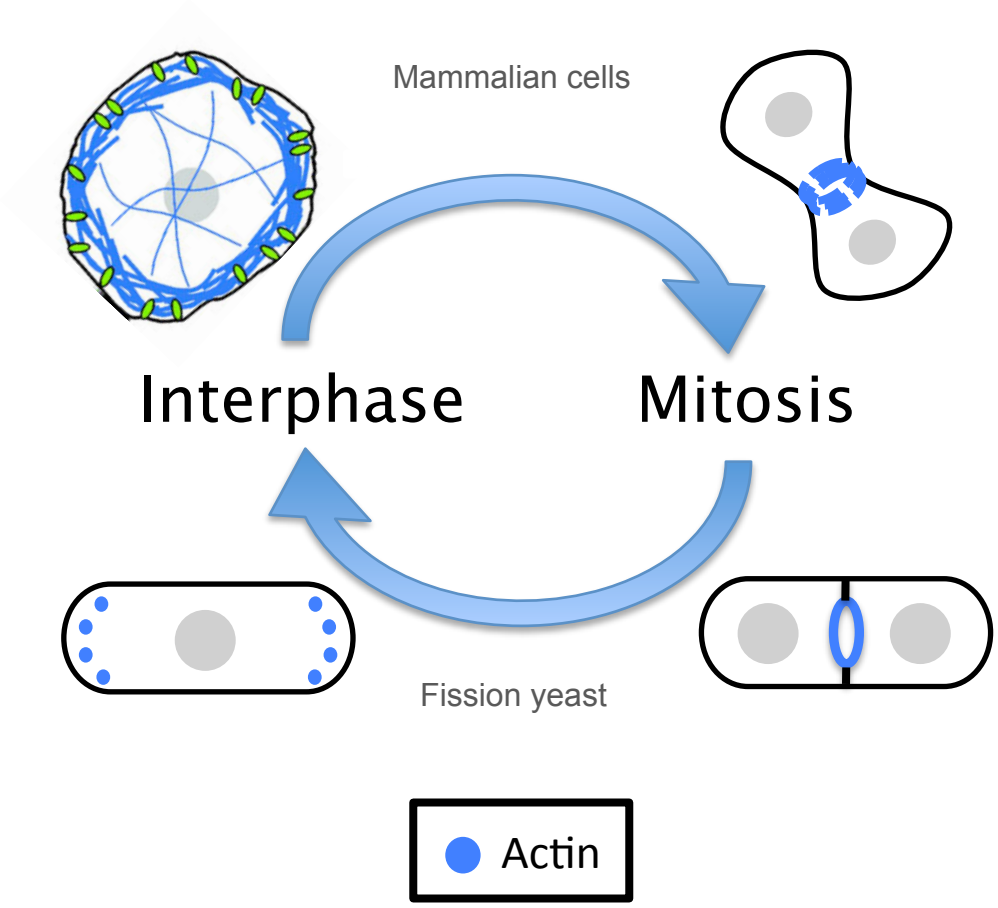
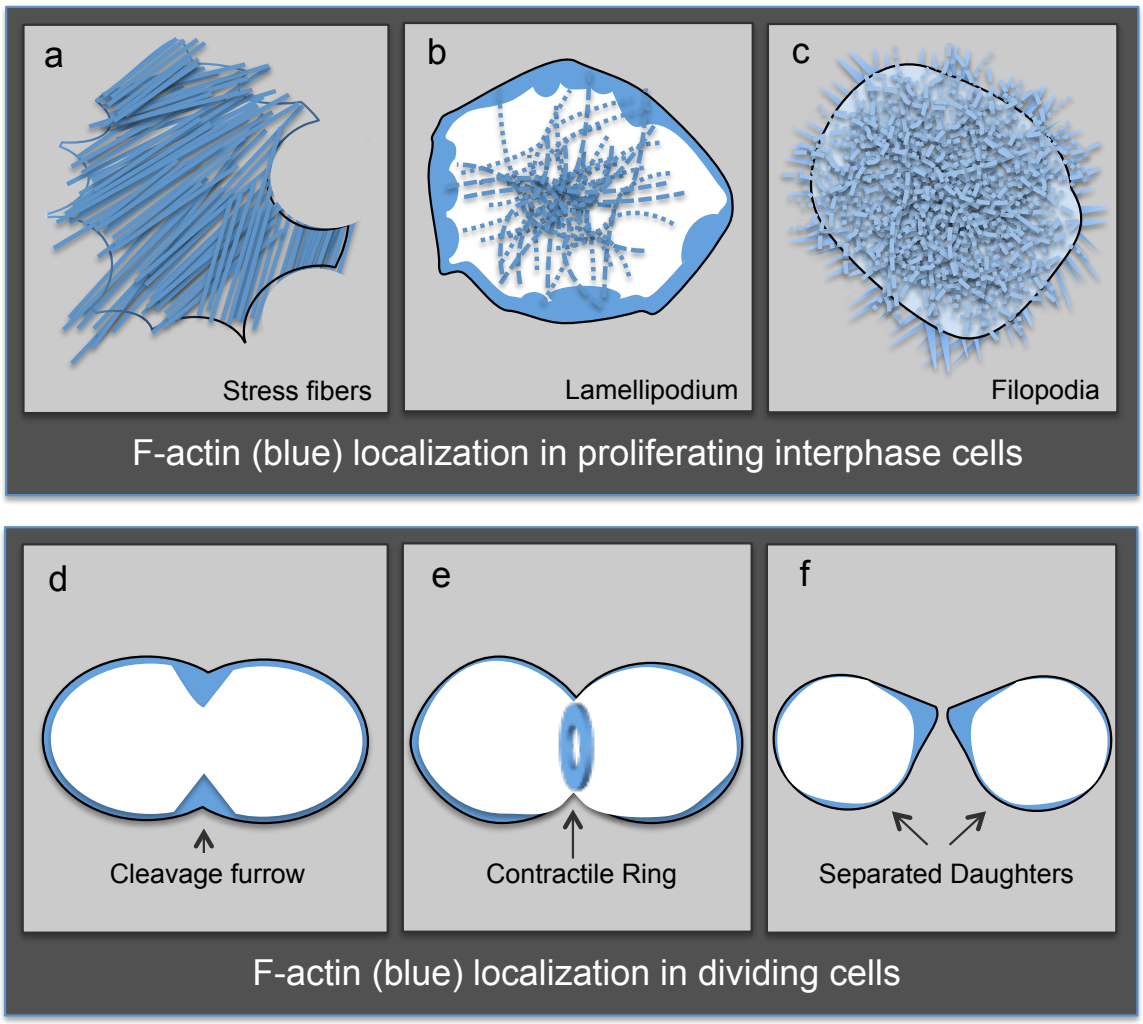


Figure 1.2 - Differential organization of the actin cytoskeleton based on cell cycle stage and external stimuli.

This figure illustrates images of distinct actin cytoskeletal structures formed in proliferating and dividing cells. Top panel shows stress fibers, lamellipodium and filopodia formation in proliferating cells. Bottom panel shows actin localization at the contractile ring in mitotic cells.

Figure 1.2 - Differential organization of the actin cytoskeleton based on cell cycle stage and external stimuli.



Role of the actin cytoskeleton in fission yeast morphogenesis

The fission yeast *Schizosaccharomyces pombe* are rod-shaped, unicellular organisms. Cell growth in *S. pombe* occurs through tip elongation and can be analyzed simply through measurement of increase in length, as the cells maintain a constant diameter. Cell division takes place by constriction of a medial actomyosin ring, much like the cleavage furrow in animal cells, a process easily visualized through microscopic techniques. Over the past two decades, identification and functional characterization of several fission yeast genes involved in growth and morphogenesis (Verde et al., 1995; Balasubramanian et al., 1998), with conserved roles in mammals, has spurred significant research in this area. In combination with a recently sequenced genome and availability of deletion libraries, *S. pombe* has become an excellent model system for studying morphogenetic changes through genetic as well as biochemical analysis.

In fission yeast, distribution of actin, which is essential for cell growth, coincides precisely with sites of cell growth and division (Marks et al., 1986). In fact, rate of cell growth at a particular cell site is directly proportional to the amount of actin present there (Gachet et al., 2004). Three distinct F-actin structures are present during the cell cycle, namely actin patches, cables and the ring. Each structure is associated with a different set of accessory proteins and is believed to perform distinct functions in cell growth and morphogenesis (McCollum et al., 1999; Subramanian et al., 2013; Kovar et al., 2011). For instance, Arp2/3, For3 and

Cdc12 are the different actin nucleation factors for patches, cables and ring formation, respectively. While actin patches are well established as sites of polarized secretion and cell growth, actin cables serve as tracks along the long axis of the cell on which a variety of cargo for cell growth and polarity are delivered either to the poles or to the equator depending on the cell cycle stage (Marks et al., 1986; Bendezu and Martin, 2011; La Carbona et al., 2006). The contractile actomyosin ring (CAR) is a structure that forms exclusively during mitosis and it determines the site of septum formation. Further, constriction of the ring in a centripetal fashion in combination with septum synthesis provides the "force" required for ingression of the cleavage furrow (Proctor et al., 2012). Following cell division, cells reorganize growth components at the old pole and initiate growth in a monopolar fashion. During G2, (at a length of about 9.5 microns) they undergo a transition from monopolar to bipolar growth, a process termed "New End Take-Off" or NETO (Mitchison and Nurse, 1985; Martin and Chang, 2005). Cells undergoing bipolar growth have actin localized to both poles and continue to grow until progression into mitosis (at a length of about 14 microns). Mitotic entry is accompanied by redistribution of actin from poles to the cell middle and any further growth ceases until division is complete (Figure 1.3).

It is evident that performing the tasks of cell growth and division requires significant restructuring of the actin cytoskeleton. Therefore, coordination of actin redistribution as cells transition between interphase and mitosis is presumably

important to keep competing actin polarity programs from interfering with each other. In fission yeast, two homologous NDR kinase pathways regulate actin polarity during the cell cycle. The Morphogenesis Orb6 Network (MOR) controls polarized growth required for cell tip elongation throughout interphase, and cell separation via septum degradation in the G1 phase after completion of cytokinesis. The Septation Initiation Network (SIN) is in charge of executing the final steps of cell division. Specifically, it carries out constriction of the contractile actomyosin ring that forms in early mitosis and is also required for deposition of the division septum. The two signaling networks direct the actin cytoskeleton towards completely different tasks. Consistent with this, duration of activation of these two signaling networks is mutually exclusive suggesting an underlying regulatory mechanism that prevents simultaneous activation of the two pathways (Figure 1.4).

Figure 1.3 – Actin rearrangement in the fission yeast cell cycle

Schematic representation of the fission yeast cell cycle phases (G1, G2 – Growth phases, S - DNA replication phase; M – Mitosis). The figure illustrates different actin structures (in blue) accompanied by change in actin dynamics with cell cycle transitions. Arrows indicate directionality of cell wall growth.

Figure 1.3 – Actin rearrangement with cell cycle in fission yeast

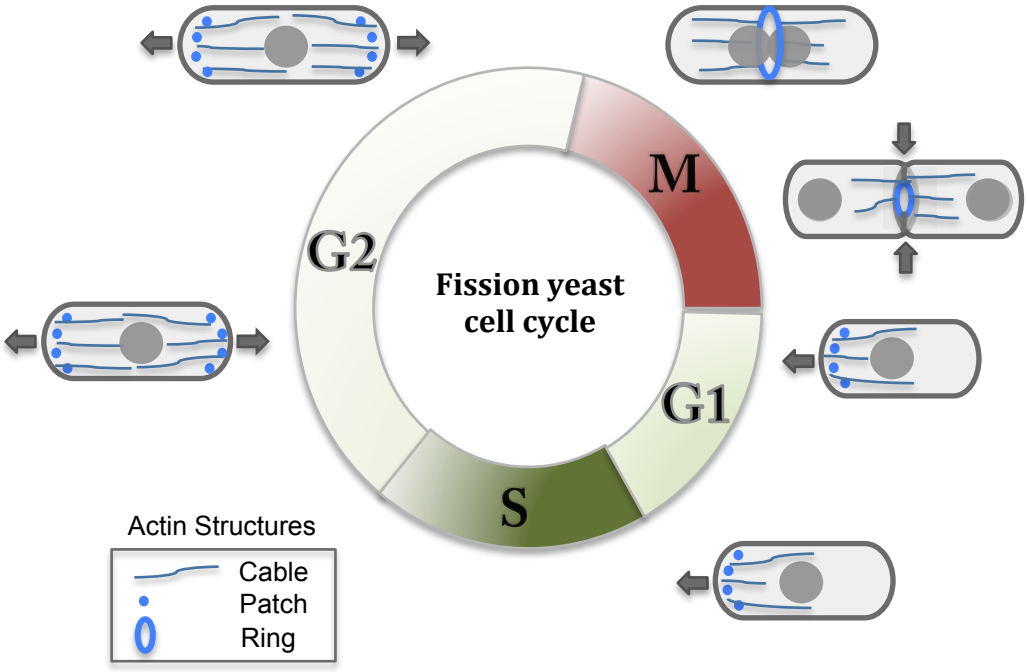
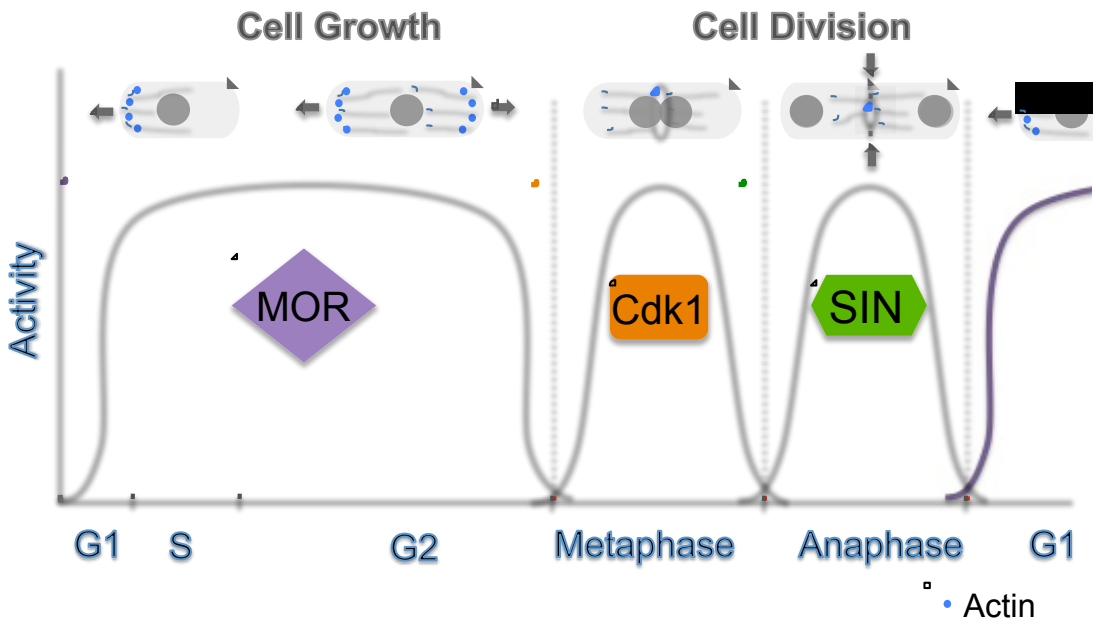


Figure 1.4 – Activation periods of SIN and MOR are mutually exclusive

A schematic of the cell cycle that represents activation times of MOR, Cdk1 and SIN signaling is diagrammed. Activity of the MOR pathway is high during interphase growth. Mitotic entry coincides with decrease in MOR activity and increase in Cdk1 activity. Upon anaphase progression, Cdk1 activity subsides accompanied by activation of the SIN. Finally, completion of cytokinesis results in SIN inactivation thereby concluding one cycle of cell division. Actin localization in the cell corresponding with indicated activation times is also shown.

Figure 1.4 – Activation periods of SIN and MOR are mutually exclusive



Regulation of the mitotic cytoskeleton and roles of the Septation Initiation Network (SIN)

Cell division is comprised of a series of events from selection of the division site and deposition of the division septum in mitosis to degradation of the primary septum that physically separates the daughter cells post mitotic exit. To accomplish these tasks, this cell-cycle juncture entails significant restructuring of the cytoskeleton to enable construction of the medial actomyosin ring, redirection of cell wall building cargo from the cell ends to cell middle and targeting of cell separation proteins to the newly formed cell wall. These events need to be coordinated precisely for successful cell division. The Septation Initiation Network (SIN), one of the two conserved NDR kinase pathways in fission yeast, is activated during late mitosis where it plays an essential role towards the end of the cell cycle in the process of cytoplasmic division called cytokinesis. Primary functions of the SIN include constriction of the centrally placed actomyosin ring and deposition of the septum wall that divides the cytoplasm equally. Execution of these late mitotic functions requires maintaining a mitotic configuration of the cytoskeleton (Figure 1.5). How the SIN accomplishes this is still not very well understood.

The mitotic cytoskeleton in *S. pombe* includes large multi-protein complexes called nodes that localize to the medial cortex and function to regulate both mitotic entry and actomyosin ring assembly. Entry into mitosis has been coupled

with cell length through an elegant mechanism involving spatial separation of the cell tip localized Pom1 kinase and its medial node localized target the Cdr2 kinase. Cdr2 in turn, inhibits Wee1, a kinase that prevents mitotic Cdk (Cdc2) activation through phosphorylation at Tyr15. In short cells, a gradient of Pom1 emanating from cell tips overlaps with Cdr2 containing medial nodes. As cells elongate in G2, Pom1 localization is maximized at the cell tips and decreases progressively towards the cell equator. Cells commit to mitosis when they achieve a sufficient length, as Pom1 can no longer inhibit mitotic entry via Cdr2 (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009). However, more recent reports argue that Pom1 may not act as a direct sensor of cell size (Wood and Nurse, 2013) and there may be an unidentified mechanism by which Cdk activity and mitotic entry is regulated.

Besides regulating Wee1 activity, Cdr2 is also required for anchoring of Mid1, a pleckstrin homology (PH) domain protein involved in a pathway that dictates division site placement to the medial cell cortex (Almonacid et al., 2009; Oliferenko et al., 2009). Release of Mid1, which shuttles between the nucleus and the cell cortex, from the nucleus upon mitotic entry is most likely mediated by the Plo1 kinase which both phosphorylates and directly binds Mid1 and is required for exit of Mid1 from the nucleus (Bähler et al., 1998; Almonacid et al., 2011). Mid1 appears to provide the initial spatial cue for recruitment of early contractile ring components (Rincon and Paoletti, 2012). Assembly of the

contractile actomyosin ring (CAR) components takes place in a precise and sequential manner. Two models have been proposed for CAR assembly. The Node model describes ring formation under normal wild-type conditions and involves assembly of protein nodes in the form of a band around the cell equator after Mid1 release from the nucleus (Pollard, 2008). Some of the early ring components recruited to the nodes include Mid1, Myo2, IQGAP (Rng2) and Formin (Cdc12) (Wu et al., 2003). Cdc12 mediated actin polymerization and capture of nearby actin filaments by Myo2 in the nodes (search and capture method) results in condensation of the nodes into a ring. In late anaphase (anaphase B), the ring matures through addition of components like tropomyosin (cdc8), α-actinin (Ain1) and Myp2. While initial assembly of the ring in early mitosis is believed to be largely independent of the SIN (Wu et al., 2003), the SIN is necessary for stabilization and maintenance of the ring in anaphase, which requires recruitment of the PCH-family protein Cdc15 (Hachet and Simanis, 2008). The SIN is also capable of driving ring formation, independent of cortical nodes through the aster model. Cells that form ectopic septa due to hyperactivation of the SIN have been shown to form orthogonal rings, albeit at slower rates, through a Mid1 independent mechanism although the rings are often mis-positioned. The aster model involves formation of a leading actin cable that arises from one side of the cell cortex and eventually condenses into a functional ring (Roberts-Galbraith and Gould, 2008; Hachet and Simanis, 2008; Huang et al., 2008). Furthermore, these cells display a loss of polarized growth

characterized by absence of tip localization of actin (Ray et al., 2010). Therefore, an active SIN is capable of driving cytoskeletal components away from the cell ends, inhibiting the interphase cytoskeletal conformation and reassembling the actin in the cell middle to form actomyosin rings.

It is important to keep in mind that SIN activity peaks only in late anaphase once chromosome segregation has begun. This timing is particularly necessary to avoid dividing the cytoplasm with unequal distribution of the genetic material (Balasubramanian et al., 2000). Mitotic Cdk1-CyclinB (Cdc2-Cdc13) represses SIN activity and decrease in Cdk1 activity is essential for initiation of SIN signaling (Bardin and Amon, 2001). However, the SIN does not directly regulate decrease in Cdk1 activity through Cdc13 degradation (Guertin et al., 2000). Therefore, early mitotic events such as division site selection, and initial CAR assembly take place prior to the activation of the SIN signaling pathway.

Upon progression into anaphase, a lowering of mitotic Cdk activity is accompanied by increase in SIN activity. SIN then initiates ring constriction coupled with deposition of the division septum. The division septum is formed centripetally and consists of a primary septum composed mainly of 1,3-β-glucan, which is sandwiched on either side by the secondary septum composed of sugars such as 1,3-α-glucan, 1,6-branched 1,3-β-glucan, 1,6-β-glucan and galactomannans (Ishiguro, 1998). Deposition of the division septum requires

enzymes for cell wall synthesis such as Cps1/Bgs1 (1,3-β-glucan synthase), which is essential for septum formation in *S. pombe* (Liu et al., 1999). Additionally, it requires redirection of polarized secretion away from the cell tips and towards the cell equator. Therefore, the process of division septum formation dictates reorganization of relevant protein complexes at the cell middle required for actin patch assembly as well as initiation of actin cable polymerization and preventing localization of these components to the cell tips, which is consistent with cessation of growth observed in mitosis. Additionally, although it was initially believed that constriction of the ring provides the mechanical force required for cell cleavage, recent studies show that ring constriction is only necessary to in the initial stages of cytokinesis. The process of septum deposition provides a majority of the force necessary to counter the turgor pressure within the cell and promote ingression (Proctor et al., 2012). Completion of septation is accompanied by SIN inactivation through an unknown mechanism that relies on SIN asymmetry (García-Cortés and McCollum, 2009).

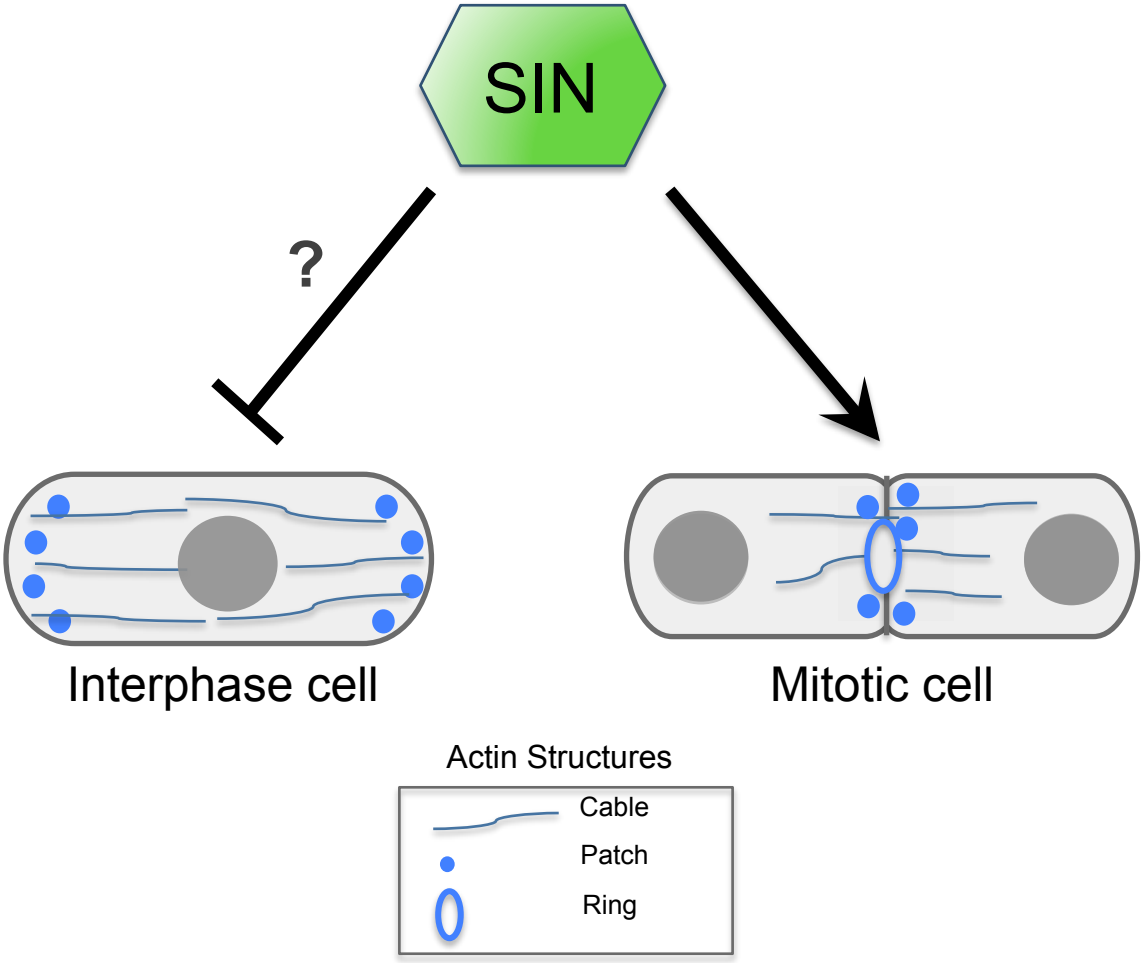
Loss of SIN activity causes inability to maintain a stable actomyosin ring as well as septation failure resulting in long multinucleate cells. This phenotype indicates that the cell is able to maintain growth and nuclear division in the absence of cytokinesis for a few cycles until the cells eventually lyse. (Balasubramanian et al., 1998; Sparks et al., 1999). Hyperactivation of the SIN (either through overexpression of SIN components, Plo1, Cdc7 or through mutation of its

negative regulator Cdc16), on the other hand, results in formation of ectopic septa in interphase as well mitotic cells (Schmidt et al., 1997; Minet et al., 1979). As mentioned earlier, this form of septation occurs through initial ring assembly in a SIN dependent manner that does not require assembly of cortical nodes. Also, activation of the SIN ectopically in interphase cells can suppress interphase polarity. Overall, these observations emphasize the importance of the SIN in maintaining cytokinesis proteins, which includes common cytoskeletal proteins that also constitute the interphase polarized growth machinery, at the cell equator and preventing the redirection of these shared components to the cell ends. Regulation of the cytoskeletal components in this manner appears to be fundamental for the execution of SIN functions in mitosis.

Figure 1.5 – The SIN maintains a mitotic cytoskeletal configuration

The schematic represents our understanding of regulation of the actin cytoskeleton by the Septation Initiation Network during mitosis.

Figure 1.5 – The SIN maintains a mitotic cytoskeletal configuration



Assembly and organization of SIN pathway components

All SIN components are anchored to the spindle pole body (Figure 1.6) through scaffolding proteins Cdc11, Sid4 and Ppc89 (Krapp et al., 2001; Morrell et al., 2004; Tomlin et al., 2002; Rosenberg et al., 2006). Core SIN components includes the upstream GTPase, Spg1, which localizes to the SPBs throughout the cell cycle and regulates the SIN signaling cascade. It is inhibited by a bipartite GAP consisting of Cdc16 and Byr4, (Furge et al., 1998; Cerutti and Simanis, 1999; Krapp et al., 2008). Association of Cdc16/Byr4 complex with the SPB in interphase is believed to keep SIN inactivated during interphase. Activation of Spg1 is thought to be carried out by Etd1, a putative GEF which localizes to the cell tips and therefore activates Spg1 only when the spindle is fully elongated and closer to the cell tips adding an additional level of regulation (García-Cortés and McCollum, 2009).

Upon activation, GTP bound Spg1 then sets in motion a protein kinase cascade composed of Cdc7, Sid1 (both GC/STE 20-like kinases) and Sid2 (an NDR kinase). Cdc14 and Mob1 are additional factors associated with Sid1 and Sid2 kinase respectively (Guertin et al., 2000; Hou et al., 2004; Salimova et al., 2000). Upon entry into mitosis, the GAP complex dissociates from both SPBs resulting in Spg1 activation and recruitment of Cdc7 kinase (Li et al., 2000). As cells enter Anaphase B, the GAP complex relocalizes to the old SPB resulting in asymmetric activation of the SIN and localization of the Cdc7 kinase to the new SPB lacking

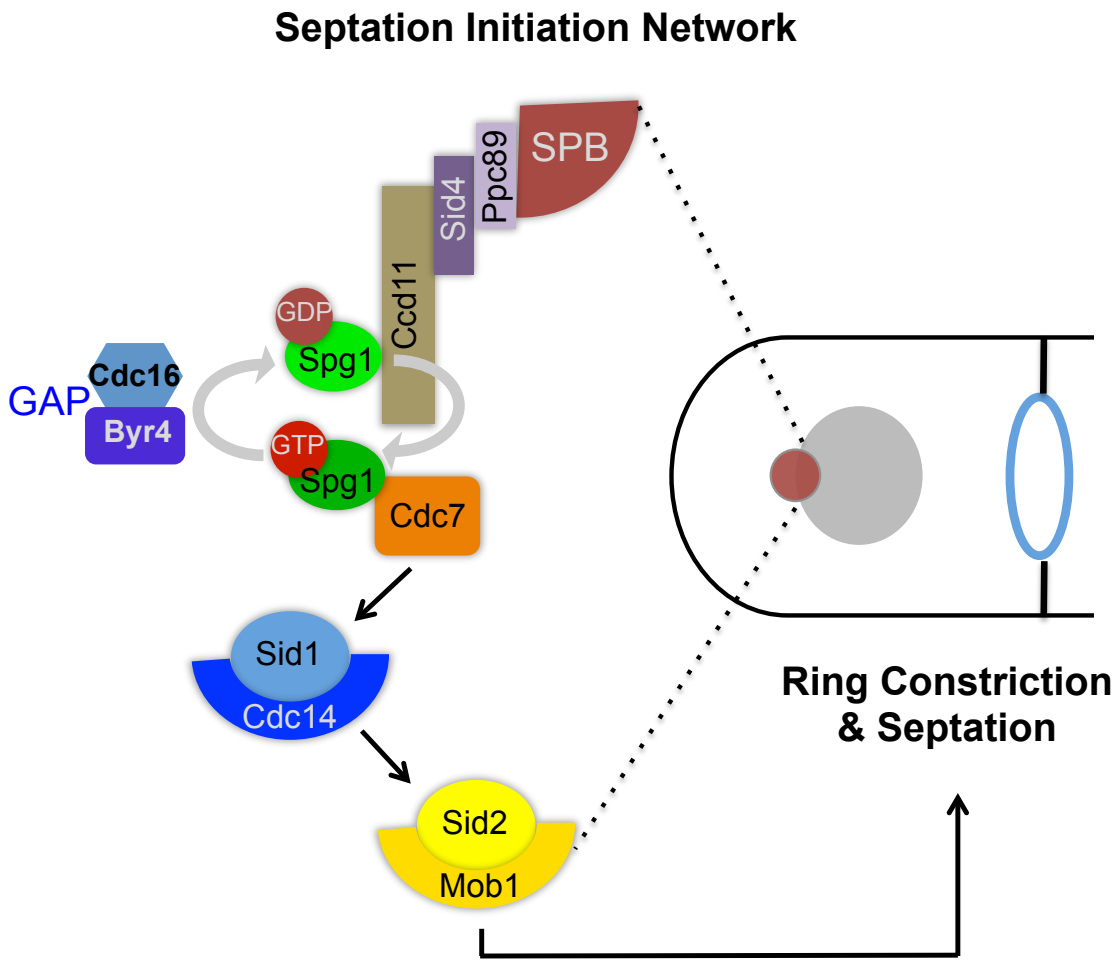
the GAP (Cerutti and Simanis, 1999). Recent work shows that asymmetric localization of Cdc7 kinase is mediated by Cdc11 dephosphorylation by the SIP (SIN Inhibitory Phosphatase) complex (Singh et al., 2011). This results in Byr4 recruitment and consequent depletion of SIN activity at the old SPB. An asymmetrically localized Cdc7 then recruits the Sid1 kinase along with the associated Cdc14 protein. Recruitment of Cdc7 and Sid1 are needed for the complete activation of the downstream SIN kinase Sid2 (bound to its binding partner Mob1), which localizes to the SPB throughout the cell cycle. It has been demonstrated that Sid2 mediated Cdc11 phosphorylation forms a positive feedback loop that promotes robust SIN signaling at the new SPB. Coupled with inactivation of the SIN at the old SPB, this enforces asymmetric activation the SIN that is essential for normal cytokinesis. A complete understanding of the significance of SIN asymmetry, while outside the scope of this thesis, is a fascinating area still open to further investigation.

Notably, the Sid2/Mob1 complex is the only SIN component, which upon activation by the upstream kinase Sid1, translocates from the SPB to the medial ring to carry out primary downstream functions of the SIN. These functions include the final steps in actomyosin (AM) ring assembly, ring constriction and formation of the division septum (Sparks et al., 1999).

Figure 1.6 – Components of the Septation Initiation Network (SIN)

A graphical representation of the core components of the SIN is illustrated in the figure. All SIN components are associated with the Spindle Pole Body (SPB) as indicated and carry out downstream functions of Contractile Actomyosin Ring (CAR) constriction (indicated in blue) and deposition of the medial septum.

Figure 1.6 – Components of the Septation Initiation Network (SIN)



Targets of the SIN

To completely understand the roles of SIN, identification of its targets, particularly targets of its downstream effector kinase, Sid2, which localizes to the medial ring and septum, was crucial. Sid2 kinases show a preference for the RXXS phosphorylation motif (Yaffe et al., 1997). The first Sid2 target identified was Clp1, a Cdc14 like phosphatase, with a conserved role in facilitating mitotic exit through dephosphorylation of Cdk1 and its substrates (Chen et al., 2008). Clp1 is also involved in mediating the cytokinesis checkpoint observed in *cps1* mutants (Liu et al., 2000). *cps1* mutants that fail cytokinesis complete the next cycle of replication and arrest as binucleates in the following G2 phase with an actomyosin ring. Clp1 plays a role in cytokinetic checkpoint by preventing further rounds of nuclear division as well in maintaining actomyosin rings to enable successful completion of cytokinesis (Mishra et al., 2004). Further, Clp1 is also known to interact with Mid1 at the ring and is essential for maintaining CAR integrity through execution of Cdc15 dephosphorylation (Clifford et al., 2008). We now know that dephosphorylation of Cdc15 is necessary step as hypophosphorylated Cdc15 assumes an open conformation believed to be recruited to the ring (Roberts-Galbraith et al., 2010). Clp1 localizes to the SPB and the nucleolus in interphase and is released from the nucleolus into the cytoplasm as cells enter mitosis. Chen *et. al.* demonstrated that Sid2 mediated phosphorylation of Clp1 maintains Clp1 in the cytoplasm through its association with the 14-3-3 binding protein, Rad24. Furthermore, upon perturbation of the

actin ring, the SIN maintains its own activity by keeping Clp1 in the cytoplasm and preventing Cdk1 activation.

Cdc11, a scaffold protein required for localization of SIN components to the SPB is another protein regulated by Sid2 phosphorylation (Feoktistova et al., 2012). Cdc11 phosphorylation by Sid2 promotes its interaction with the upstream SIN kinase Cdc7 at the new SPB. Cdc7 recruitment, in turn, forms a positive feedback loop that further enhances Sid2 activity. Simultaneously, deactivation of the SIN occurs at the old SPB through displacement of Cdc7. This is mediated by the newly identified SIN inhibitory Complex (SIP) which dephosphorylates Cdc11 (Singh et al., 2011). Therefore, Cdc11 phospho-regulation plays an important role in asymmetric activation of the SIN.

Additional function of the SIN in spindle elongation and telophase nuclear positioning was highlighted when the kinesin-14, Klp2 was identified as a Sid2 target (Mana-Capelli et al., 2012). Klp2, a minus end directed motor protein, localizes to microtubules (MTs) and plays a role in interphase nuclear positioning as well as mitotic chromosome alignment. Sid2 phosphorylation of Klp2 inhibits its localization to MTs by preventing its interaction with Mal3, an EB1 homologue that is required for loading Klp2 onto MT plus ends. This regulation has a two fold significance. Firstly, it is important to promote anaphase spindle elongation,

which requires plus-end directed movement. Secondly, during telophase, Klp2 is kept off the MTs to prevent clustering of the nuclei before septation is complete.

Bohnert *et al.* identified Cdc12, a fission yeast formin specifically required for cytokinesis, as one of the actomyosin ring components targeted by the SIN (Bohnert et al., 2013). Cdc12 localizes to the actomyosin ring and promotes nucleation and polymerization of F-actin filaments. Sid2 phosphorylates Cdc12 to prevent multimerization of the protein mediated by a previously unrecognized C-terminal FH2 domain on Cdc12 that provides F-actin bundling activity. This activity was found to be important for SIN dependent actomyosin ring maintenance.

Finally, Sid2 was found to phosphorylate the NIMA kinase Fin1 (Grallert et al., 2012). This study is the first demonstration of a Sid2 function outside of mitosis and independent of the rest of the SIN components. Specifically, Fin1 phosphorylation in G2 activates the kinase, which in turn regulates the Pom1/Cdr1/Cdr2/Wee1 cell geometry network to promote mitotic commitment. Activation of Fin1 however is short-lived, as a phosphorylated Fin1 quickly promotes its own destruction via ubiquitination thereby tightly regulating the window of G2-M progression.

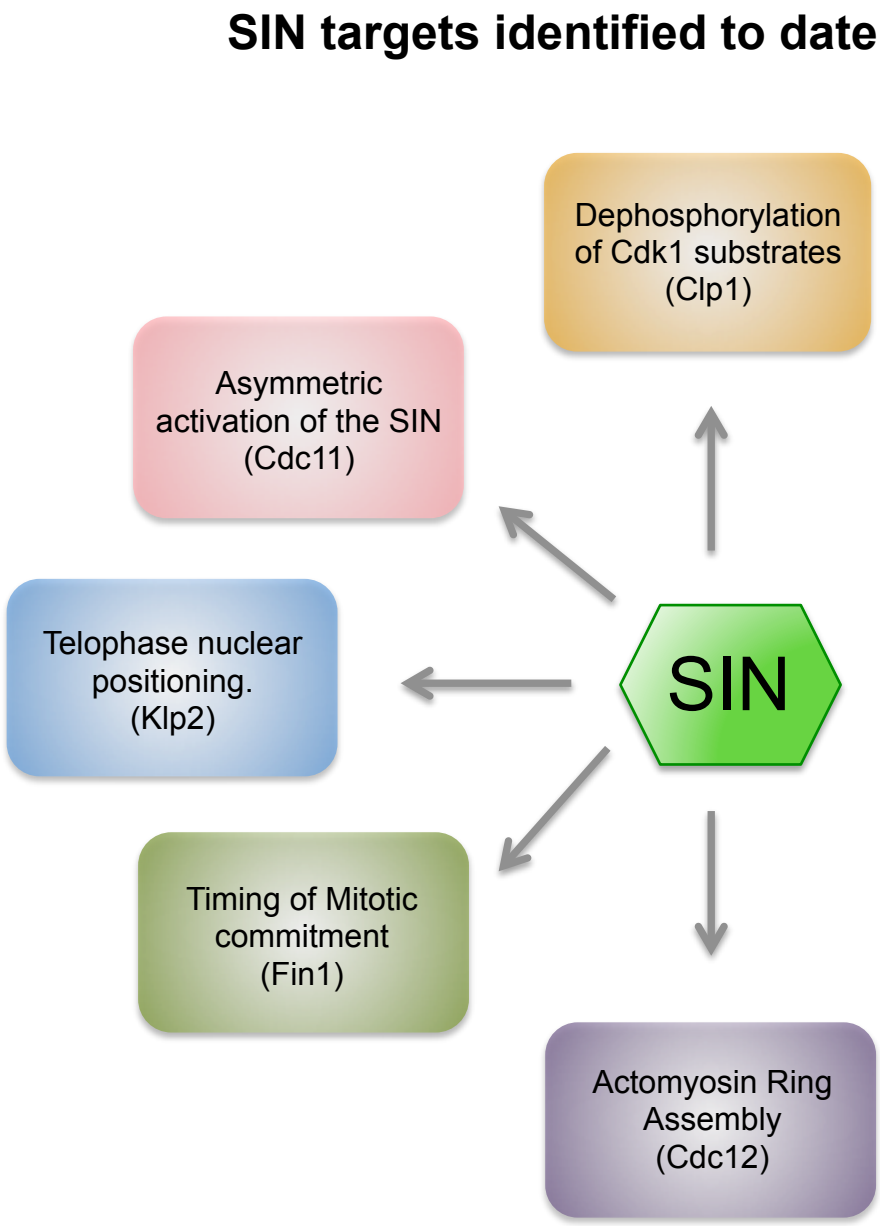
Over the past 5 years, our knowledge of SIN targets and functions has advanced

considerably (Figure 1.7). While rigorous analysis of SIN targets has furthered our understanding of the roles of the SIN pathway in processes such as the cytokinetic checkpoint, asymmetric activation of the SIN, anaphase spindle elongation, telophase nuclear positioning, actomyosin ring assembly and even timing of mitotic commitment, we still need to identify downstream effectors that allow the SIN to inhibit polarized configuration of the cytoskeleton and prevent interphase growth during mitosis.

Figure 1.7 – Known targets of the SIN

The figure illustrates five known targets of the SIN kinase, Sid2 identified to date. It also describes the respective functions regulated by the SIN in the fission yeast cell cycle.

Figure 1.7 – Known targets of the SIN



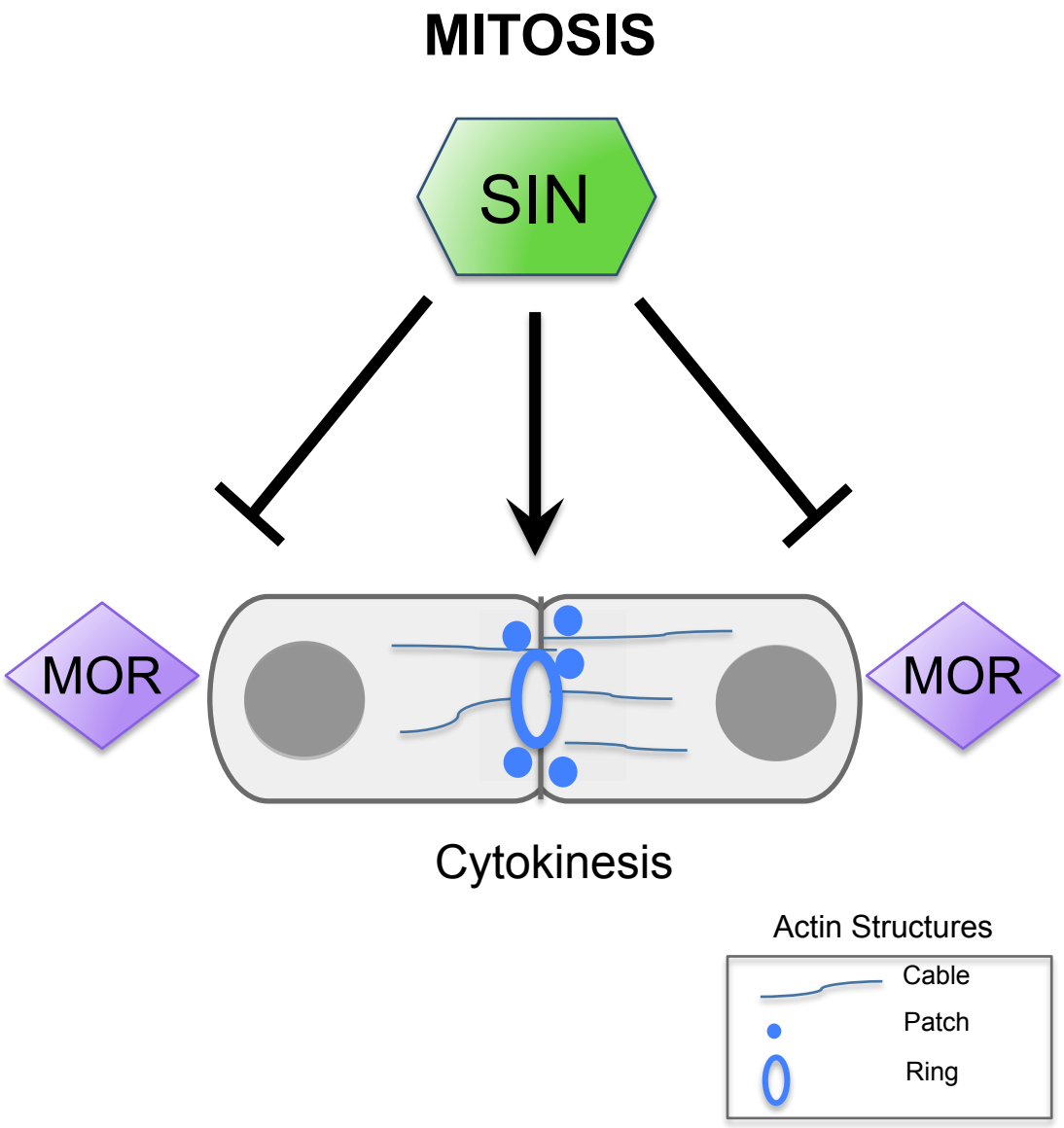
Morphogenesis Orb6 Network (MOR)

The Morphogenesis Orb6 Network (MOR), the other NDR kinase pathway in fission yeast, is active throughout interphase and controls cell morphology and tip growth through accretion of actin to sites of cell growth (Verde et al., 1998; Kanai et al., 2005; Kovar et al., 2011). Consistent with its function, mutants in any of the MOR components fail to grow in a polarized manner resulting in loss of rod-shaped morphology of the cells. Moreover, MOR mutants show dispersed actin localization that causes the round/pear shaped phenotype observed(Verde et al., 1995). It is interesting to note that ectopic activation of the SIN results in the same phenotype in interphase cells. Furthermore, like the SIN, the MOR pathway is also an NDR signaling pathway with a conserved kinase cascade. Hence, it is possible that SIN directly inhibits the MOR to prevent interphase configuration of the cytoskeleton. Previous work from our lab corroborates the presence of such crosstalk (Figure 1.8) by demonstrating that the SIN inhibits the MOR through reduction of the activity of its downstream kinase, Orb6 (Ray et al., 2010). However, no information is available on the mechanism of this inhibition or the molecular players that are involved in this cross-regulation.

Figure 1.8 – SIN inhibits the interphase polarity pathway, MOR

The schematic shows that SIN carries out cytokinesis as well as inhibits interphase polarity simultaneously during mitosis. Specifically, polarized growth is prevented through inhibition of MOR pathway activity.

Figure 1.8 – SIN inhibits the interphase polarity pathway, MOR



Core components of the MOR

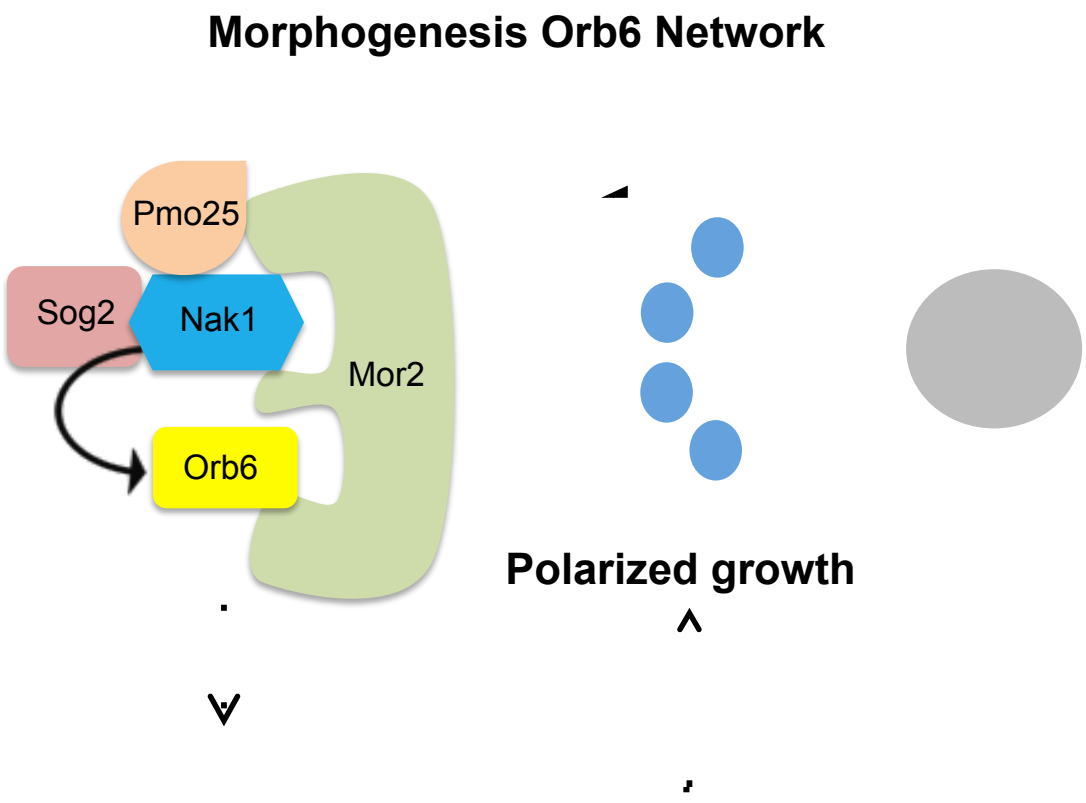
Like the Septation Initiation Network (SIN), the MOR signaling pathway comprises of two kinases belonging to the STE20-like/GC and the NDR kinase families at its core (Figure 1.9). Nak1 (STE20-like/GC kinase), like Sid1, constitutes the upstream kinase (Huang et al., 2003; Leonhard and Nurse, 2005). Pmo25 was identified as a binding partner of Nak1 and is essential for the activities of both kinases in the pathway (Kanai et al., 2005). Sog2 is a leucine rich repeat protein whose budding yeast homolog is known to interact with the Nak1 equivalent Kic1 (Bogomolnaya et al., 2006). Nak1 interacts with and activates the Sid2 counterpart, Orb6 (an NDR family kinase), the downstream effector of the pathway (Kanai et al., 2005). Mob2 is an Orb6 associated protein that is crucial for its full activity (Hou et al., 2003). Finally, Mor2, which is a homolog of the *Drosophila* Furry protein, acts as a scaffold that interacts with both kinases (Hirata et al., 2002). It is believe to be important for promoting the activation of Orb6 by Nak1. All MOR components localize to the cell ends (sites of polarized growth) during interphase. Some of the MOR proteins display localization at the SPB, the site of assembly for SIN proteins, during mitosis. Specifically, Nak1 is seen at the SPB in early mitosis whereas Pmo25 localizes to the SPB during late mitosis (Kanai et al., 2005). Finally, towards the end of cytokinesis, all MOR proteins make an appearance at the cell division site suggesting a possible role in cell division. Interestingly, MOR mutants not only display a loss of polarized morphology but also display cell separation defects

(Kanai et al., 2005). Therefore, carrying out cell separation through dissolution of primary septum may be one of the MOR functions. Consistent with this, the role of MOR pathway equivalent in budding yeast called RAM (Regulation of Ace2 and Morphogenesis) in cell separation is well characterized (Brace et al., 2011).

Figure 1.9 – Components of the Morphogenesis Orb6 Network (MOR)

A graphical representation of the identified components of the MOR is shown. All MOR components are localized to the cell ends during interphase growth where they carry out polarized cell growth. Actin patches (shown in blue) form the sites of polarized secretion and are essential for tip elongation. Not indicated in the figure is the additional role of MOR in mediating cell separation following the formation of the division septum.

Figure 1.9 – Components of the Morphogenesis Orb6 Network (MOR)



MOR Signaling Pathway: Its role in interphase growth

Both actin and microtubule cytoskeleton are important for polarized cell growth in interphase. Although the microtubule cytoskeleton is primary involved in marking the site of growth (Martin, 2009), the actin cytoskeleton is necessary for its execution. The MOR pathway controls interphase polarized growth through regulation of the actin cytoskeleton. Actin is required at the cell tips for formation of F-actin patch structures as well as actin cables. Actin patches constitute sites of endocytosis and polarized secretion and play a role in cell wall synthesis and remodeling. MOR signaling pathway has been implicated in F-actin patch assembly (Huang et al., 2005). Hob1 and Wsp1 are two patch proteins whose localization to the cell tips or the medial division site is dependent on the MOR kinase, Nak1. Further, *in vitro* F-actin formation by Wsp1 requires Nak1. Actin cables on the other hand are necessary for directed transport of secretory vesicles containing necessary cargo proteins for cell shape maintenance and morphogenesis (Marks et al., 1986; Bendezu and Martin, 2011; La Carbona et al., 2006).

Small Rho GTPases are one of the conserved proteins families with central roles in growth and morphogenesis in conjunction with the actin cytoskeleton. In fission yeast, key regulators belonging to this family include Cdc42, Rho1, Rho2, Rho3, Rho4 and Rho5, of which only Cdc42 and Rho1 are essential. Rho1 localizes to places of active growth and regulates tip extension and septation (Nakano et al.,

2005; Mutoh et al., 2005). Rho3 localizes to both the cell tips and the cell middle as well as interacts with the exocyst (Wang et al., 2003). Rho4 has a dedicated role in cytokinesis, particularly in cell separation where it regulates polarized secretion of hydrolytic enzymes such as Agn1 and Eng1 necessary for septum degradation (Santos et al., 2003, 2005).

The MOR signaling cascade has so far been shown to control localization of one Rho family GTPase, namely Cdc42 (Das et al., 2009), a master regulator of establishment and maintenance of cell polarity and growth (Estravís et al., 2011; Miller and Johnson, 1994; Rincon et al., 2007). Cdc42 activates For3 (Formin), an F-actin cable polymerization factor by relieving For3 autoinhibition (Martin et al., 2007). MOR regulates For3 localization at the cell tips via spatial control of the Cdc42 GTPase. Specifically, it prevents localization of the Cdc42 GEF, Gef1 at the cell middle thereby limiting an active GTP bound Cdc42 population to the cell ends where it recruits For3 and mediates actin cable polymerization necessary for polarized cell growth. Interestingly, Gef1 also contains putative NDR kinase phosphorylation sites and could likely be an Orb6 target. Another candidate Orb6 effector could be Rga4, the Cdc42 GAP, which also contains 2 putative NDR phosphorylation sites and shows physical interaction with Orb6 by two-hybrid analysis (Das et al., 2009). Further, Pak1/Shk1 kinase, a key effector of Cdc42, acts upstream of Orb6 suggesting a possible feedback mechanism to maintain Cdc42 at the cell tips during interphase growth. Aside from For3

localization, Cdc42 also regulates other molecules such as the exocyst complex to control polarized secretion (Bendezu and Martin, 2011). However, further investigation is needed to determine whether MOR plays role in the regulation of other such polarity factors.

MOR Signaling Pathway: Its role in cell separation

The fission yeast, *S. pombe* creates a medial cell wall septum orthogonal to the long axis of the cell in order to accomplish cytoplasmic division (Sipiczki, 2007). This septum is composed of three layers. The primary septum is deposited in the center and surrounded by the secondary septum on either side of it. Dissolution of the primary septum coupled with the removal of the cell wall material (called septum edging) that flanks it, is then required to physically separate the two daughter cells (Figure 1.10). This process of cell wall degradation occurs after mitotic exit and has to be temporally coordinated with the process of cytokinesis in order to successfully complete the cell division process. Furthermore, precise targeting of the cell wall degradation apparatus, which includes hydrolytic enzymes, is required for the execution of this process without causing cell lysis.

In fission yeast, the cell separation proteins are under the control of a transcriptional program called the Ace2 Regulatory Network. The Ace2 transcriptional wave, one of the four major transcriptional waves in the cell cycle, results in expression of a group of genes with a function in cell separation

(Alonso-Nuñez et al., 2005). The expression of these genes peaks in late mitosis and coincides with septation in cells. These cell separation genes includes Agn1, a **(**1,3)-α-glucanase that is required for degradation of the septum edging, the cell wall material at the intersection of the cell cortex and the septum (Dekker et al., 2004; García et al., 2005; Dekker et al., 2006). Eng1, an endo-1,3-β-glucanase is the other hydrolytic enzyme transcribed, which is needed for the breakdown of the primary septum. Other proteins include Mid2 (Tasto et al., 2003; Berlin et al., 2003), an anillin homolog that forms part of the Septin-Mid2 Ring (SMR), which acts as a positional marker for the exocyst and guides the secretion of the hydrolytic enzymes like Agn1 and Eng1 to the right region of the septum where they show a ring like localization (Sipiczki, 2007). In the absence of Mid2, these enzymes are mislocalized and demonstrate a disc-like localization and cell separation fails (Martín-Cuadrado et al., 2005). Finally, proteins involved in the cell separation process but not directly regulated by the Ace2 transcriptional network includes components of the exocyst complex, which is involved in tethering secretory vesicles to specific sites on the plasma membrane and is essential for carrying out cell separation (Martín-Cuadrado et al., 2005).

MOR pathway mutants demonstrate a defect in cell separation along with loss of polarity as suggested by their spherical and septated phenotype (Kanai et al., 2005). Reports in budding yeast have shown that the Orb6 kinase counterpart, Cbk1 directly controls Ace2 transcriptional activity through phosphorylation

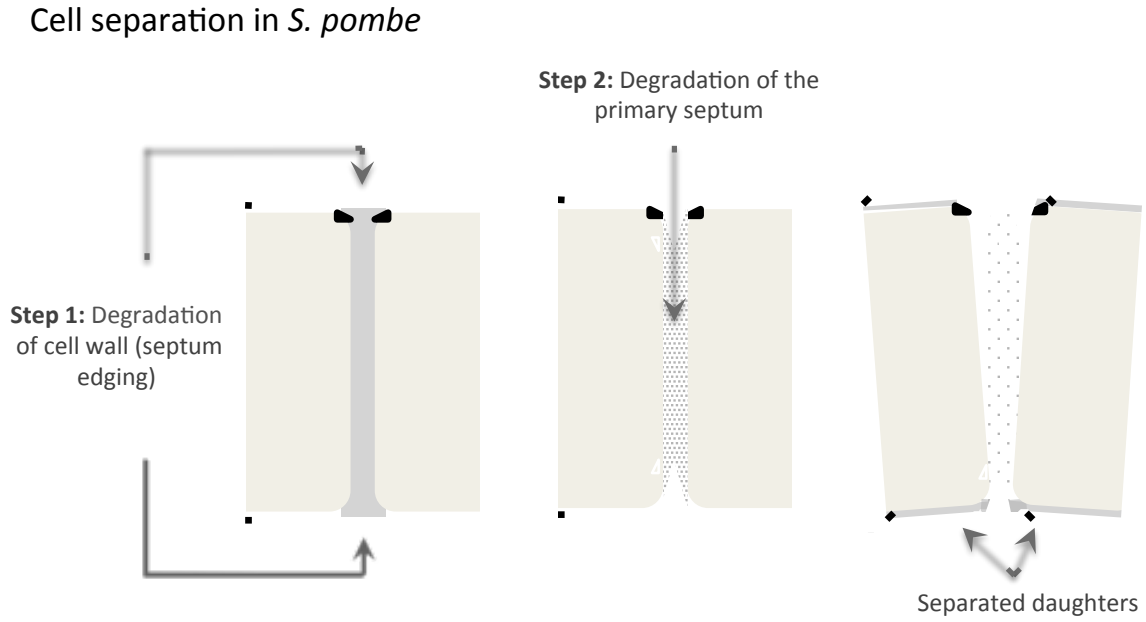
(Mazanka et al., 2008). Since the Ace2 network is highly conserved between the two species, it is possible that Ace2 might function downstream of the MOR pathway to regulate cell separation in fission yeast as well.

The SIN pathway performs cytokinesis in late mitosis when polarized cell growth is inhibited. Since SIN inhibits MOR activity, it is possible that MOR inhibition is required to prevent tip growth while cells are trying to perform cytokinesis. However why this inhibition is necessary is unclear. Further investigation is needed to understand the biological impact of carrying out polarized growth and cytokinesis simultaneously.

Figure 1.10 – Fission yeast cell separation

A graphical representation of the steps involved in fission yeast cell separation is illustrated. Medial region of a fission yeast cell with a completely formed septum is shown. Arrows indicate steps involved in separation of the daughters.

Figure 1.10 – Fission yeast cell separation



Conservation of the NDR kinase pathways in other eukaryotes

NDR kinases, a subclass of the AGC (protein kinase A/G/C like) serine/threonine kinases, are involved in the regulation of important biological processes such as mitotic exit, morphogenesis, growth, proliferation and apoptosis. Despite their involvement in a variety of cellular functions, the framework of the signaling networks they constitute is remarkably conserved from yeast to humans (Hergovich et al., 2006) (Table 1.1). Members of the Ste20-like/GC kinase family, MOB adaptor proteins and scaffolding proteins form core signaling components that function in all NDR kinases pathways.

Ste20 kinases act upstream of the NDR kinase. Studies in different experimental systems have now confirmed their role in interacting with and activating the NDR kinase through phosphorylation. MOB adapter proteins are conserved NDR kinase interacting proteins that are known to facilitate NDR kinase activation. It has been suggested that this activation takes place through release of autoinhibition or by preventing inhibitory self-association between kinase molecules. Finally, large scaffolding proteins perform the conserved function of promoting NDR kinase activation by linking it to the upstream Ste20/GC family kinase in the pathway.

The MEN and RAM represent NDR kinase pathways in budding yeast

Both MOR and SIN pathways have homologous equivalents in the budding yeast system. In this system they are referred to as the RAM (Regulator of Ace2 and Morphogenesis) and the MEN (Mitotic Exit Network). Cbk1 and Dbf2 kinases are the NDR kinase counterparts that promote signaling through RAM and the MEN, respectively. The MEN pathway controls the process of mitotic exit and is required to coordinate chromosomal segregation with cytokinesis (Bardin and Amon, 2001). Like the SIN, triggering MEN activation requires an upstream GTPase called Tem1, which is regulated by the Bub2-Bfa1 GAP complex and the GEF Lte1. The scaffold protein Nud1 facilitates association of the MEN components with the SPB. Tem1 activation promotes activity of the Ste20-like/GC kinase Cdc15, which then stimulates activation of the NDR kinase Dbf2. Dbf2 is bound to the MOB adapter protein Mob1. Phosphorylation of the Cdc14 phosphatase by the activated Dbf2 kinase promotes relocalization of the phosphatase from the nucleolus to the cytoplasm where it dephosphorylates and inactivates the mitotic CDKs which allows exit from mitosis.

The RAM signaling cascade is the second NDR kinase pathway in budding yeast and plays a role in polarized growth and cell separation (Nelson et al., 2003). The downstream NDR kinase Cbk1 is activated by the upstream Ste20-like/GC kinase Kic1. Kic1 also interacts with Hym1 and Sog2 and the scaffold protein Tao3/Pag1. While the RAM pathway controls cell separation through regulation

of the Ace2 transcriptional network, its role in polarized growth is independent of Ace2. Mechanism of polarized growth regulation by the RAM is still being investigated. So far, reports suggest that the RAM controls polarized secretion by targeting GTPase modules of the Rab as well Rho families (Maerz and Seiler, 2010). Further, it also regulates cell wall remodeling via inhibition of RNA binding proteins such as Ssd1, which known to bind and suppress mRNAs that encode cell wall remodeling proteins (Jansen et al., 2009).

NDR kinases pathways in higher eukaryotes: Signaling modules and corresponding functions

Homologs of some of the essential regulators of NDR pathways have been identified in higher eukaryotes where they are involved in similar and diversified physiological functions. Work from model systems like *C.elegans* and *D. Melanogaster*, shows that respective NDR kinases identified in these systems, namely SAX-1 and Trc (Tricornered) have roles in neuronal growth, morphology and differentiation (Gallegos and Bargmann, 2004; Zallen et al., 2000; Emoto et al., 2004). For instance, work in flies demonstrates that Trc together with the scaffold protein Fry (Furry) regulates dendritic tiling in neurons (Emoto et al., 2004). Interestingly, further reports suggested that the upstream kinase Hpo (Hippo) functions in neuronal morphology through regulation of both the downstream NDR kinases, Trc and Wts (Warts) (Emoto et al., 2006). Moreover, it regulates complementary aspects of dendrite development through the two

distinct downstream signaling modules. The Hpo-Trc kinase module controls dendritic tiling, whereas the Hpo-Wts module controls dendritic maintenance.

The NDR kinase, Wts/Lats was first identified in *drosophila* as a tumor suppressor (Justice et al., 1995; Xu et al., 1995). Following identification of this kinase, several additional proteins have been identified as Hippo tumor suppressor pathway components (Hergovich and Hemmings, 2009). Like its counterparts in the yeast system, the Hippo signaling pathway consists of the upstream Ste20-like/GC family kinase called Hippo, the downstream NDR family kinase Warts/Lats and its co-activator Mats (Mob1-like protein). The Warts/Lats kinase negatively regulates the proto-oncogene Yorkie by phosphorylation (Zhao et al., 2008). Phosphorylation of Yorkie causes it release from the nucleus and its cytoplasmic retention via 14-3-3 binding. Yorkie acts as a transcriptional co-activator that interacts with the TEAD transcription factor to regulate expression of targets like Cyclin E (required for G1/S progression), dIAP (drosophila inhibitor of apoptosis) and the bantam microRNA.

Work in mammalian systems revealed that the Hippo/Lats pathway was conserved in vertebrates as well. The human Hippo/Lats tumor suppressor pathway is composed of the Hippo homologs MST1/2, MOB adapter proteins MOB1/2 and the NDR kinase homologs LATS1/2. As seen in flies, LATS1/2 phosphorylates the Yorkie-related transcriptional co-activators YAP and TAZ

(Hergovich and Hemmings, 2009) and regulates expression of genes involved in cell proliferation and apoptosis. MST1/2 is regulated by an upstream tumor suppressor protein called RASSF1A that which causes LATS1 phosphorylation and functions in controlling mitotic exit and cytokinesis in cells (Guo et al., 2007). Consistent with it's tumor-suppressive role, LATS1 deficient mice develop soft tissue sarcoma and ovarian tumors (St John et al., 1999). Mice deficient in MST1/2 developed massive overgrowth, and hepatocellular carcinoma (Zhou et al., 2009). In an independent study, Mst1/2 mutant mice displayed an embryonic lethal phenotype suggesting that these proteins play essential roles in early mouse development (Oh et al., 2009).

Contrary to LATS1/2, studies on NDR1/2 have identified their pro-tumorigenic roles. For instance, NDR1/2 kinases were found to function in G1/S progression by controlling protein stability of the Cdk-Cyclin inhibitor protein p21. Moreover, both NDR1/2 mRNA levels are up-regulated in certain cancers (Hergovich et al., 2008). In contrast, NDR1/2 mRNA levels have been reported as down regulated in some other cancers. Therefore, mammalian NDR1/2 kinases may function as both, tumor suppressors as well as oncogenes. Aside from G1/S progression, NDR1/2 were also implicated in other cell cycle functions such as centrosome duplication and mitotic chromosome alignment downstream of MST1 and MST2 (Cornils et al., 2011).

Table 1.1 - Conservation of NDR pathway components and their functions

The table includes key molecular players in NDR kinase pathways in different organisms. Homologs of the *S. pombe* SIN pathway proteins are indicated in green and the MOR pathway protein homologs are indicated in purple. Also, indicated are the respective physiological functions performed by the downstream NDR kinase in each pathway.

Table 1.1 - Conservation of NDR pathway components and their functions

	S. pombe (SIN,MOR)	*S. cerevisiae*	*D. melanogaster*	*H. sapiens*
STE20-like/GC Kinase	Sid1, Nak1	Cdc15, Kic1	Hpo	MST1/2, MST3
Scaffold	Mor2	Tao3(Pag1)	Furry	hFURRY1, hFURRY2
NDR kinase	Sid2, Orb6	Dbf2, Cbk1	Lats/Wts, Trc	LATS1/2, NDR1/2
NDR kinase co-activator	Mob1, Mob2	Mob1, Mob2	dMob1, dMob1-4	hMOB1, hMOB1/2
Physiological functions	Cytokinesis Polarized growth & Cell separation	Mitotic Exit Polarized growth & Cell separation	Tissue growth control, Cell cycle exit & Apoptosis; Maintenance of dendrites Dendritic tiling & branching, Maintaining integrity of cellular extensions	Cell death, proliferation & Apoptosis, Cytokinesis/ Mitotic exit Centrosome duplication, Mitotic alignment & Apoptosis; G1/S progression

Crosstalk identified between the two fission yeast NDR pathways

Despite having downstream effector proteins belonging to the NDR kinase family, SIN and MOR signaling pathways in *S. pombe* regulate polarity programs of a highly contradictory nature. This contradiction is reflected in the fact that the activation periods of the two pathways do not show any overlap. Previous work from our laboratory on the two signaling networks confirmed the presence of an antagonistic interaction between them (Ray et al., 2010). Inhibition of the MOR during cytokinesis was found to be SIN dependent. Specifically, the SIN pathway causes a drop in the activity of Orb6 kinase thereby reducing signaling through the MOR network. However, the exact molecular players involved and the mechanisms employed for this inhibition are unknown. Moreover, the physiological relevance of executing this antagonistic behavior remains an open question. Results included in the following chapters expands our knowledge of the mechanism underlying MOR inhibition by the SIN as well as highlight the significance of this inhibition during late mitosis.

CHAPTER II

Identification of Sid2 substrates in the MOR pathway

My contribution to this Chapter: Figures 2.2B, 2.2C, 2.3A, 2.3B, 2.3D, 2.4A, 2.4B, 2.4C, 2.5A, 2.5B, 2.5C and 2.5D

All tables and figures presented in this chapter (except Figure 2.5 which is unpublished data) is part of the following publication:

Gupta S, Mana-Capelli S, McLean JR, Chen CT, Ray S, Gould KL, and McCollum D. Identification of SIN pathway targets reveals mechanisms of crosstalk between NDR kinase pathways. Curr. Biol. 2013.

Summary

The Septum Initiation Network (SIN) regulates multiple functions during late mitosis to ensure successful completion of cytokinesis in *S. pombe*. However, very little is known about the downstream functions of the SIN that help coordinate important mitotic processes. Our previous study identified that SIN inhibition of the Morphogenesis Orb6 Network (MOR), an interphase polarity pathway, helps coordinate necessary cytoskeletal rearrangements during the mitosis-interphase transition. To further investigate the molecular mechanisms involved in the regulation of the MOR pathway and to discover additional targets of the SIN, we developed a new approach to identify substrates of the SIN effector kinase, Sid2. Using this approach, we were able to successfully identify several Sid2 substrates that function in various mitotic processes including Nak1, a central kinase in the MOR pathway and Sog2, its putative binding partner. Further, we identified Sid2 phospho-sites on Nak1 and Sog2 and determined their role in polarized growth inhibition. In conclusion, our results illustrate an effective method to identify substrates of Sid2-like kinases and provided insight into molecular details of SIN mediated regulation of the MOR.

Introduction

The fission yeast *Schizosaccharomyces pombe* has become a powerful model system for studying cytokinesis. Like mammalian cells, *S. pombe* divides through the use of an actomyosin contractile ring, which is composed of a set of highly conserved cytoskeletal proteins. The SIN pathway, a GTPase-kinase signaling network, which is activated in late mitosis and is required for actomyosin contractile ring and septum assembly, primarily regulates cytokinesis in *S. pombe*. In addition, it also plays a role in spindle checkpoint inactivation and telophase nuclear positioning. The various functions of the SIN are carried out by the terminal kinase in the pathway called Sid2. How Sid2 accomplishes the different functions of the SIN is just beginning to be investigated. The first substrate identified was the Cdc14-family phosphatase Clp1, required for maintenance of SIN activity during cytokinesis (Chen et al., 2006). More recently discovered Sid2 substrates include, the SIN scaffold protein Cdc11 (Feoktistova et al., 2012), the kinesin motor protein Klp2 (Mana-Capelli et al., 2012), the contractile ring formin Cdc12 (Bohnert et al., 2013) and the NIMA related kinase Fin1 (Grallert et al., 2012), which have shed light on several of the cell cycle functions of the SIN effector kinase Sid2 (See Figure 1.6). Thus, a molecular understanding of SIN function requires identification of Sid2 substrates.

We recently showed that, in addition to its other functions, the SIN promotes cytokinesis through inhibition the MOR signaling pathway (Ray et al., 2010),

which normally drives initiation of polarized growth following completion of cytokinesis (Maerz and Seiler, 2010). The terminal MOR pathway kinase Orb6, a member of the NDR family of kinases, is activated by the Nak1 kinase (GC kinase family) in a manner that depends on the scaffold protein Mor2, which binds both kinases. Previous evidence suggests that the SIN inhibits the MOR by suppressing Orb6 kinase activity (Ray et al., 2010). However the mechanism by which the SIN inhibits the MOR is not known.

In this study we developed an approach to identify substrates of the SIN effector kinase Sid2. This screen identified many candidate Sid2 substrates involved in the various known SIN functions. Amongst the identified substrates are two components of the MOR signaling pathway, namely Nak1, an upstream kinase belonging to the STE20-like/GC kinase family and Sog2, a predicted binding partner of Nak1. Identification and characterization of Sid2 mediated post-translational modification of these substrates allowed us to determine the possible mechanism by which the SIN inhibits MOR mediated polarized growth during cytokinesis.

Results

Identification of Sid2 substrates

To determine how the SIN performs its various functions in late mitosis including MOR inhibition, we developed an approach to identify new Sid2 substrates. Sid2 family kinases preferentially phosphorylate the consensus sequence RXXS (where 'S' becomes phosphorylated) (Chen et al., 2008; Feoktistova et al., 2012), which, in its phosphorylated form, is also the core consensus binding site for 14-3-3 proteins (Yaffe et al., 1997; Mah et al., 2005). Because Sid2 phosphorylation of Clp1 at RXXS sites is required for the 14-3-3 protein Rad24 to bind Clp1 (Chen et al., 2008), we hypothesized that other Sid2 substrates might be identified through SIN-dependent interaction with Rad24. Since many kinases besides Sid2 create 14-3-3 binding sites, Rad24-TAP protein complexes were purified from cells with constitutively active SIN (*cdc16-116*) and cells with inactive SIN (*sid1-239*, *cdc11-123*) for comparison using tandem affinity purification strategy (Gould et al., 2004). Protein samples were digested and analyzed by 2D LC-tandem mass spectrometry (LC-MS/MS) to identify Rad24 binding partners. The abundance (spectral counts) of each Rad24 interactor was normalized to Rad24 abundance for each experiment and then averaged over 2 biological replicates. The ratio of individual protein abundance in SIN "ON" (*cdc16-116)* versus SIN "OFF" (*sid1-239*, *cdc11-123*) cells was calculated, revealing many proteins significantly enriched in SIN "ON" conditions, with the top 13 proteins enriched at least 8 fold (Table 2.1). For a complete list see Table

2.2. Validating our methodology, two of the known Sid2 substrates Clp1 and Cdc11 were among our top hits (Chen et al., 2008; Feoktistova et al., 2012). Most other top hits were plausible Sid2 targets with annotated roles in contractile ring and septum assembly, spindle checkpoint, and/or mitosis. In addition to the identification of peptides, LC-MS/MS analysis also revealed phosphorylation sites on predicted Sid2 motifs (RXXS) in most of the putative SIN targets (Table 2.1, Table 2.3)

Table 2.1 - Identification of Sid2 kinase candidate substrates

Proteins most enriched in Rad24-TAP purifications when the SIN is activated and any RXXS phosphorylation sites identified by LC-MS/MS are shown (also see Tables 2.2 and 2.3).

Table 2.1 - Identification of Sid2 kinase candidate substrates

Protein Pombe (Human homolog)	Fold enriched when SIN ON	% coverage	RXXS phosphorylation identified by MS
Sog2 (LRRC57)	59	65	S421, S449, S665
Nak1 (MST4)	27	52	S491, S501
Spa2 (?)	22	88	S167
Clp1 (CDC14)	21	66	S396, S467/S468
Rgf1 (ARHGEF8)	21	48	S35, S68, S342
Ase1 (PRC1)	17	64	S537
Pos1 (?)	14	80	
Ppk25 (MARK2)	11	61	S38, S404
Mph1 (MPS1)	11	64	S259, S277
Ipp1 (PPA1)	9	68	
Scw1 (?)	8	79	S191, S232, S325, S361
Cdc11 (Centriolin)	8	34	
Mug72 (?)	8	75	S379, S564

Table 2.2 - Putative SIN targets identified as binding partners of the 14-3-3 protein Rad24

Proteins enriched in Rad24 purifications when the SIN is activated ("ON") versus deactivated ("OFF"). This table contains all enriched proteins with more than 100 total spectral counts (TSC) that are at least 4X enriched in SIN "ON". RXXS column indicates presence (+) or lack of (-) phosphorylation detected in LC-MS/MS. Rows shaded gray indicate proteins that were also identified in background controls.

Table 2.2 - Putative SIN targets identified as binding partners of the 14-3-3 protein Rad24

ORF	Coverage	TSC	ON	OFF	ON/OFF	Protein	RXXS
SPAC8E11.02c	97	28359	1000.0	1000.0	1.0	Rad24	
SPAC17A2.13c	99	10333	485.3	309.0	1.6	Rad25	
SPBC887.09c	65	384	49.8	0.9	58.5	Sog2	+
SPBC17F3.02	52	311	37.9	1.4	26.6	Nak1	+
SPAC3G9.05	88	1197	140.2	6.5	21.5	Spa2	+
SPAC1782.09c	66	437	51.5	2.4	21.1	Clp1	+
SPCC645.07	48	480	57.1	2.7	21.0	Rgf1	+
SPAPB1A10.09	64	373	43.2	2.6	16.6	Ase1	+
SPAC16E8.08	80	215	24.5	1.7	14.3	Pos1	-
SPBC32C12.03c	61	186	20.1	1.9	10.7	Ppk25	+
SPBC106.01	49	161	16.8	1.6	10.6	Mph1	+
SPAC23C11.05	68	178	18.6	2.0	9.2	Ipp1	-
SPCC16C4.07	76	682	66.4	8.3	8.0	Scw1	+
SPCC1739.11c	34	147	14.0	1.8	7.7	Cdc11	-
SPCC1902.02	75	2966	282.4	37.8	7.5	Mug72	+
SPBC902.03	42	268	24.4	3.6	6.7	Psp7	-
SPAC6G10.02c	52	268	25.1	3.8	6.6	Tea3	-
SPBC3B8.10c	68	847	76.5	11.8	6.5	Nem1	+
SPBC16A3.18	91	1511	135.8	22.1	6.1	Cip1	+
SPAC12G12.03	67	935	83.2	13.5	6.2	Cip2	+
SPAC29B12.07	24	240	21.3	3.8	5.7	Sec16	-
SPAC24H6.05	61	349	30.9	5.5	5.7	Cdc25	+
SPCP1E11.02	54	256	21.7	4.0	5.4	Ppk38	+
SPAC926.06c	63	1025	86.3	16.5	5.2	Lrr1	+
SPAC1006.06	43	330	27.6	5.5	5.0	Rgf2	+
SPBC1A4.05	57	214	17.9	3.6	5.0	Blt1	-
SPCP1E11.04c	83	714	57.1	12.4	4.6	Pal1	+
SPBC4F6.06	48	234	17.9	4.1	4.4	Kin1	-

Table 2.3 - List of all RxxS and associated scaffold scores for sites for putative Sid2 substrates

RXXS phosphorylation sites identified in putative Sid2 substrates (see Table 2.2). "Surrounding sequence" is the amino acid sequence surrounding the phosphoserine (indicated by lowercase s). The "Best Ascore" and "Localization Probability" are scores for the localization of the phosphorylation site and the "% Seq. cov." is the percentage of the protein identified in the Rad24 purifications

Table 2.3 - List of all RxxS and associated scaffold scores for sites for putative Sid2 substrates

ORF	Protein	Surrounding Sequence	Site	Best Ascore	Localization Probability
SPAC8E11.02c	Rad24	IGARRAsWRIVSS	S61	49.3	100%
		ASWRIVsSIEQKE	S66	51.2	100%
		GEKRQHsADQSLE	S146	2.0	65%
SPBC887.09c	Sog2	ERNRSNsTNDDYS	S421	7.3	80%
		LTNRIVsVEKPAS	S665	81.1	100%
		KEARKLsEQLPNR	S449	1000.0	100%
SPBC17F3.02	Nak1	LQSRSIsMGAFEQ	S501	13.1	98%
		PGLRMPsSFDLQS	S491	34.0	100%
SPAC3G9.05	Spa2	TEQRTTsSPTIPS	S167	23.1	99%
		NMTRAAsQPPPQV	S129	1000.0	100%
SPAC1782.09c	Clp1	VRLRRSsSQSNIE	S467	17.0	51%
		RLRRSSsQSNIEP	S468	17.0	98%
		GQPRKVsGHNPPS	S396	102.9	100%
		SAQRSVsMSSLNN	S513	0.0	49%
SPCC645.07	Rgf1	PLIRSVsEYPANV	S275	5.0	75%
		LPFRSTsLQPPSS	S68	14.0	98%
		EPARTVsTPAFME	S35	15.3	99%
		HDMRNRsFDHSTL	S342	41.5	100%
SPAPB1A10.09	Ase1	NPSRSIsAEPPSA	S537	9.4	99%
SPBC32C12.03c	Ppk25	KHQRSIsDFVGTA	S38	29.3	100%
		RHQRHHsEILGAM	S404	174.4	100%
SPBC106.01	Mph1	DTLRRQsSGATAL	S63	20.9	100%
		DVERRAsELHSRP	S259	43.7	100%
		EPQRSAsQPYESH	S277	0.0	48%
SPCC16C4.07	Scw1	DNYRKGsQTNLSD	S191	22.9	99%
		DLFRRSsNHVSAS	S232	35.3	100%
		PILRFNsLSINTN	S325	41.8	100%
		PHPRVFsANSAFS	S361	52.9	100%
SPCC1902.02	Mug72	KASRPPsIASTVH	S564	64.7	100%
		ISNRIGsVDDLLN	S379	166.8	100%
SPBC3B8.10c	Nem1	YNRRRSsYSASSL	S51	14.0	96%

SPAC12G12.03	Cip2	ALSRGMsNSVTLT	S214	0.0	39%
SPBC16A3.18	Cip1	QASRKTsGSGSSA	S57	28.0	100%
		ASSRAAsPAPSDS	S82	31.7	100%
		ALSRLQsPLNSPK	S37	115.1	100%
SPAC24H6.05	Cdc25	TLFRSLsCTVETP	S99	9.4	81%
		LRSRSSsSYSINK	S206	14.0	94%
		TFMRTKsYTFGQS	S567	14.0	95%
		PFLRSRsSSSYSI	S204	24.4	99%
		NVSRSRsSGNAPP	S192	25.8	100%
		VLRRTQsMFLNST	S359	39.8	100%
		LKVRSPsPMAFAM	S334	43.8	100%
SPCP1E11.02	Ppk38	VVGRRAsMSIAVN	S583	25.7	100%
SPAC926.06c	Lrr1	NLRRSVsLGSKDY	S271	33.9	100%
SPAC1006.06	Rgf2	NFYRRSsSTDDFG	S105	11.1	81%
		LPRRPSsALLTNP	S747	26.0	100%
		PNIRKNsVHVNAP	S151	143.3	100%
		FYRRSSsTDDFGI	S106	0.0	25%
		SLPRRPsSALLTN	S746	0.0	50%
SPCP1E11.04c	Pal1	LAERSNsSMGTFD	S89	15.3	99%
		SRHRSPsHNDSSP	S121	20.5	82%
		SSSRHRsPSHNDS	S119	22.4	99%
		KIARARsTHVASS	S108	23.1	100%

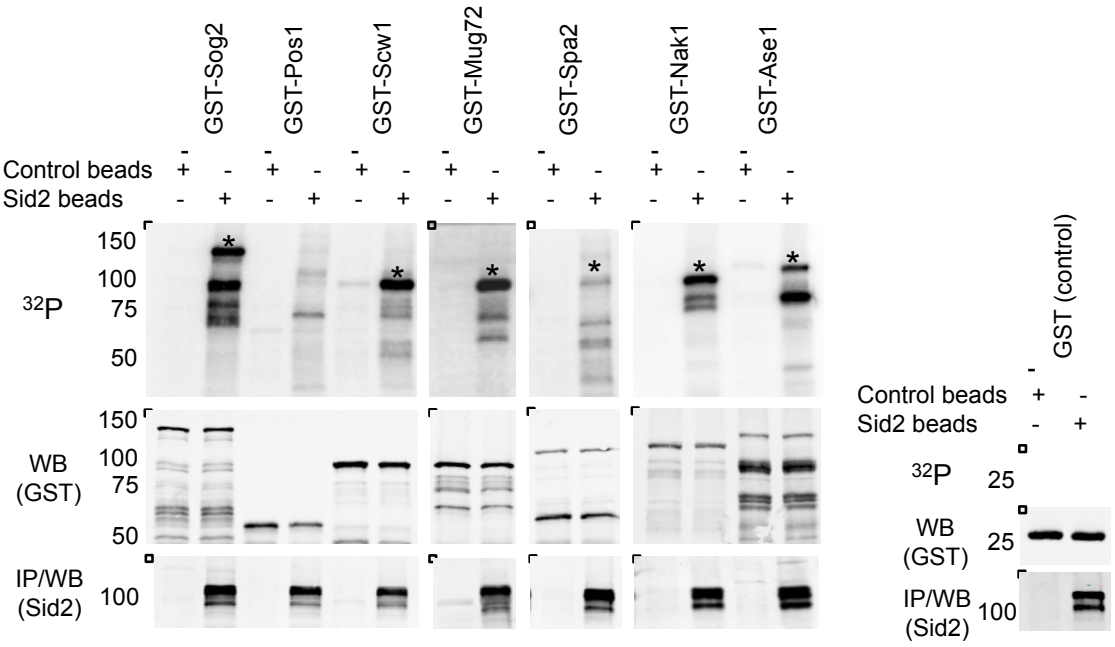
Six out of seven top Sid2 candidate targets can be phosphorylated *in vitro*

We next tested whether the SIN "ON" enriched proteins could be directly phosphorylated by the Sid2 kinase in vitro. Substrates for the Sid2 kinase assay were cloned in pDEST15 and purified as GST fusions. Seven of the top candidate Sid2 substrates could be purified as soluble recombinant proteins. Kinase assays were performed as described previously (Sparks et al., 1999; Ray et al., 2010). Briefly, beads were incubated in kinase buffer supplemented with yeast protease inhibitor cocktail along with 1 mM PMSF, 2uM ATP, 5 uCi γ-^{32}P-ATP, and 0.5 to 1 ug of purified protein substrate at 30°C for 30 min. Reactions were stopped by the addition of 20 µl of 2x SDS sample buffer followed by boiling. Samples were subjected to SDS-PAGE, gels were dried, and the signal was quantified using a Phosphorimager. All but one of the seven top candidate targets was phosphorylated by Sid2 kinase purified from yeast (Figure 2.1). The exception was Pos1, which was likely co-purified with its binding partner and Sid2 substrate Spa2 (Kathy Gould; unpublished observation). As expected, the GST only control does not show any phosphorylation by Sid2 (Figure 2.1, small panel on the right).

Figure 2.1 - Validation of Sid2 kinase candidate substrates

Sid2 phosphorylates candidate substrates *in vitro*. Sid2 kinase assays were performed using the indicated substrates purified from bacteria as GST-fusions. Substrate-GST fusions or GST alone were incubated with (Sid2 beads) or without (control beads) Sid2. Half of the kinase reaction was used to detect phosphorylation by autoradiography (^{32}P) and half was used in Western blots to determine the levels of substrate (GST) and kinase (Sid2). Asterisks mark phosphorylation of the indicated full-length substrate.

Figure 2.1 - Validation of Sid2 kinase candidate substrates



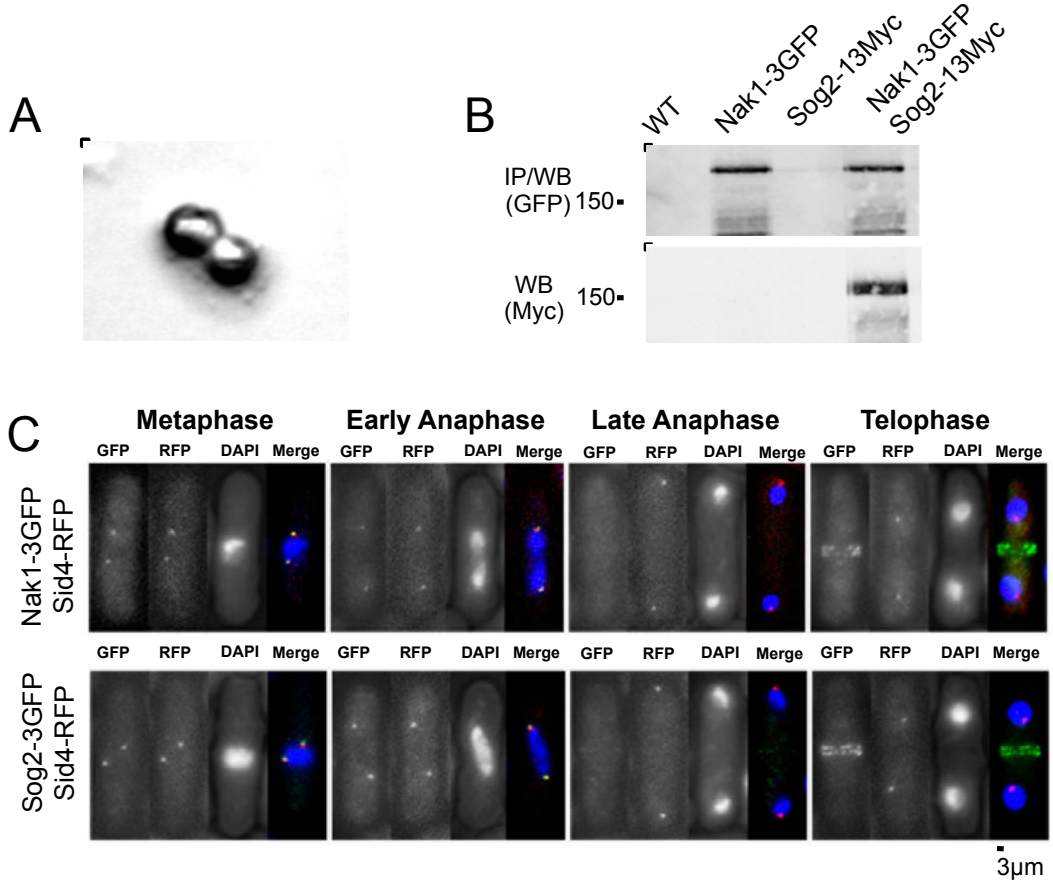
Nak1 and Sog2 form part of a complex and display a similar localization pattern

The top two hits in our screen for Sid2 substrates were the GC family kinase, Nak1 and its putative binding partner Sog2 (based on the known function of its homolog in budding yeast (Nelson et al., 2003)). Further analysis showed that *sog2*, like *nak1*, is an essential gene and germinating *sog2*Δ spores, which stop proliferation after two to four rounds of division, had a round cell morphology like other MOR pathway mutants (Figure 2.2A) (Gupta and McCollum, 2011; Maerz and Seiler, 2010). To test whether Nak1 and Sog2 are in a complex, we performed a co-immunoprecipitation using triple GFP tagged Nak1. As shown in (Figure 2.2B) Sog2-13Myc co-immunoprecipitates with Nak1 indicating that the two proteins are part of a complex. We also analyzed localization of the two proteins during mitosis by utilizing expression of a triple GFP tagged Nak1 and Sog2 protein. Both proteins show a very similar localization pattern; they are localized to SPBs in early mitosis, then leave the SPBs in anaphase when the SIN gets activated, and localize to the division site late in cytokinesis (Figure 2.2C) (Leonhard and Nurse, 2005).

Figure 2.2 - Nak1 and Sog2 are part of a complex and show similar localization at the SPB during mitosis

(A) Phenotype of *sog2Δ* cells. Diploid cells heterozygous for the *sog2*Δ*::ura4+* mutation were sporulated, the tetrads dissected and allowed to germinate. Haploid cells carrying *sog2*Δ*::ura4+* divided once or twice then stopped dividing and died with a round phenotype. An example of the *sog2*Δ*::ura4+* phenotype is shown. (B) Sog2-13Myc co-immunoprecipitates with Nak1-3GFP. Extracts were prepared from cells of the indicated genotypes following overnight growth in YE at 25°C. Nak1-3GFP was immunoprecipitated using an anti-GFP antibody and the immunoprecipitates were analyzed by immunoblotting for GFP and Myc. (C) Localization of Nak1-3GFP and Sog2-3GFP during mitosis. Sid4-RFP was used as a marker for SPBs. Cells were grown at 25°C in YE, fixed in methanol and then imaged. Representative images are shown. GFP (Green), RFP (Red) and DAPI (Blue) are shown in merged images.

Figure 2.2 - Nak1 and Sog2 are part of a complex and show similar localization at the SPB during mitosis



Construction and characterization of Nak1 phosphorylation mutants

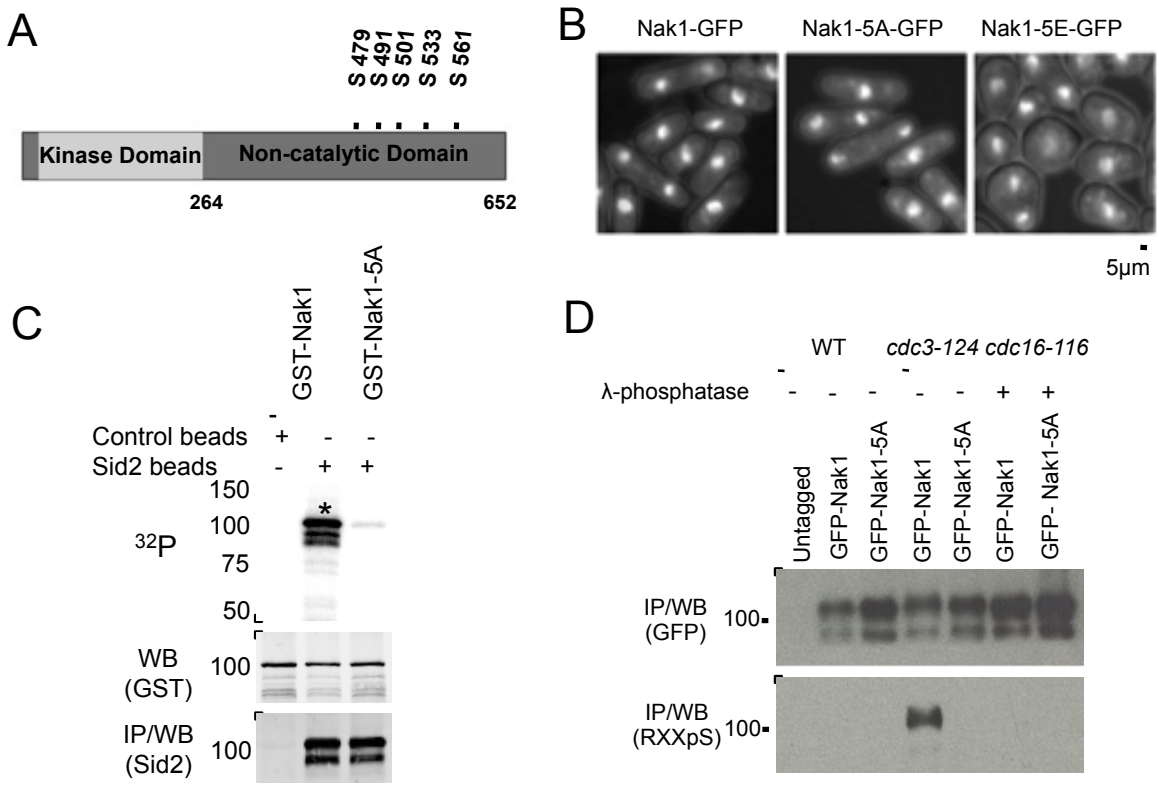
To understand whether Sid2 phosphorylation of Nak1 played a role in modulating signaling through the MOR (Ray et al., 2010) we sought to create mutations in the Sid2 phosphorylation sites on Nak1. Nak1 contains 7 Sid2 consensus phosphorylation motifs (RXXS), two of which (S491, S501) we identified as phosphorylated in vivo by LC-MS/MS (Table 2.1, Table 2.3). Three other Sid2 consensus sites (S479, S533, S561) were identified in phosphoproteomic mass spectrometry studies (Beltrao et al., 2009; Wilson-Grady et al., 2008; Koch et al., 2011). These 5 sites, which cluster in an 82 amino acid region of the Nak1 C-terminal non-catalytic domain (Figure 2.3A), were mutated to either alanine (*nak1-5A*) or glutamic acid (*nak1-5E*) to create a non-phosphorylatable or a phospho-mimetic mutant respectively. Since we expected Sid2 phosphorylation to inhibit Nak1, it was not surprising that the Nak1-5A protein was functional, as judged by its ability to rescue the viability and shape defects of the *nak1*Δ deletion mutant, whereas the *nak1*Δ cells expressing Nak1-5E were viable but had defects in cell shape (Figure 2.3B). Unlike Nak1, recombinant Nak1-5A could not be phosphorylated by Sid2 *in vitro* (Figure 2.3C). We also examined the *in vivo* phosphorylation status of Nak1-5A compared to Nak1 using phospho-specific antibodies that recognize the RXXS motif. Western blot analysis of Nak1 immunoprecipitates from asynchronously growing wild-type cells and cells in which the SIN pathway was activated using the *cdc16-116* mutation (Note that for all experiments where the SIN is activated, the *cdc3-124* mutation, which

blocks septum formation (Balasubramanian et al., 1994), was also present to block non-specific effects on cell growth triggered by ectopic septation upon SIN activation) showed that wild-type Nak1, but not Nak1-5A, was phosphorylated on RXXS sites specifically in cells with activated SIN signaling (Figure 2.3D, lanes 4 and 5). Treatment of Nak1 immunoprecipitates with λ-phosphatase results in disappearance of the protein band indicating that its detection is phosphorylation specific (Figure 2.3D, lanes 6 and 7).

Figure 2.3 - The Nak1-5A mutant cannot be phosphorylated by Sid2

(A) Diagram of Nak1. The five Sid2 phosphorylation sites (RXXS) mutated to either alanine (A) or glutamic acid (E) are indicated. (B) Rescue of the *nak1*Δ deletion mutant by the Nak1 phosphorylation site mutants. Plasmids for the Nak1 phosphorylation site mutants were integrated into *nak1*Δ heterozygous diploids, sporulated, and clones carrying the Nak1 phosphorylation site mutants and the *nak1*Δ deletion were isolated and grown at 36°C for 4h. (C) Sid2 phosphorylates GST-Nak1 *in vitro*, but not GST-Nak1-5A. Kinase assays were performed on GST-Nak1 and GST-Nak1-5A as described in Figure 1B. (D) GFP-Nak1, but not GFP-Nak1-5A is phosphorylated *in vivo*. GFP-Nak1 and GFP-Nak1-5A were immunoprecipitated from extracts prepared from cells of the indicated genotypes expressing GFP-Nak1 or GFP-Nak1-5A from a thiamine repressible promoter. Cells were grown in the absence of thiamine for 16 hours, then the SIN pathway was activated by shifting *cdc3-124 cdc16-116* cells to 36°C for 3h. In addition, extracts were prepared from cells with activated SIN for treatment with λ -phosphatase following Immunoprecipitation. Levels of Nak1 RXXpS phosphorylation and GFP-Nak1 were detected by western blotting with anti-GFP antibody and a phospho-specific antibody against to the RXXS motif respectively.

Figure 2.3 - The Nak1-5A mutant cannot be phosphorylated by Sid2



Phosphorylation of Nak1/Sog2 complex by the Sid2 kinase is required for SIN mediated inhibition of polarized growth

SIN activation causes cessation of polarized cell growth by inhibiting the activity of the downstream MOR pathway kinase, Orb6 (Ray et al., 2010). To determine whether Sid2 phosphorylation of Nak1 mediates its inhibition of the MOR, we tested if Nak1-5A could bypass SIN-mediated inhibition of polarized cell growth. Wild-type Nak1, Nak1-5E, or Nak1-5A were expressed in *cdc16-116* mutant cells where the SIN is constitutively active. Nak1-5A but not wild-type Nak1 or Nak1-5E bypassed SIN-mediated inhibition of cell elongation (Figure 2.4A-B). Cells expressing the non-phosphorylatable form of Nak1 were approximately twice as long as control cells (Figure 2.4A). While the cell elongation phenotype indicated that MOR was functional in the cells, we also set out to obtain a biochemical readout to support this. We performed *in vitro* kinase assays to measure Orb6 activity by utilizing the commercially available substrate, Myelin basic protein (MBP) [Note: In these assays, Orb6 was not directly pulled down, instead its binding partner Mob2-13Myc was immunoprecipitated for technical reasons (Hou et al., 2003)]. Unlike Nak1 or Nak1-5E, Nak1-5A expression in cells with active SIN was able to partially restore Orb6 kinase activity (Figure 2.4C, lane 4), which is normally inhibited by the SIN (Ray et al., 2010). It is of note that expression of the Nak1-5A mutant in WT cells did not result in any observable defects in cytokinesis. Taken together, these results indicate that the SIN inhibits Orb6

kinase activity and MOR mediated polarized growth at least in part through phosphorylation of the Nak1 kinase.

The lack of cytokinesis defects in cells expressing the Nak1-5A mutant might be because the SIN interferes with MOR activity through additional mechanisms and thus Nak1-5A is only partially resistant to SIN inhibition. Sog2, a Nak1 associated essential protein, which functions in the MOR pathway was also identified in our screen for Sid2 substrates (Gupta et al., 2013; Kume et al., 2013). To test whether secondary regulation of MOR mediated polarized growth was occurring through phosphorylation of Sog2, we attempted to create a non-phosphorylatable form of Sog2. Sog2 contains 11 RXXS motifs, three of which (S421, S449, S665) were identified as phosphorylated in vivo using mass spectrometry in our study (Figure 2.1, Table 2.3). Two additional RXXS sites (S312, S657) were identified as phosphorylated using mass spectrometric analysis in an independent study (Cipak et al., 2013). All 5 sites were mutated to alanine to create a Sog2-5A mutant (Figure 2.5A). To test whether the non-phosphorylatable form of Sog2 can bypass SIN inhibition, we expressed it in a *cdc3-124 cdc16-116* mutant strain where SIN is constitutively active. Expression of Sog2-5A alone was insufficient to bypass polarized growth arrest implemented upon SIN activation and resembled results observed on expression of a vector control (Figure 2.5B, C). Expression of Nak1-5A mutant showed a significant increase in cell length over time consistent with our previous observation. Interestingly, co-expression

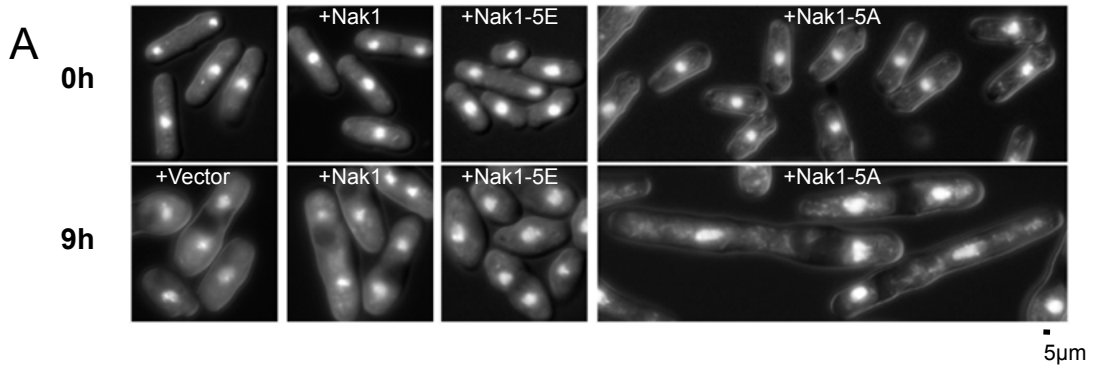
of both the Sog2-5A and Nak1-5A non-phosphorylatable mutants produced a cumulative effect absent on co-expression of WT Sog2 with the Nak1-5A mutant (Figure 2.5B,C**)**. This observation suggests that phosphorylation of Sog2 is another mechanism by which SIN inhibits MOR function in late mitosis. Since expression of Nak1-5A alone did not cause any cytokinesis defects in WT cells, we also assessed the effect of expressing non-phosphorylatable versions of both Sid2 targets in the WT background. Co-expression of Sog2-5A and Nak1-5A resulted in lysis of cells at the cell middle suggesting that they lyse while undergoing cytokinesis (Figure 2.5D). This phenotype has been further characterized in Chapter IV.

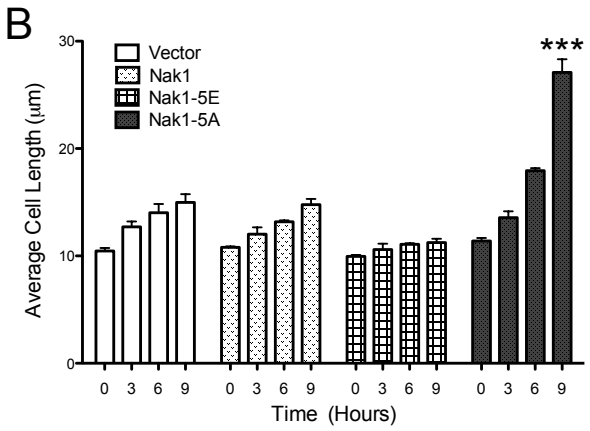
Figure 2.4 - Elimination of Sid2 phosphorylation of Nak1 bypasses SIN mediated inhibition of polarized growth

(A-B) Expression of Nak1-5A bypasses SIN inhibition of cell elongation. *cdc16-116 cdc3-124* cells with the indicated plasmids were grown at 25°C in the absence of thiamine for 19 hours (to induce expression of the indicated proteins) and then shifted to 36°C to activate the SIN. Samples were collected every 3 hours. (A) Images representing the indicated cells at 0 hour and 9 hour time points are shown. Cells were stained with DAPI and visualized by a combination of fluorescence and DIC microscopy. (B) Cell lengths of 50 cells were measured for each time point. Error bars denote SD values obtained from the average cell lengths measured from three separate experiments. Statistical analysis using unpaired t tests (as indicated by asterisks for the 9h time point) shows that the difference in cell lengths between cells expressing the vector control and the Nak1-5A construct was found to be statistically significant (***p-value <0.001). (C) Nak1-5A and the Nak1-Mor2 fusion (See Chapter III) cause Orb6 kinase activation even when the SIN is active. All strains except the control (untagged) express the indicated Nak1 proteins and Mob2-13Myc, which was used to pull down its associated kinase Orb6. The temperature sensitive (ts) *cdc3-124 cdc16-116* background was used to activate the SIN. Cells were grown at 25°C then shifted to 36°C for 3 hours. Orb6 immune complex kinase assays were performed by first pulling down the Orb6 regulatory subunit Mob2. Myelin basic protein (MBP) was used as substrate. Half of the kinase reaction was used to

detect phosphorylation by autoradiography (^{32}P) and half was used in Western blots to determine the levels of Mob2 (lower panel). Orb6 kinase activity for each strain was normalized to Mob2 level and relative to the activity of GFP-Nak1 expressing *cdc3-124 cdc16-116* cells.

Figure 2.4. Elimination of Sid2 phosphorylation of Nak1 bypasses SIN mediated inhibition of polarized growth





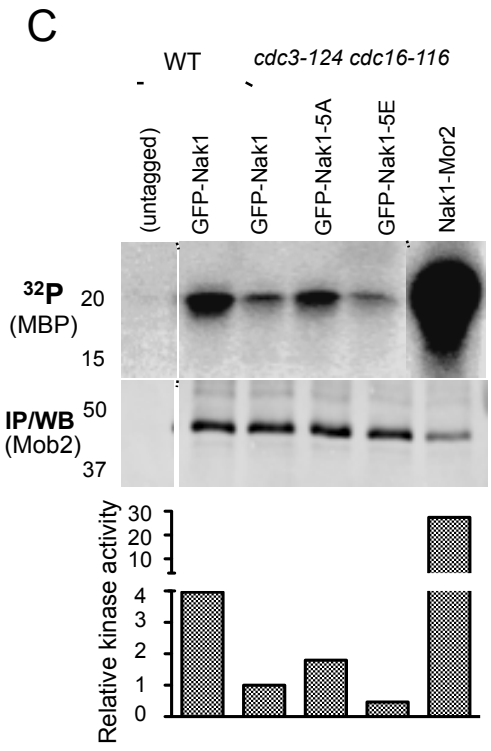
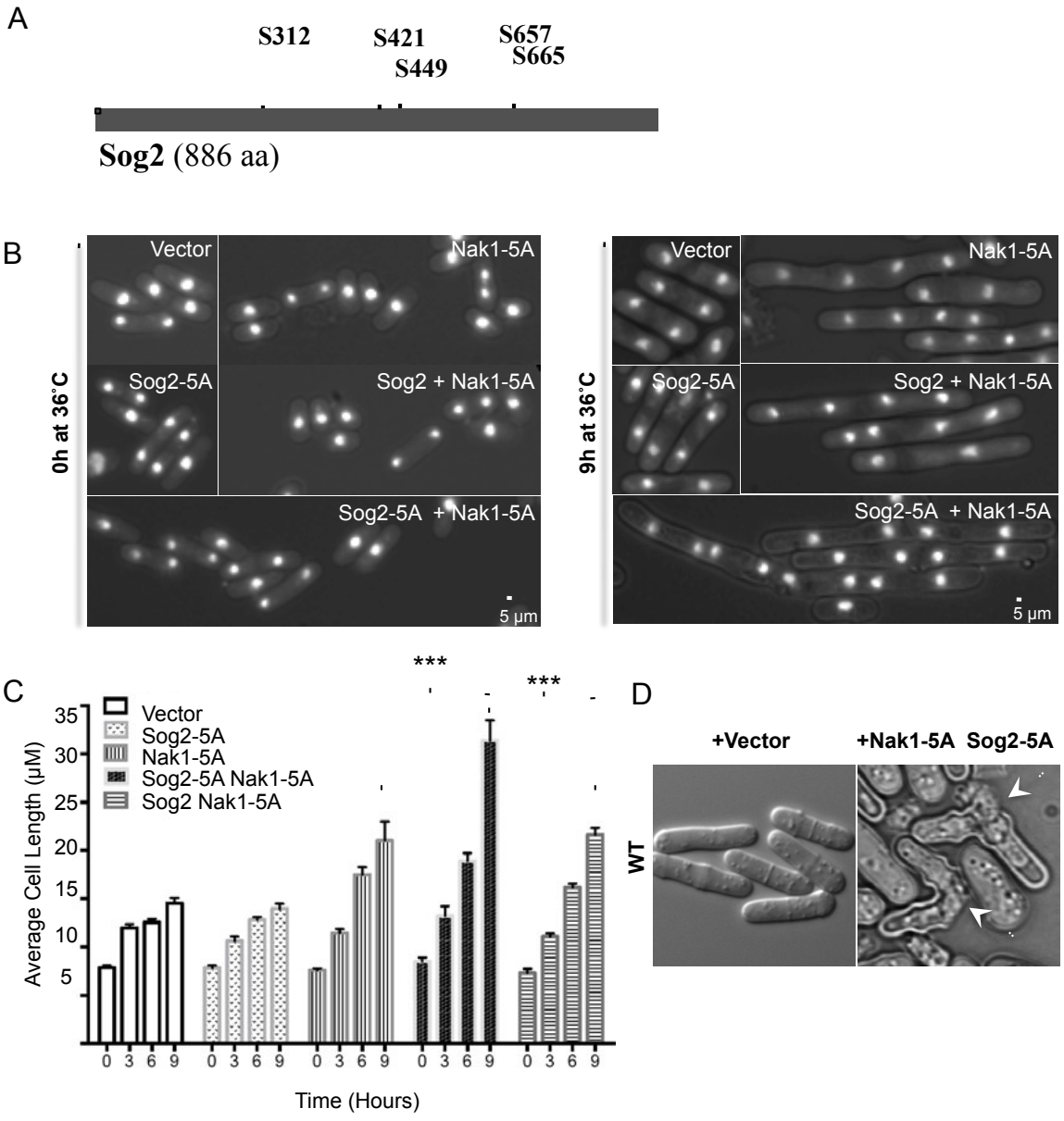


Figure 2.5 - SIN inhibition of polarized growth occurs in part through phosphorylation of Sog2

(A) Diagram of Sog2. The five Sid2 phosphorylation sites (RXXS) mutated to either alanine (A) or glutamic acid (E) are indicated. (B-C) Co-expression of Sog2-5A and Nak1-5A enhances bypass of SIN inhibition of cell elongation. *cdc16-116 cdc3-124* cells with the indicated plasmids were grown at 25°C in the absence of thiamine for 19 hours (to induce expression of the indicated proteins) and then shifted to 36°C to activate the SIN. Samples were collected every 3 hours. (B) Images representing the indicated cells at 0 hour and 9 hour time points are shown. Cells were stained with DAPI and visualized by a combination of fluorescence and DIC microscopy. (C) Cell lengths of 50 cells were measured were measured for each time point. Error bars denote SD values obtained from the average cell lengths measured from three separate experiments. Statistical analysis using unpaired t tests (as indicated by asterisks) shows that the difference in cell lengths between cells expressing the Nak1-5A mutant and the ones co-expressing both Nak1-5A and Sog2-5A construct was found to be statistically significant (***p-value <0.001). Differences in cell lengths between cells co-expressing Nak1-5A and Sog2-5A and the ones co-expressing Nak1-5A and Sog2 were also statistically significant (p-value <0.001) (D) Representative images of WT cells expressing either vector or co-expressing the Sog2-5A and NAk1-5A proteins 19h at 25˚C in medium lacking thiamine to induce expression of the proteins are shown. *White arrows* indicate point of lysis in cell middle.

Figure 2.5 - SIN inhibition of polarized growth occurs in part through phosphorylation of Sog2



Discussion

Although the SIN pathway has been studied for many years and is known to carry out multiple functions in late mitosis, substrates of the SIN were unidentified until recent reports identified Clp1, Cdc11, Cdc12, Klp2 and Fin1 as targets of the SIN kinase, Sid2 (See Figure 1.6). Our 14-3-3 based strategy identified multiple candidate substrates of the SIN effector kinase Sid2. Examination of the top ten hits from the screen suggests that many of these candidates are likely to be real Sid2 substrates. First, many of the proteins fall into pathways regulated by the SIN such as interphase polarity (Sog2, Nak1), septum assembly (Rgf1, Scw1), spindle checkpoint (Mph1), actomyosin ring assembly (Clp1) and nuclear positioning (Ase1) (Figure 2.6). Most of the proteins were phosphorylated by Sid2 in vitro and displayed phosphorylation on RXXS sites in vivo, with the exception of Pos1, which likely came down in our screen because it is a stoichiometric binding partner of Spa2 (K. Gould; unpublished observations). Furthermore we identified the known Sid2 substrate Clp1, and detailed characterization of Nak1 confirmed that it is a Sid2 substrate in vivo. Although the screen has been remarkably successful, technical limitations likely caused some Sid2 substrates to be missed. For example, as with any biochemical approach, low abundance proteins will likely be overlooked, as would proteins that are constitutively phosphorylated by other kinases that create 14-3-3 binding sites, or proteins that do not bind 14-3-3 after Sid2 phosphorylation. However this approach has greatly expanded our knowledge of Sid2 substrates and should be useful for

identification of substrates of other kinases, especially those that prefer to phosphorylate the 14-3-3 binding motif RXXS. We expect that characterizing hits from our screen will illuminate how the SIN regulates additional mitotic events.

These results also provide a molecular insight into how the SIN inhibits the MOR pathway. Our previous work indicated that SIN signaling inhibited the ability of Nak1 to activate the terminal kinase in the MOR pathway, Orb6 (Ray et al., 2010; also see Chapter III). Data presented here show that Sid2 phosphorylation of the carboxy-terminal (non-kinase) half of Nak1 likely affects its ability to activate Orb6 as demonstrated by the Orb6 kinase assays. Consistent with this model, non-phosphorylatable Nak1 (Nak1-5A) blocked the ability of the SIN to inhibit the MOR and polarized growth. Moreover, we determined that Sid2 phosphorylation of the RXXS sites identified on Sog2 might serve as an auxiliary mechanism to Nak1 phosphorylation in order to ensure MOR inactivation during cytokinesis. This conclusion is verified by the observation that co-expression of non-phosphorylatable Sog2-5A with Nak1-5A improves the cell elongation phenotype observed by expressing the Nak1-5A mutant alone. Moreover, bypassing SIN inhibition of the MOR through individual expression of either Nak1-5A or Sog2-5A does not cause any observable effect on SIN function in WT cells. But co-expressing the two proteins interferes with cytokinesis and causes lysis in cells suggesting that both ways of MOR inhibition must be circumvented in order for

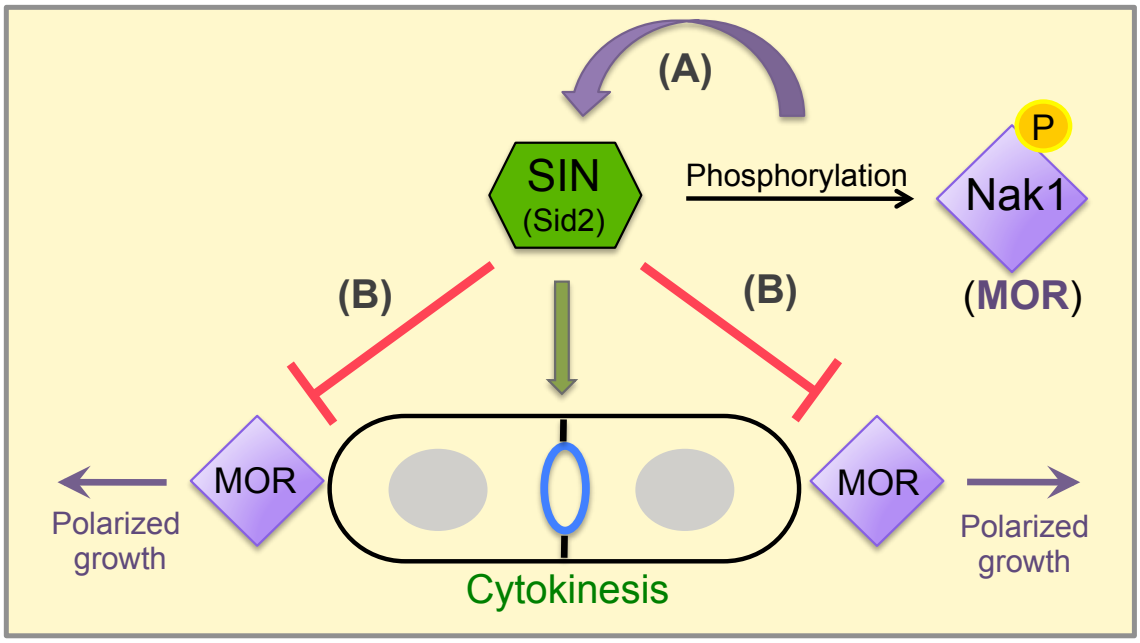
unregulated MOR to hamper cytokinesis. However, the specifics of how the lysis occurs needs to be determined.

Taken together, these results further our understanding of the key players involved in SIN pathway mediated inhibition of the MOR. It also demonstrates that this inhibition is required to prevent polarized growth during cytokinesis. Because MOR pathway components are highly conserved in mammalian cells, it is possible that similar mechanisms could govern cross-talk between the pathways in higher organisms even if the overall functions of the pathways have diverged. Analysis of the other Sid2 substrates identified in this study should shed light on how the SIN promotes its various functions. Furthermore we expect that application of our system for identifying kinase substrates via the 14-3-3 based strategy will be useful for revealing substrates of other kinases and can be applied to other organisms.

Figure 2.6 – SIN regulates a variety of mitotic tasks

This figure illustrates the possible roles of the SIN pathway during mitosis based on newly identified targets of the SIN kinase, Sid2. The top two identified candidate targets, Nak1 and Sog2 function in the interphase polarized growth pathway, MOR and appear to be targeted by the SIN for inhibition of MOR activity during cytokinesis.

Figure 2.6 – SIN regulates a variety of mitotic tasks



CHAPTER III

Molecular mechanisms of MOR inhibition during cytokinesis

My contribution to this chapter: Table 3.1, Figures 3.1A, 3.1B, 3.2, 3.3A, 3.3B, 3.3C, 3.4A, 3.4B, 3.5, 3.6A, 3.6B, 3.6C, 3.7, 3.8B

Table 3.1 and Figures 3.1, 3.2, 3.8A are part of the following publication:

Ray S, Kume K, **Gupta S**, Ge W, Balasubramanian M, Hirata D, McCollum D. The mitosis-to interphase transition is coordinated by cross talk between the SIN and MOR pathways in Schizosaccharomyces pombe. J. Cell Biol. 2010; 190:793–805.

Figures 3.3, 3.5, 3.6 have been included in the following publication:

Gupta S, Mana-Capelli S, McLean JR, Chen CT, Ray S, Gould KL, and McCollum D. Identification of SIN pathway targets reveals mechanisms of crosstalk between NDR kinase pathways. Curr. Biol. 2013.

Figures 3.4, 3.7, 3.8B are unpublished results.

Summary

Regulation of cytoskeletal remodeling is essential to co-ordinate the transition between mitosis and interphase. In fission yeast, the MOR signaling pathway promotes actin polarization at the cell tips in interphase, whereas the SIN signaling pathway drives actomyosin ring constriction and cytokinesis during mitosis. The precise mechanism by which cells coordinate the activities of the two signaling pathways during transitions between interphase and mitosis is unknown. Previous results from our lab indicated that in addition to regulating cytokinesis, the SIN pathway also inhibits MOR signaling in mitosis by interfering with the activity of the most downstream MOR component, the NDR-family kinase Orb6. In Chapter II, I identified MOR pathway components, Nak1, an upstream MOR pathway kinase, and its binding partner Sog2 as top hits in a biochemical screen to find substrates of the SIN effector kinase, Sid2. Further, observations included in Chapter II demonstrated that mutation of identified and putative Sid2 phosphorylation sites on Nak1 and Sog2 reinstates MOR mediated polarized growth in an active SIN background. In this chapter, I show that SIN phosphorylation of Nak1/Sog2 complex interferes with its interaction with Mor2, a scaffold protein known to interact with both Nak1 and Orb6 kinases and promote Nak1 mediated activation of Orb6. In addition, I demonstrate that Nak1 phosphorylation is also required for Nak1 removal from the SPB and consequent activation of the SIN.

Introduction

Schizosaccharomyces pombe are rod-shaped cells that grow by elongation at cell ends and divide by medial fission. These cells form an ideal system for the study of biochemical signaling pathways that underlie cell polarity and morphogenesis. The NDR kinase signaling cascades that control various aspects of growth and division in fission yeast are MOR (Morphogenesis Orb6 Network) and SIN (Septation Initiation Network) respectively. These pathways are conserved in higher eukaryotes where they perform similar but more diverse roles (Cornils et al., 2011b; Hergovich et al., 2008, 2006).

The SIN pathway, which is active during late anaphase, is essential for stable formation of the contractile actomyosin ring (CAR), ring constriction, and septum formation during cytokinesis (Krapp et al., 2004; Goyal et al., 2011). Sid2, which is part of the NDR family kinase in fission yeast, is responsible for performing downstream functions of the SIN (Sparks et al., 1999). During mitosis, execution of SIN functions requires that the cytoskeletal components be maintained at the cell middle where they form the cytokinetic apparatus. SIN plays a role in maintenance of the CAR in anaphase via regulation of one of its targets, Clp1, and prevents actin polarization toward cell tips (Chen et al., 2008; Mishra et al., 2004). Understandably, SIN mutants display an inability to maintain the CAR, as these cells form an initial ring, which eventually falls apart. Consequently, repeated cytokinetic failure occurs resulting in long multinucleate cells that

perform constitutive polarized growth (Pollard, 2008; Wu et al., 2003). Conversely, during interphase growth, when the cytoskeleton exhibits a polarized morphology, SIN mediated septum formation is inhibited by keeping the Spg1 GTPase in an inactive GDP-bound state. Activation of the SIN using the temperature-sensitive Spg1 GAP mutant *cdc16-116*, results in an arrest in cell elongation coupled with an arrest in nuclear division (Minet et al., 1979; Fankhauser et al., 1993). Actin distribution reflects this arrest in cell elongation since the actin cytoskeleton is dispersed throughout the cell instead of having a polarized configuration (Sparks et al., 1999). This further confirms that an active SIN inhibits actin polarization at the cell tips. Additionally, SIN activation also blocks mitotic entry in interphase cells likely through regulation of Wee1 kinase (Ray et al., 2010). MOR mutants also show a depolarized actin distribution, a block in cell elongation and a G2 arrest, suggesting that SIN activation mimics the absence of MOR activity (Hirata et al., 2002). Overall, the observations from ectopic activation of the SIN raised the possibility that the SIN disrupts the interphase actin cytoskeleton by inhibiting the MOR.

Identification of the Sid2 substrates, Nak1 (a STE20-like/GC family kinase) and its binding partner Sog2, in the MOR pathway suggests that SIN targets these proteins to regulate polarity of the cytoskeleton during mitosis. Previous experiments monitoring the activity of the MOR pathway kinase Orb6 through the cell cycle revealed that Orb6 activity is reduced during mitosis (Kanai et al.,

2005). Results from our lab further clarified that this reduction in Orb6 activity was dependent on the SIN (Ray et al., 2010). Results from Chapter II illustrate that this SIN dependent decrease in Orb6 kinase activity is a consequence of Sid2 mediated phosphorylation of identified MOR components. However, the molecular consequences of SIN phosphorylation of the Nak1/Sog2 complex and their roles in promoting SIN function during mitosis remain unclear. In this chapter, I present results that explore new molecular players in the SIN-MOR crosstalk. Our experiments suggest that interaction of Nak1 with Mor2, a scaffold protein that interacts with both the core kinases, Nak1 and Orb6, appears to be the key interaction inhibited by the SIN. In addition, our results show that SIN mediated phosphorylation of Nak1/Sog2 complex has an additional role in regulating mitotic localization of Nak1/Sog2 at the SPB, which in turn boosts SIN function during early mitosis. Therefore, SIN mediated phosphorylation of MOR proteins appears to have a twofold role in promoting mitotic SIN function.

Results

SIN prevents polarized growth through inhibition of Nak1 mediated Orb6 activation

Since Sid2 kinase phosphorylates Nak1 (as well its interactor Sog2), we wanted to test the precise mechanistic outcome of this modification on MOR function during cytokinesis. It has been previously shown that Nak1 protein level remains constant throughout the cell cycle. Additional results suggest that Nak1 activity is unchanged throughout the cell cycle (Leonhard and Nurse, 2005) . Also, previous *in vitro* kinase assays indicate that an active SIN does not have a significant effect on Nak1 kinase activity (Ray et al., 2010). Since Nak1 is suggested to physically interact with and activate Orb6 (Kanai et al., 2005; Liu and Young, 2012), we hypothesized that the SIN might inhibit association of Nak1 with Orb6. A fusion of the two protein kinases (Nak1-Orb6) was created to test this possibility. To ascertain that the fusion is functional, rescue of growth defects in various MOR pathway mutants was tested. The fusion successfully rescued defects in *nak1-167* and *orb6-25 ts* mutants (Table 3.1). Interestingly, the fusion but not the individual proteins, rescue defects in the temperature sensitive mutant of Mor2 (*mor2-786*), a scaffold protein in the MOR pathway (Table 3.1).

Next, by expressing the Nak1-Orb6 fusion in *cdc3-124 cdc16-116* background, we tested the ability of the fusion to bypass the block in cell elongation upon SIN activation. 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 displayed a long cell phenotype (Figure 3.1B). Simply co-expressing Nak1 and Orb6 proteins did not replicate the elongated cell phenotype observed with the fusion. To test whether Nak1 activity was required for the fusion to be functionally active, we also made a fusion of the Nak1 kinase dead allele and Orb6 (Nak1*-Orb6). This fusion also did not show bypass of SIN inhibition of growth suggesting that Nak1 kinase activity was required in the fusion to drive the polarized growth phenotype (Figure 3.1B). On observing cellular actin, we could see that *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. This distribution of actin is consistent with the cells trying to achieve both mitotic and interphase cytoskeletal distribution (Figure 3.1A).

In addition to bypassing the inhibition of cell elongation, cells expressing the Nak1-Orb6 fusion protein were also able to partially override the block in nuclear division as seen by the reduction in mononucleate and increase in binucleate and tetranucleate cells over time. (Figure 3.2, 3.1A). These results indicated that polarized cell growth as well as nuclear division is prevented during cytokinesis, at least in part, through SIN mediated inhibition of the MOR pathway.

Table 3.1 – Rescue of MOR pathway mutants by Nak1-Orb6 fusion

The ability (+) or inability (-) of the Nak1-Orb6 fusion protein to rescue different MOR pathway ts mutants at the restrictive temperature of 36˚C is shown. N/A indicates conditions that were not tested.

Table 3.1 – Rescue of MOR pathway mutants by Nak1-Orb6 fusion

Strain	Vector	Nak1	Orb6	Nak1–Orb6 fusion
pmo25-35	–	–	N/A	–
nak1-167	–	+	–	+
orb6-25	–	N/A	+	+
mor2-786	–	–	–	+

Figure 3.1 – Fusion of Nak1 kinase to Orb6 kinase bypasses SIN mediated inhibition of polarized growth

(A) *cdc3-124 cdc16-116* cells carrying the indicated plasmids were induced for 19 h in media lacking thiamine and shifted to 36°C; then cells were collected at 2, 4, and 7 h. The plasmids used were the pRep41 vector, or the pREP41 vector carrying Nak1, Orb6, the Nak1–Orb6 fusion, the Nak1*–Orb6 fusion (* indicates kinase dead allele), or the Nak1 and Orb6 genes on separate plasmids (Nak1+Orb6). *cdc3-124 cdc16-116* cells with the indicated transgenes were grown and processed for actin staining. Montage of representative cells with actin (phalloidin staining) and nuclei (DAPI staining) staining are shown for the 7-h time point.

(B) 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. A statistically significant increase in cell length (based on unpaired *t* test analysis) was observed only in cells expressing the fusion construct (*p-value <0.05; ****p-value ≤0.0001). At least 100 cells were analyzed for each time point. Error bars in the cell length plot denote SD of the length measurements obtained from three separate experiments.

Figure 3.1 – Expression of Nak1-Orb6 fusion bypasses SIN mediated inhibition of polarized growth

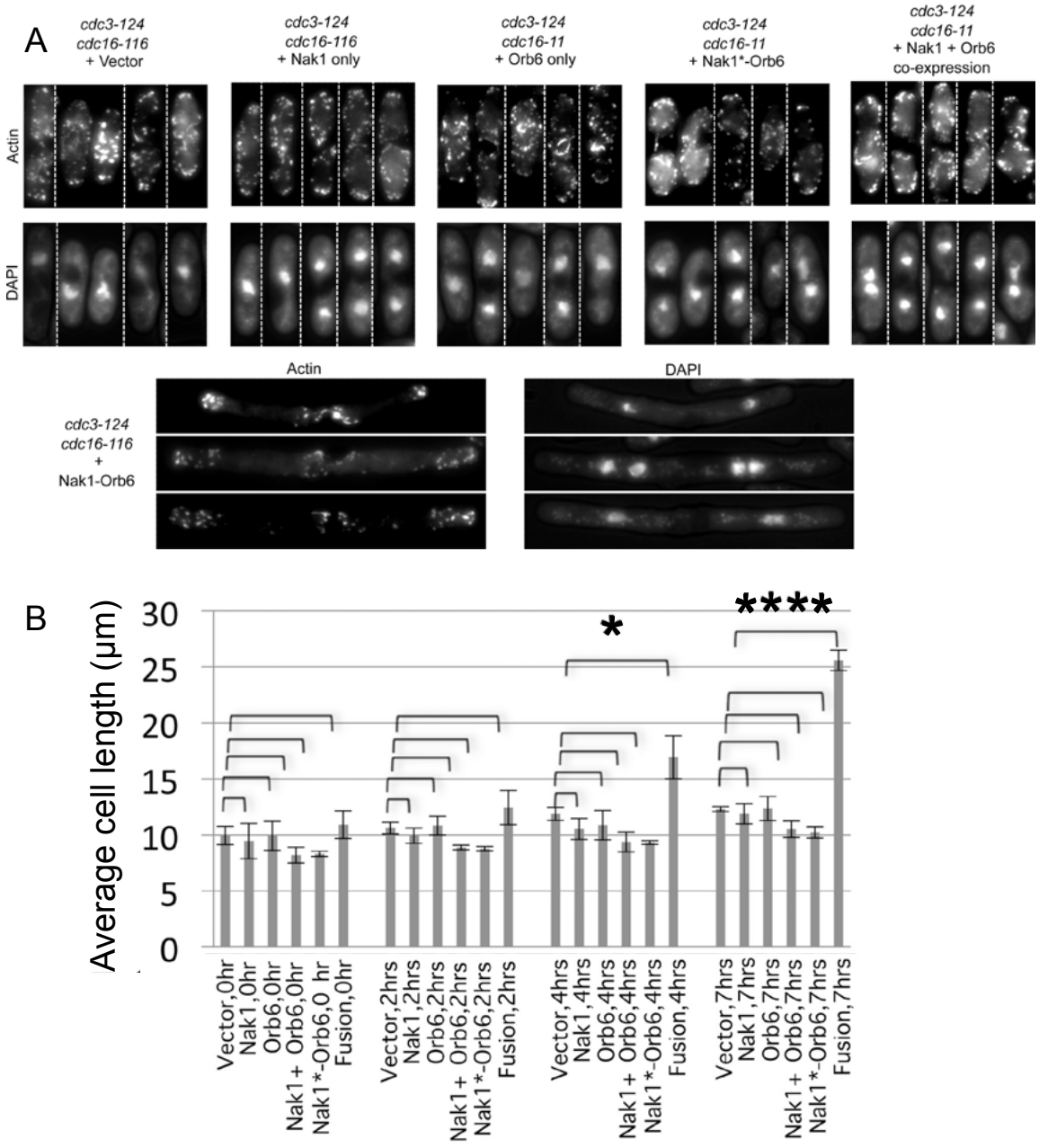
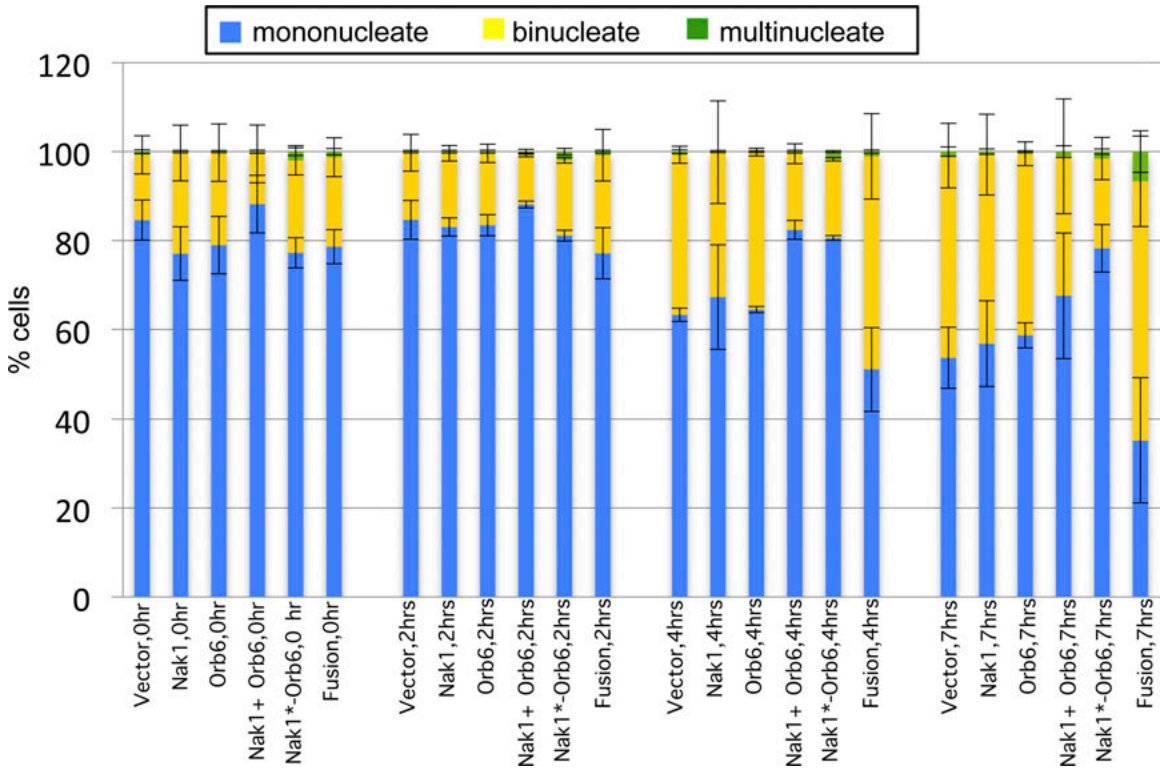


Figure 3.2 – Expression of Nak1-Orb6 fusion bypasses SIN mediated inhibition of nuclear division arrest

Cells were cultured as mentioned in Figure 3.1A. Nuclear count was measured and cells were classified as mononucleate, binucleate or tetranucleate. At least 100 cells were analyzed for each time point. Error bars in the denote SD obtained from three separate experiments.

Figure 3.2 – Expression of Nak1-Orb6 fusion bypasses SIN mediated inhibition of nuclear division arrest



Fusion of Nak1 to Mor2 bypasses SIN mediated inhibition of the MOR

Both Nak1 and Orb6 kinase are known to interact with homolog of the *Drosophila* furry-like protein called Mor2 (Kanai et al., 2005). Mor2 functions as a scaffold that promotes interaction between the two kinases (Hergovich et al., 2006). Consistent with this idea, results from Table 3.1 and Figure 3.1 show that fusion of Nak1 to Orb6 bypasses the requirement for Mor2 in cells and can partially bypass SIN inhibition of polarized growth. Given our identification of Nak1 as a Sid2 target, we hypothesized that Sid2 phosphorylation of Nak1 might inhibit Orb6 activation by blocking interaction between Nak1 and Mor2. If this were the case, then fusion of Nak1 kinase to the Mor2 scaffold would be predicted to bypass SIN inhibition. Therefore, we constructed a Nak1-Mor2 fusion (Figure 3.3A) that was expressed on a plasmid using a thiamine repressible promoter. This fusion was able to rescue both *nak1-167* and *mor2-286* temperature sensitive mutants indicating that both proteins in the fusion remained functional. To determine whether this fusion could bypass SIN mediated inhibition of polarized growth, its expression was induced in cells with activated SIN (*cdc3-124 cdc16-116* cells) (Figure 3.3B,C). (Note that induced expression of the Nak1-Mor2 fusion causes a cell lysis defect (See Chapter IV), but blocking cell division using the cdc3-124 mutation eliminates the lysis phenotype.) Cells with vector control or unfused Nak1 or Mor2 did not elongate. Cells co-expressing the Nak1 and Mor2 proteins also maintained constant cell length. In contrast, expression of the Nak1-Mor2 fusion caused a remarkable increase in cell length both under

induced and repressed conditions (Figure 3.3B,C). Quantification of cell length data showed that cells expressing the Nak1-Mor2 fusion grew over three times the length of control cells after 9h of SIN activation, supporting the model that the SIN inhibits cell elongation by interfering with the interaction between Nak1 and Mor2. We also tested the ability of the Nak1-Mor2 fusion to activate Orb6 kinase using the *in vitro* kinase assay described in Chapter II (See Figure 2.4C). Compared to Nak1-5A (the version of Nak1 that cannot be phosphorylated by Sid2), expression of the Nak1-Mor2 fusion in *cdc3-124 cdc16-116* (active SIN background) cells caused a huge increase in Orb6 kinase activity (Figure 2.4C, lane 6). Furthermore, to directly demonstrate that the SIN regulates the interaction between Nak1 and Mor2, we performed a co-immunoprecipitation analysis. Although, Nak1 and Mor2 showed an interaction *in vivo,* we were unable to determine whether this interaction was inhibited upon SIN activation (*cdc3 cdc16* cells), as the Mor2 protein appeared to undergo degradation under these conditions (Figure 3.4).

Figure 3.3 – Interaction between the Nak1 kinase and Mor2 scaffold is inhibited by the SIN

(A) Schematic diagram of the construct encoding the Nak1-Mor2 and Nak1-5E-Mor2 fusion protein under the control of a medium strength thiamine repressible promoter, *nmt41*.

(B) Expression of both the Nak1-Mor2 and the Nak1-5E-Mor fusions bypassed the SIN mediated inhibition of cell elongation. *cdc16-116 cdc3-124* cells with the indicated plasmids were grown at 25°C in the absence of thiamine for 16 hours (to induce expression of the indicated proteins) and then shifted to 36°C to activate the SIN. Samples were collected every 3 hours and cell lengths of 50 cells were measured. Error bars denote SD values obtained from the average cell lengths measured from three separate experiments. Statistical analysis using unpaired t tests (as indicated by asterisks for the 9h time point) shows that the difference in cell lengths between cells expressing the vector control and either the Nak1-Mor2 or the Nak1-5E-Mor2 fusions were found to be statistically significant (***p-value <0.001).

(C) Images representing the indicated cells from part (B) at 0h and 9h time points are shown. Cells were stained with DAPI and visualized by a combination of fluorescence and DIC microscopy.

Figure 3.3 – Interaction between the Nak1 kinase and Mor2 scaffold is inhibited by the SIN

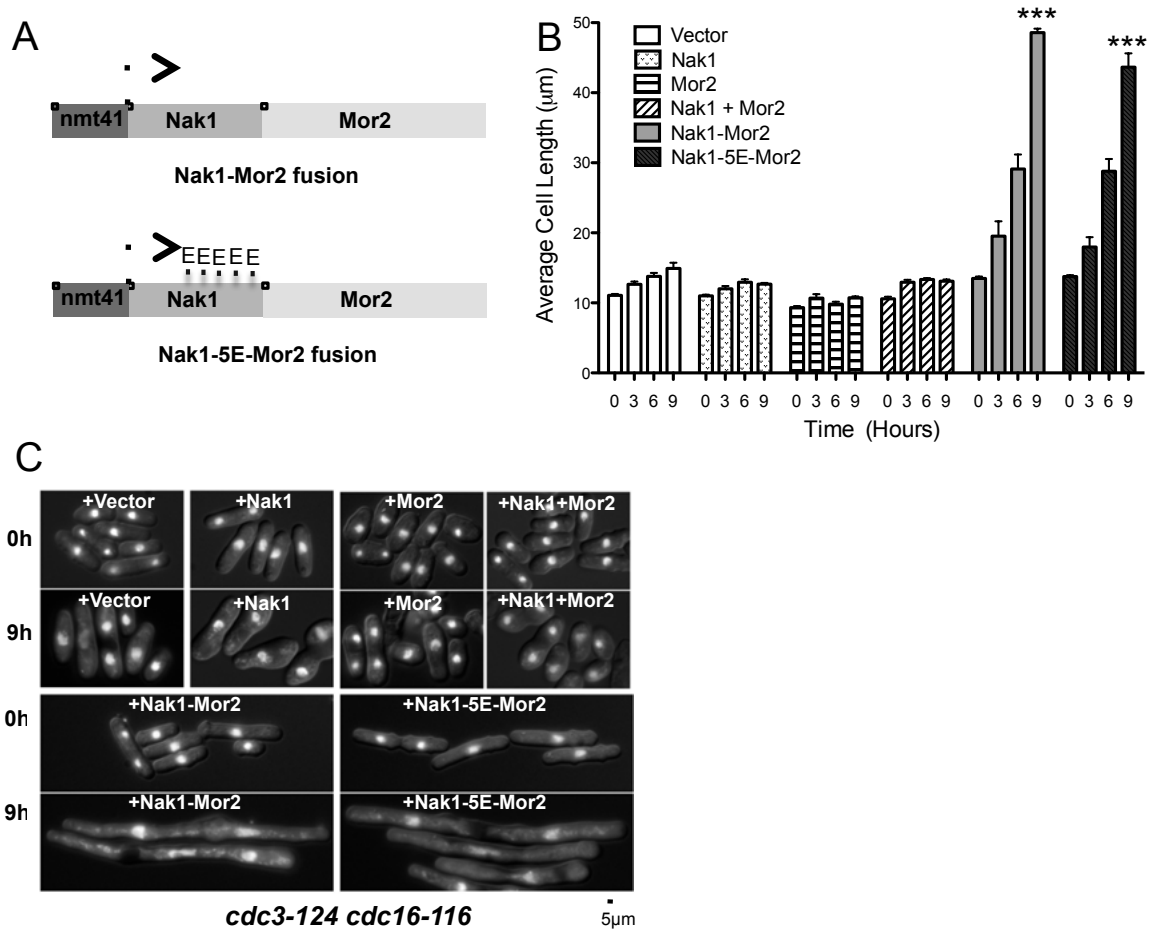
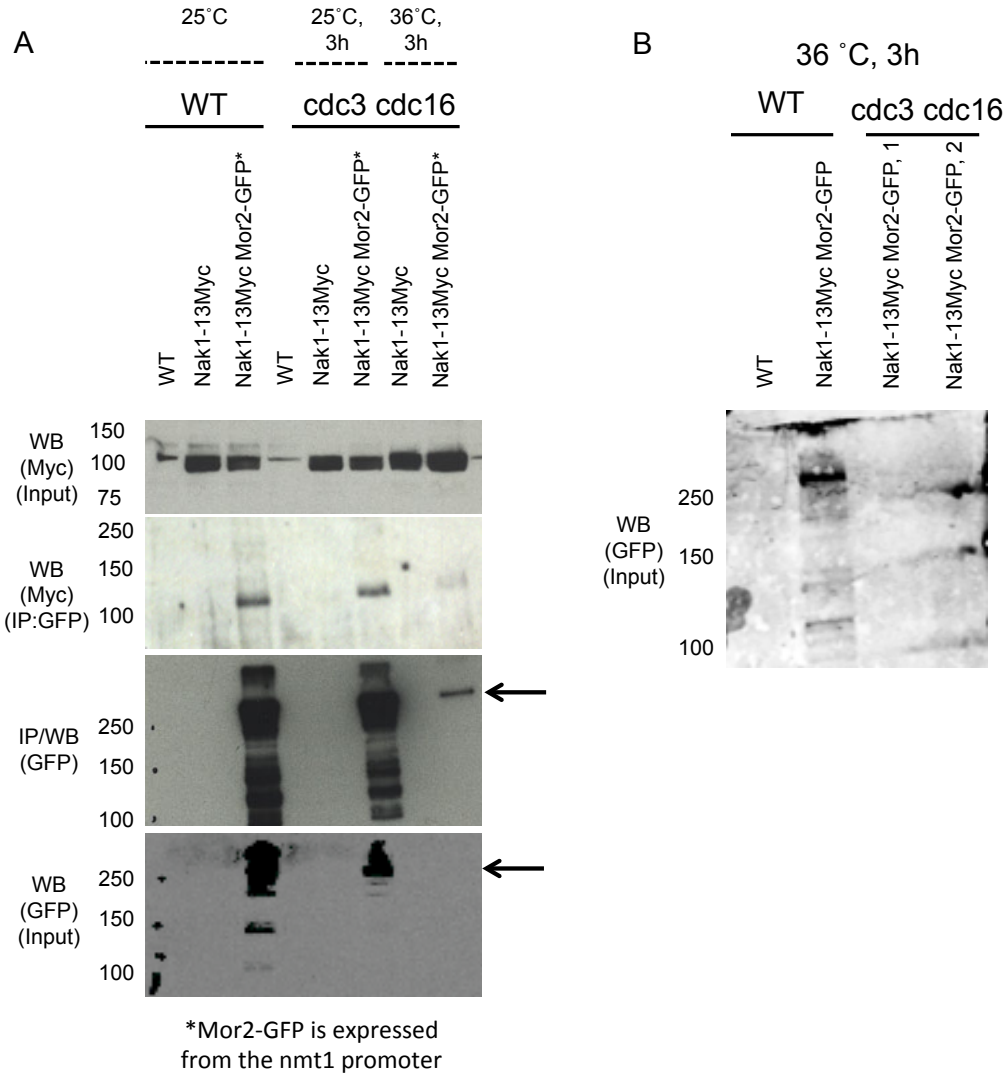


Figure 3.4 – Nak1 interacts with Mor2 *in vivo;* this interaction is disrupted upon ectopic SIN activation due to degradation of Mor2

(A) Co-immunoprecipitation was performed using *nak1-13Myc mor2-GFP* and *nak1-13Myc mor2-GFP cdc3-124 cdc16-116* strains. Immunoprecipitation was done using anti-GFP antibody to pull down Mor2. Precipitated proteins were then immunoblotted using anti-GFP and anti-Myc antibodies. Experiments were performed at 25˚C for WT cells, whereas, *cdc3-124 cdc16-116* cells were either maintained at 25˚C or shifted to 36˚C for 3h before cell lysis and protein sample preparation.

(B) Western blotting was done to determine change in Mor2 protein levels in WT and *cdc3-124 cdc16-116* (SIN active) backgrounds. Immunoprecipitation was performed using anti-GFP antibody and the blot was probed for Mor2 by using anti-GFP antibody.

Figure 3.4 – Nak1 interacts with Mor2 *in vivo;* this interaction is disrupted upon ectopic SIN activation due to degradation of Mor2



Sid2 phosphorylation of Nak1 inhibits its interaction with the scaffold protein Mor2

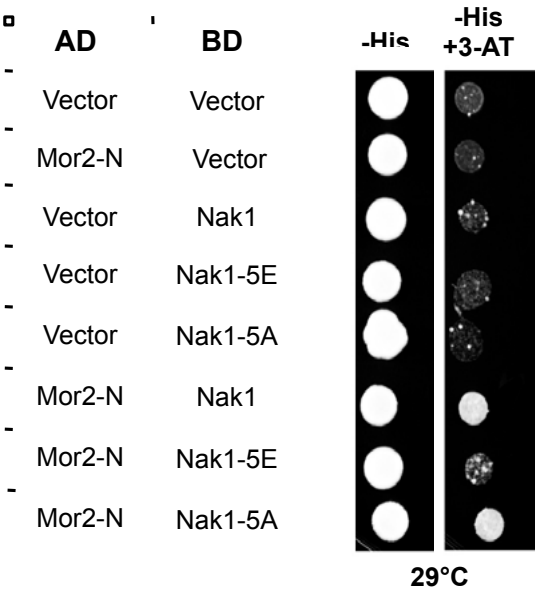
To test more directly whether Sid2 mediated phosphorylation of Nak1 was responsible for disrupting the interaction between Nak1 and Mor2; we created a phospho-mimetic version of the Nak1 protein by mutating the five Sid2 phosphorylation sites on Nak1 to phospho-mimetic glutamic acid residues to make Nak1-5E. Expression of the Nak1-5E mutant protein was unable to completely rescue the polarized growth and cell shape defects in the *nak1-167* mutant like wild-type Nak1 or Nak1-5A (Figure 2.3B). Unlike the Nak1-5A protein, expression of Nak1-5E could not bypass SIN mediated inhibition of cell elongation (Figure 2.4A,B). However, fusion of Nak1-5E to Mor2 (Nak1-5E-Mor2, Figure 3.3A) could bypass SIN inhibition of polarized cell growth similar to the Nak1-Mor2 fusion (Figure 3.3B,C). This indicates that Sid2 kinase mediated phosphorylation of Nak1 most likely disrupts the interaction of Nak1 with the Mor2 scaffold. To test this notion more directly, we examined whether phospho-mimetic mutations in Nak1 could disrupt the previously described two-hybrid interaction between Nak1 and Mor2 (Kanai et al., 2005). Although we observed an interaction between Nak1 and the Mor2 amino-terminus as previously seen, this interaction was greatly reduced with the Nak1-5E mutant (Figure 3.5). Furthermore, the Nak1-5A non-phosphorylatable mutant retained its interaction with the Mor2-N protein (Figure 3.5). Together these results support the model

that Sid2 mediated phosphorylation of Nak1 interferes with its interaction with the Mor2 scaffold.

Figure 3.5 - Nak1, but not the Nak1-5E mutant, shows a physical interaction with the Mor2-N terminus by yeast two-hybrid analysis

Saccharomyces cerevisiae Y190 cells expressing the Mor2-N terminus (aa 1–1095) fused to the Gal4 activation domain (AD) along with either Nak1, Nak1-5E, or Nak1-5A fused to the Gal4 DNA-binding domain (BD) were spotted on SD agar plates lacking histidine +/- 3-aminotriazole (3-AT). Cell growth was observed after culturing at 29˚C for 3 days.

Figure 3.5 - Nak1, but not the Nak1-5E mutant, shows a physical interaction with the Mor2-N terminus by yeast two-hybrid analysis.



Sid2 phosphorylation of Nak1 displaces Nak1 and associated Sog2 protein from the SPB in late anaphase

Nak1 and Sog2 display a curious localization pattern in mitosis. Both proteins are seen at the SPBs in metaphase and remain there until activation of the SIN in late anaphase (Chapter II, Figure 2.2C). Since SIN activation coincided with Nak1 and Sog2 displacement, we hypothesized that this displacement may be SIN dependent. To test if the SIN regulates Nak1 and Sog2 localizations, we observed their GFP fusions in a *sid2-250* mutant after incubation at the semi-permissive temperature of 33°C (incubation at full restrictive temperature (36°C) impaired GFP fluorescence). Interestingly, both proteins persisted at the SPBs longer in anaphase in *sid2-250* mutants compared to wild-type cells and the distance between the SPBs when either SPB contained either Nak1 or Sog2 was significantly longer in *sid2-250* mutants (Figure 3.6A). Moreover, displacement of the two proteins from the SPB was brought about by Sid2 phosphorylation since Nak1-5A and Sog2-5A non-phosphorylatable versions of the proteins also demonstrated persistent localization at the SPB (Figure 3.6A, rightmost panel and 3.6B). These results raise two questions: 1) what does Nak1/Sog2 complex do at SPBs in early mitosis? and 2) why is it important for the SIN to remove Nak1/Sog2 complex from the SPBs in anaphase?

The SIN is normally kept inactive in early mitosis to keep cells from initiating cytokinesis before chromosome segregation has initiated (Guertin et al., 2000;

Feoktistova et al., 2012). We therefore reasoned that SPB-localized Nak1 may inhibit the SIN (which also localizes to SPBs) in early mitosis, and SIN dependent removal of Nak1 from the SPBs in anaphase may enhance SIN activity. To test whether Nak1 inhibits SIN activity in early mitosis, Nak1 was inactivated using the *nak1-167* mutation in cells that were first arrested in metaphase by overexpression of the spindle checkpoint protein Mad2 (He et al., 1998). Although some wild-type cells eventually begin to septate despite the metaphase arrest, premature septation was significantly increased in *nak1-167* mutant cells (Figure 3.6B). The ectopic septation in *nak1-167* cells was blocked by the *sid2-250* mutation (Figure 3.6B) indicating that increased septation in *nak1-167* mutants can be attributed to premature SIN activation. Together these experiments are consistent with a model where Nak1 helps prevent premature SIN activation in metaphase, and SIN dependent removal of Nak1 from the SPBs in anaphase promotes full SIN activation.

Figure 3.6 - Mitotic localization of Nak1 and Sog2 to the SPB is inhibited by the SIN

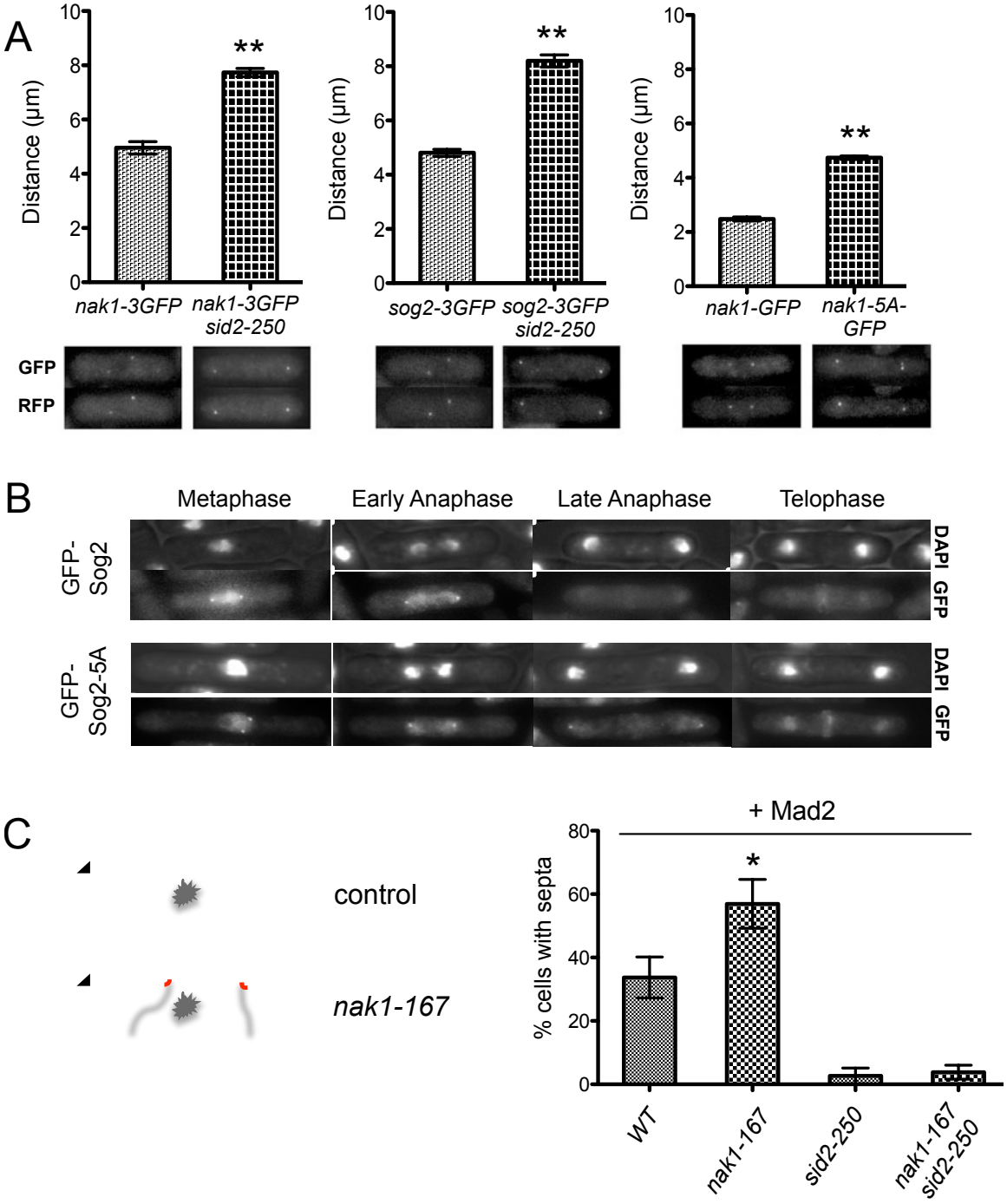
(A) Sid2 promotes loss of Nak1 and Sog2 from anaphase SPBs. Upper panels depict the average distance between SPBs showing localization of the indicated proteins. Lower panels show representative images of the cells examined above. Sid4-RFP marked SPBs. Cells were grown at 25°C in YE followed by a shift to 33°C for 2h, fixed in methanol and then imaged (left and middle panels). In the right panel, *nak1-GFP* and *nak1-5A-GFP* cells were grown overnight in YE at 25°C and then fixed and imaged. Error bars denote SD values obtained from the average distance measured from three separate experiments. Statistical analysis using paired t tests indicates that the difference in SPB separation between the control and the experimental values is statistically significant (all **p-values <0.01, a minimum of 25 cells were scored for every experiment).

(B) Localization of GFP-Sog2 and GFP-Sog2-5A during mitosis. Cells were grown at 25°C in YE and imaged live. Representative images are shown. DAPI (Blue) was used to stain the DNA.

(C) Nak1 inhibits SIN dependent ectopic metaphase septation. Cells of the indicated genotypes were grown at 25°C in the absence of thiamine for 16 hours to induce expression of the *mad2* gene under control of the full strength thiamine inducible promoter (*nmt1*) and then shifted to 36°C for 3 hours. Cells were then fixed in methanol, imaged and a minimum of 100 cells were scored for the presence of ectopic septa (cartoon). Error bars denote SD values obtained from

percentages measured from three independent experiments. Statistical analysis using unpaired t tests indicates that the difference in ectopic septation between the WT control and the *nak1-167* cells is statistically significant (*p-value <0.05).

Figure 3.6 - Mitotic localization of Nak1 and Sog2 to the SPB is inhibited by the SIN



Discussion

Transition from interphase to mitosis results in a major reorganization of the cytoskeleton from the cell tips to the cell middle (Heng and Koh, 2010). In early mitosis, through a pathway initiated by the anillin related Mid1 protein, some cytoskeletal components are required for initial assembly of the actomyosin ring (Wu et al., 2003; Huang et al., 2008). In late mitosis, a decrease in Cdk1 activity is accompanied by activation of the SIN, which regulates actomyosin ring stability, ring constriction and septum formation. Previous studies have shown that perturbation of the ring causes a Clp1 mediated checkpoint arrest that allows the SIN to remain active, maintain the mitotic cytoskeleton, prevent reversion to interphase polarity and complete cytokinesis (Liu et al., 2000; Trautmann et al., 2001; Mishra et al., 2004). The mechanism by which SIN carries out these functions is however unclear. Previous results from our lab show that an active SIN reduces the activity of the NDR family kinase, Orb6, a downstream component of the MOR pathway involved in polarized growth during interphase. This result presented a potential mechanism by which an active SIN maintains the cytoskeletal components in a mitotic configuration. In support of this idea, MOR kinase Nak1, which is required for activation of Orb6 and the Nak1 binding partner Sog2 were identified as in vivo targets of the SIN kinase Sid2 (See Chapter II). Further, mutation of Sid2 phosphorylation sites on the Nak1/Sog2 complex allowed the cells to bypass SIN mediated arrest in polarized growth. Expression of the Nak1-5A non-phosphorylatable mutant also showed a rescue

of decreased Orb6 activity observed upon SIN activation verifying that Sid2 mediated phosphorylation of Nak1/Sog2 complex was a mechanism employed by the SIN to suppress MOR signaling (Chapter II). Results in this chapter show that Nak1/Sog2 phosphorylation by the SIN interferes with the ability of the Nak1 kinase to associate with and activate Orb6 as indicated by the Nak1-Orb6 fusion experiments (Figure 3.1, 3.2). Specifically, it prevents interaction of Nak1 with the Mor2 protein, an evolutionarily conserved scaffold that binds both protein kinases and promotes Nak1 mediated Orb6 activation. (Figure 3.3, 3.5). Upon expression of the Nak1-Mor2 fusion, we not only see reestablishment of polarized growth despite an active SIN but also observe a remarkable increase in Orb6 activity (Figure 2.4C). It is interesting to note that the ability of different transgenes (Nak1-5A, Nak1-Orb6, Nak1-Mor2) to bypass SIN mediated polarized growth arrest varies. Fusion of Nak1 kinase to the Mor2 scaffold was most effective in activating the Orb6 kinase and bypassing SIN induced growth arrest, which suggests that it may be the key interaction that the SIN pathway targets to shutdown the MOR activity. Fusion of Nak1 kinase with the Orb6 kinase or mutating Sid2 kinase phosphorylation sites on Nak1 to alanine could not precisely replicate this phenotype. Also, the Mor2 scaffold appears to degrade upon ectopic SIN activation suggesting that SIN may be regulating the stability of this protein to modulate MOR activity. Mor2 is an evolutionarily conserved protein with homologs in *S.cerevisiae*, *C.elegans* and *D.melanogaster* called Tao3/Pag1, SAX-2 and Fry respectively (Du and Novick, 2002; Gallegos and Bargmann,

2004; Cong et al., 2001). These are large proteins (approximately 300 kDa) that have been observed to function as scaffolds for protein complexes containing NDR kinases (Tamaskovic et al., 2003; Hergovich et al., 2006). Furthermore, as seen in yeast and *drosophila* they interact with these NDR kinases and are important for their function (Kanai et al., 2005; Du and Novick, 2002; Fang et al.; Emoto et al., 2004). It would be interesting to identify regulation of this interaction in other higher organisms and understand the roles of scaffolds in mediating NDR kinase pathway functions.

Mitotic entry is accompanied by a switch to cell growth arrest and assembly of the division apparatus. SIN is inactive in early mitosis, when Cdk1 activity is high, and only turns on in late mitosis upon decline in Cdk1 activity. This regulation of SIN activity allows chromosomal segregation to occur before the division of cytoplasm ensuring equal distribution of nuclear material. Further, MOR proteins Nak1 and associated Sog2, both of which localize to the cell tips during interphase, show a change in localization as cells enter mitosis. These proteins localize to the SPB early in mitosis and disappear from the SPB when the SIN gets activated in late anaphase. On exploring this mitotic localization of Nak1/Sog2 complex, our work also clarified another mechanism of crosstalk between the SIN and MOR pathways. We found that Nak1 (possibly in conjunction with Sog2), through its SPB localization in early mitosis helps prevent premature activation of the SIN. Sid2 mediated phosphorylation of Nak1/Sog2 is

responsible for displacing the complex from the SPB. This regulatory mechanism in combination with decreased Cdk1 activity may be needed for full activation of the SIN in late anaphase. Further investigation will be required to determine how Nak1/Sog2 at the SPB inhibits the SIN.

Interestingly, both Nak1 and Sog2 contain putative Cdk1 phosphorylation sites some of which have been identified by Mass spectroscopy (unpublished work of the Gould Lab, Cipak et al., 2013). Moreover, some of these sites show an overlap with Sid2 sites identified on these proteins. Since Cdk1 activity is high during mitosis, it is conceivable that Cdk1 mediated phosphorylation of these proteins regulates polarized growth during early mitosis. Preliminary data generated by utilizing the Nak1-Mor2 fusion supports this possibility. We expressed the Nak1-Mor2 in *nda3-km311* cells, a cold sensitive tubulin mutant strain that arrests in metaphase at 19˚C (Toda et al., 1983). Expression of the fusion in the metaphase arrested cells resulted in elongated cells indicated a bypass of cell growth arrest (Figure 3.7). Therefore, it appears that inhibiting Nak1 and Mor2 interaction, potentially by Cdk1 phosphorylation of Nak1 could be a possible SIN-independent mechanism for maintaining a constant cell length during early mitosis. Further experiments need to be performed to identify the specifics of this mechanism.

Finally, while our results have successfully demonstrated that SIN inhibits the

MOR by decreasing Orb6 activity, we also verified the effect of uninhibited MOR on SIN activity by utilizing our MOR protein fusions. Using in vitro kinase assays, earlier results from our lab have shown that overexpression of the Nak1-Orb6 fusion does not cause a significant decrease in Sid2 activity (Figure 3.8A,Ray et al., 2010). As a complement to this result, we also noticed that cells that express the Nak1-Mor2 fusion maintain asymmetric Cdc7 localization, an indicator of active SIN, throughout cytokinesis (Figure 3.8B). Moreover, monitoring Cdc7 localization in *orb6-25* ts cells showed that Cdc7 did not persist at the SPB in these MOR mutants after completion of cytokinesis suggesting that SIN inactivation in the following interphase was not MOR dependent (Ray et al., 2010). In conclusion, while SIN activation during interphase inhibits MOR activity resulting in loss of polarity, increase in MOR signaling in cells with an active SIN does not affect SIN activity directly, but acts downstream of SIN signaling pathway to affect SIN function of growth inhibition.

Figure 3.7 – Cdk1 mediated inhibition of Nak1 and Mor2 interaction could be the mechanism of growth inhibition in early mitosis

This figure contains representative images of metaphase arrested, cold-sensitive *nda3-km311* cells expressing either the vector control (pREP41) or the Nak1-Mor2 fusion protein (pREP41-Nak1-Mor2). Both empty vector and the Nak1-Mor2 fusion were expressed either under repressed (upper panels) or induced (lower panels) conditions for 24h. While cells expressing the control plasmid maintain a growth arrest at the restrictive temperature of 19˚C, cells expressing the fusion show cell significant increase in cell length.

Figure 3.7 – Cdk1 mediated inhibition of Nak1 and Mor2 interaction could be a growth inhibition mechanism in early mitosis.

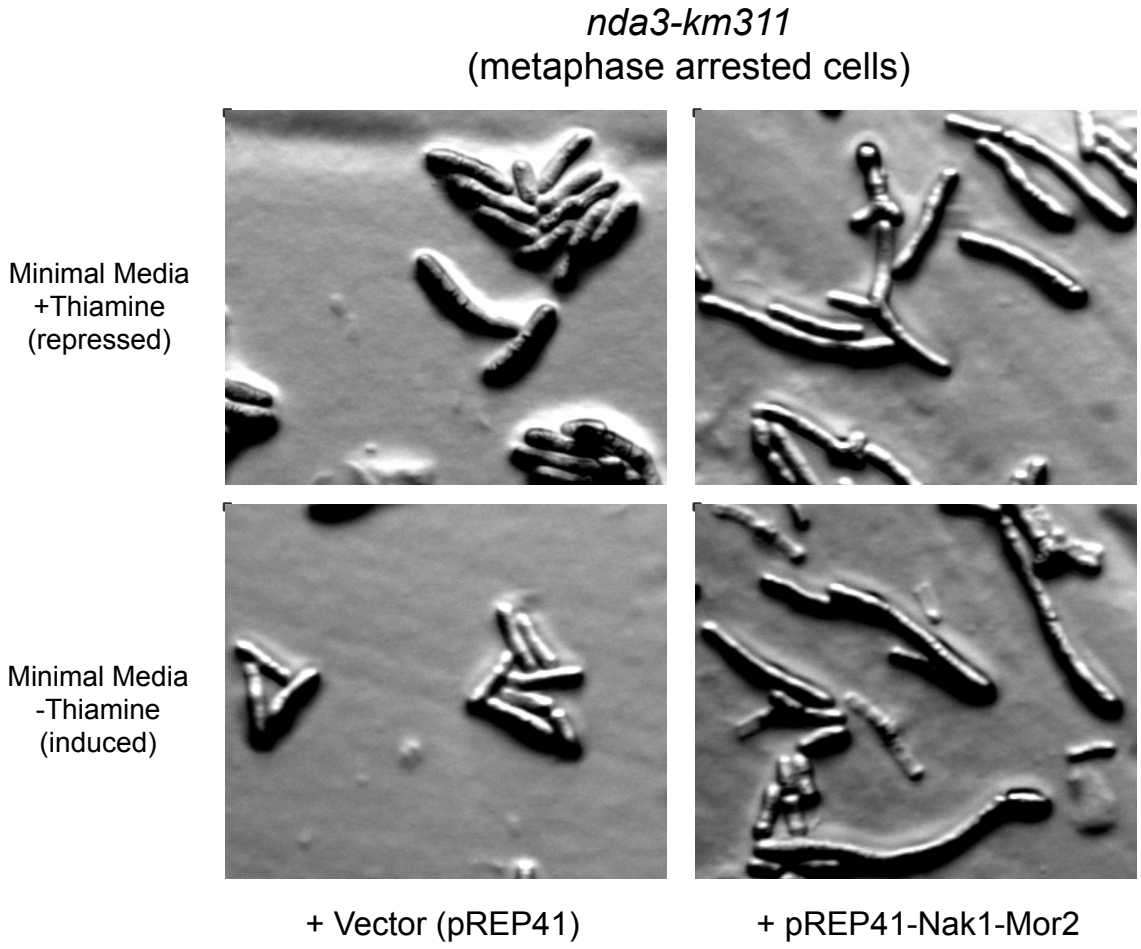
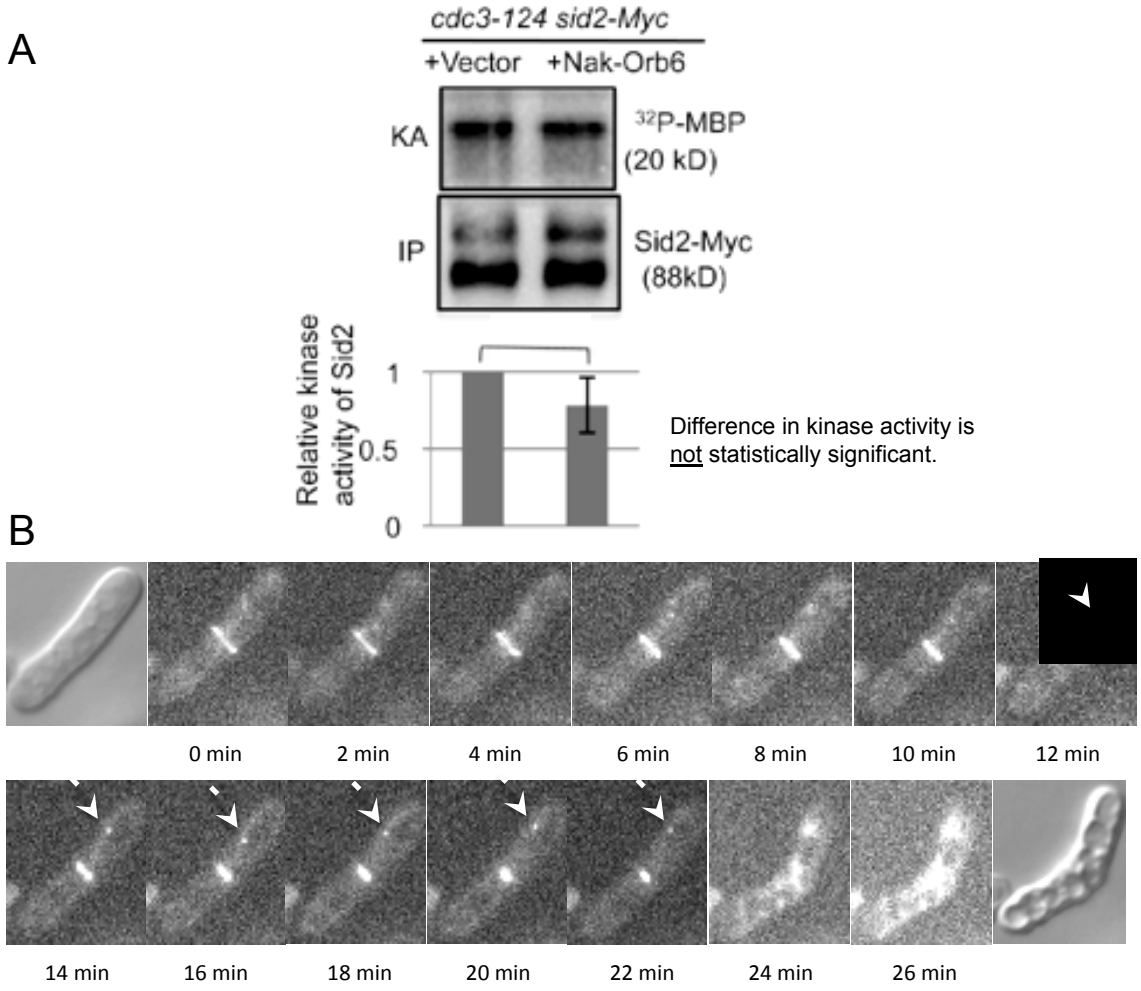


Figure 3.8 – MOR does not affect SIN activity

(A) *cdc3-124 sid2-13Myc* cells expressing either the empty vector control or the Nak1-Orb6 fusion protein were grown in media lacking thiamine for 19 h at 25°C and then shifted to 36°C for 3h. Cell extracts were prepared and Sid2-13Myc was immuno-precipitated with anti-Myc antibody. The immunoprecipitates were split, with one portion used for Western blotting using Myc antibodies (IP) and the other portion used for in vitro kinase assays using myelin basic protein (MBP) as an artificial substrate (Ray et al., 2010). The kinase activity (KA) was measured using a PhosphorImager, quantified using 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 three different experiments. p value = 0.1047.

(B) Nak1-Mor2 fusion was expressed in cells with GFP tagged cdc7 kinase and GFP tagged Rlc1 (regulatory light chain protein) to monitor the cytokinetic ring. Cdc7-GFP (indicated by white arrows) can be seen localized to the SPB as cells undergo ring constriction (lysis seen is discussed in Chapter IV).

Figure 3.8 – MOR does not affect SIN activity



rlc1-GFP cdc7-GFP + pREP41-Nak1-Mor2

CHAPTER IV

Significance of MOR inhibition during cytokinesis

My contribution to this chapter: Figures 4.1A, 4.1B, 4.1C, 4.2A, 4.2B, 4.2C, 4.3A, 4.3B, 4.3C, 4.4, 4.5A, 4.5B, 4.5C, 4.5D

All figures in this chapter are part of the following manuscript under preparation: **Gupta S**, McCollum D. Crosstalk between NDR kinase pathways co-ordinates cytokinesis with cell separation in *S. pombe.*

Summary

NDR (Nuclear Dbf-2 related) kinases constitute key regulatory nodes in signaling networks that control multiple biological processes such as growth, proliferation, mitotic exit, morphogenesis and apoptosis. Two NDR signaling cascades exist in *S. pombe* called the Septation Initiation Network (SIN) and the Morphogenesis Orb6 Network (MOR), which regulate the cytoskeletal machinery during cytokinesis and polarized interphase growth, respectively. Previous results show that during late mitosis, an active SIN inhibits signaling through the MOR via phosphorylation of the MOR pathway kinase Nak1 and its binding partner Sog2. This reinforces the premise that mechanisms must exist to prevent the two distinct regulatory programs from interfering with one another. Work documented in this chapter reveals that failure to inhibit MOR signaling in a WT background is lethal because cells initiate septum degradation/cell separation before completing cytokinesis. Overall, we conclude that SIN inhibition of MOR pathway during late mitosis plays an important role in coordinating cell separation with completion of cytokinesis.

Introduction

Cytokinesis in *S. pombe* is primarily regulated by the SIN pathway, an NDR kinase signaling network, which is activated in late mitosis and is required for constriction of the actomyosin ring and septum assembly. Like mammalian cells, *Schizosaccharomyces pombe* divides through the use of an actomyosin contractile ring, which is composed of a set of highly conserved cytoskeletal proteins some of which have been recently identified as SIN targets (See Figure 1.6, Figure 2.6). In addition, the SIN pathway has been implicated in numerous other cell cycle roles such as inhibition of polarized cell growth during late mitosis, spindle checkpoint inactivation, telophase nuclear positioning and timing of mitotic commitment (Figure 1.6, Figure 2.6). We have previously determined that the SIN inhibition of polarized cell growth during cytokinesis occurs through crosstalk with a second NDR kinase pathway in fission yeast called the MOR, which regulates cell separation and polarized growth following completion of cytokinesis (Gupta et al., 2013; Ray et al., 2010, Also see Chapters II and III). Such crosstalk between NDR kinase pathways is a conserved phenomenon that is observed across species (See General Discussion). MOR pathway proteins, Nak1 kinase and its binding partner Sog2 were identified as two targets of the SIN effector kinase Sid2 by mass spectrometric analysis in our study (Gupta et al., 2013). We also showed that Sid2 mediated phosphorylation of Nak1/Sog2 plays a twofold role in regulation of the MOR during mitosis. At anaphase onset, phosphorylation of the MOR proteins is required to displace these proteins from

the spindle pole body, which is required for full activation of the SIN in late mitosis. SIN phosphorylation of Nak1 kinase is also necessary to inhibit MOR signaling in order to prevent reversion to polarized growth during cytokinesis. While mechanisms of SIN-MOR crosstalk have been delineated, SIN inhibition of the MOR was shown to be crucial only when the cytokinetic machinery was compromised (Ray et al., 2010). Also, circumventing SIN inhibition of the MOR by utilizing the non-phosphorylatable alanine mutant Nak1-5A did not show any observable cytokinetic defects in WT cells. Therefore, a comprehensive understanding of the mechanisms of SIN inhibition of MOR functions during cytokinesis is still incomplete. Moreover, the significance of the cross-regulation between the fission yeast NDR kinase pathways needs to be elucidated.

In this chapter, our results demonstrate that an ectopic activation of the MOR by using a fusion of the Nak1 kinase to the Mor2 scaffold protein causes severe lysis defects during cytokinesis. We characterized the precise cytokinetic defects through extensive time-lapse analysis and determined that failure of the SIN to inhibit the MOR results in cell lysis because an active MOR initiates the cell separation program prematurely. We further identified some of the molecular players that may responsible for these cell division defects. Taken together, our work reveals the importance of coordinating SIN and MOR activities during cytokinesis.

Results

Unregulated MOR signaling causes cell lysis during cytokinesis

In comparison to non-phosphorylatable mutants or the Nak1-Orb6 fusion, the Nak1-Mor2 fusion was much better at bypassing SIN-mediated inhibition of polarized growth (See Chapter III). Expression of Nak1-Orb6 fusion in cells with a compromised SIN (sid2-250 ts mutants at a semi-permissive temperature of 29˚C) or in cells where the cytokinetic apparatus was perturbed (using Latrunculin B, an actin depolymerizing drug) resulted in growth defects (Figure 4.1A, Ray et al., 2010). However, expression of the Nak1-Orb6 fusion in WT cells did not result in any significant cytokinesis defects. Moreover, *in vitro* kinase assay measuring Orb6 activity indicated that the Nak1-Mor2 fusion protein was more effective at activating signaling through the MOR cascade (See Figure 2.4C). Therefore, to better understand the consequences of inappropriate MOR activation during cytokinesis, we examined the effect of Nak1-Mor2 expression in wild-type cells. Interestingly, expression of the Nak1-Mor2 fusion was severely toxic in WT cells. A high frequency of septating cells expressing the fusion protein showed cell lysis (Figure 4.1B,C). Cells grown in a liquid EMM showed an increased rate of lysis with increased time of induction. Addition of 1.2M sorbitol to the culture medium was found to rescue this lysis phenotype (Figure 4.1B,C). Since sorbitol is well known as an osmotic stabilizer that has been shown to rescue similar lysis phenotypes in cells (Ribas et al., 1991; Santos et al., 2003), the lysis phenotype presumably reflected a cell wall defect. Interestingly, SIN

temperature sensitive mutants display a similar lysis phenotype when subjected to a semi-permissive temperature of 33 ˚C (Jin et al., 2006). This lysis phenotype in SIN mutants is likely caused by failure to inhibit the MOR, since it can be rescued by partial inhibition of the MOR (Ray et al., 2010). Therefore, the phenotype we observe upon ectopic MOR activation using the Nak1-Mor2 fusion could be indicative of a weakened ability of SIN to inhibit the MOR. Hence, when SIN pathway is unable to completely restrict MOR signaling, an uninhibited MOR can cause cell wall defects resulting in lysis.

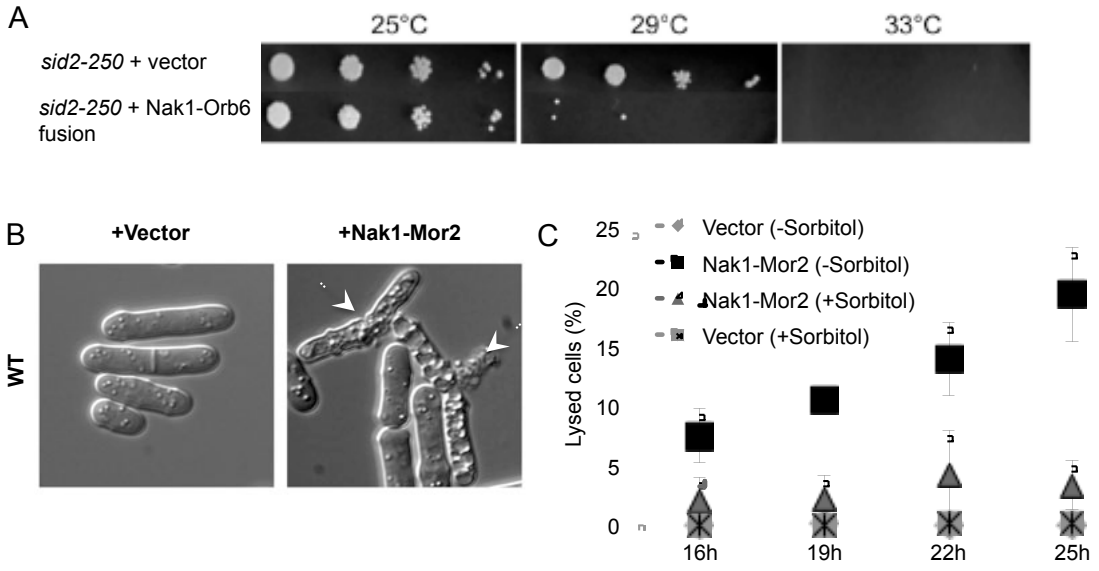
Figure 4.1 – Inability to inhibit the MOR during late mitosis results in growth defects due to cytokinetic failure

(A) *sid2-250* cells expressing the vector alone or the Nak1–Orb6 fusion were grown in medium lacking thiamine for 14 h 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) Representative images of WT cells expressing either vector or Nak1-Mor2 fusion for 19h at 25˚C in medium lacking thiamine to induce expression of the proteins are shown. *White arrows* indicate point of lysis in cell middle and expulsion of cytoplasmic material.

(C) Quantitation of the lysis defect in cells, grown as indicated in B, is represented as percentage of cells that lyse at different times of induction (16h, 19h, 22h, 25h). The experiment was performed both in medium containing sorbitol (+sorbitol) and medium lacking sorbitol (-sorbitol).

Figure 4.1 – Inability to inhibit the MOR during late mitosis results in growth defects due to cytokinetic failure



Nak1-Mor2 expression induces cell lysis at different stages of cell division

To determine the precise stage of cytokinesis/septation during which lysis occurs, we performed time-lapse analyses on cells expressing the fusion along with the contractile actomyosin ring markers YFP-Myo2 or Rlc1-GFP. In separate time-lapse experiments, septum formation was also followed using DIC. Cytokinesis in *S. pombe* normally follows an orderly series of events (García-Cortés and McCollum, 2009; Goyal et al., 2011). The actomyosin ring forms in early mitosis, then in anaphase the SIN becomes active and causes the division septum to form behind the constricting actomyosin ring. Completion of ring constriction and septum formation causes SIN inactivation. Once the primary septum is completed the cell forms new cell wall behind the septum and secretes enzymes to degrade the septum and bring about cell separation. Our time-lapse data suggested that this process was becoming disrupted at various points in Nak1-Mor2 expressing cells since cell lysis was observed during ring constriction (10 out of 14 cells), after completion of ring constriction (3 out of 14 cells) and before initiation of constriction (1 out of 14 cells) (Figure 4.2A,C). Consistent with these results, time-lapse analysis of septum formation in Nak1-Mor2 expressing cells using DIC microscopy showed that cells lysed both during septum ingression (5 out of 10 cells) and following completion of septum formation (5 out of 10 cells) (Figure 4.2B,C). Therefore, inappropriate MOR activity causes cell lysis during cytokinesis, but cell lysis does not seem to be restricted to just one phase of cytokinesis.

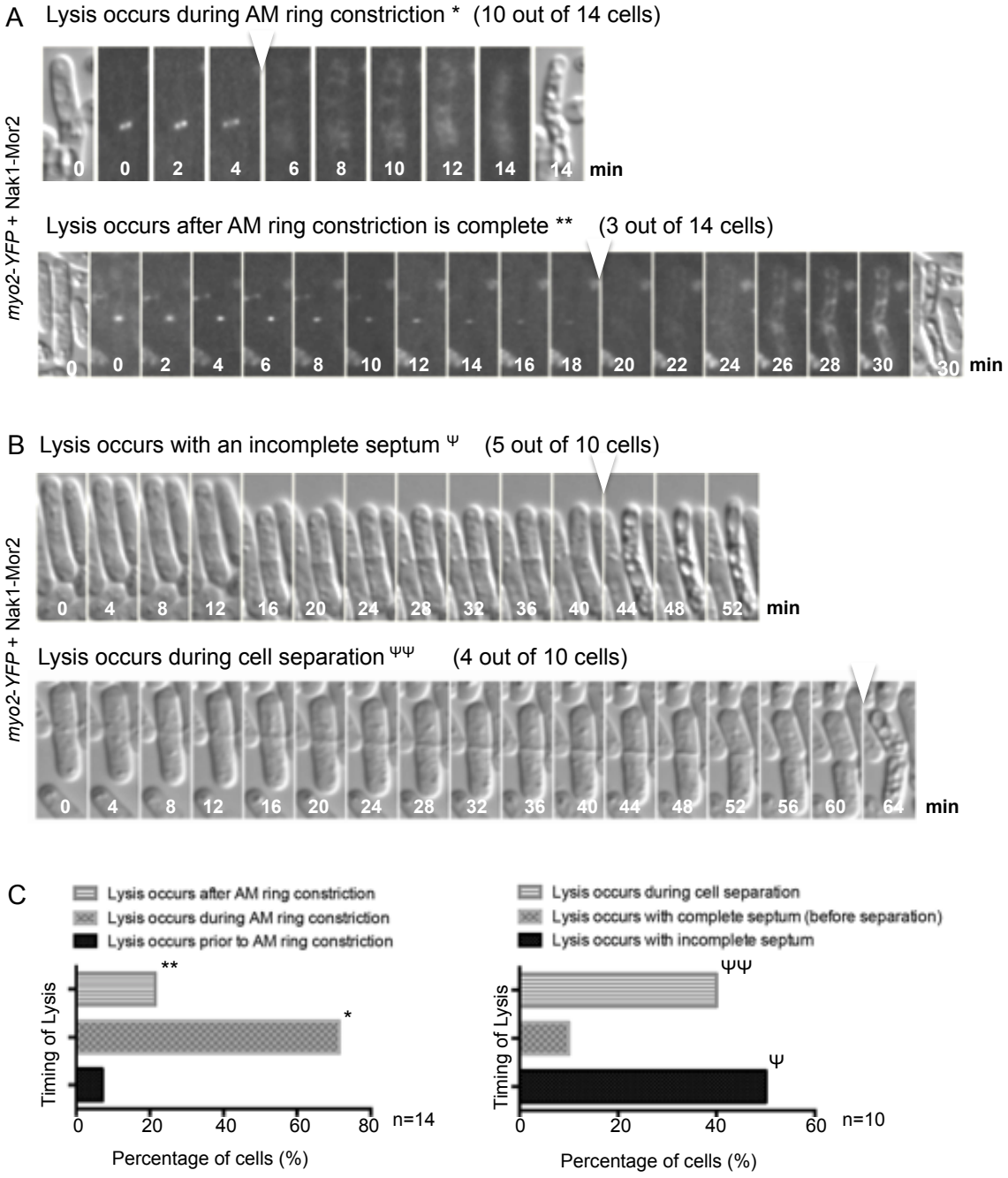
Figure 4.2 – Characterization of the timing of cell lysis seen in WT cells expressing the Nak1-Mor2 fusion

(A) Cells were grown in medium lacking thiamine for 19h at 25˚C to induce the expression of the empty vector control (pREP41) or the Nak1-Mor2 fusion and then monitored by fluorescence and DIC time-lapse microscopy. Images were taken at every 2 min interval of the time-lapse movie. The actomyosin (AM) ring dynamics were followed in WT cells by monitoring YFP tagged Myo2 protein. The upper panel shows a representative cell (of the 10 out of the 14 cells monitored) that undergoes lysis in the middle of ring constriction. The lower panel shows a representative cell (of the 3 out 14 cells monitored) that undergoes lysis following completion of ring constriction. Single DIC images of the cells at the initial and final timepoint are also included for clarity.

(B) Cells were grown as indicated in A. Septum dynamics were followed in the same cells by DIC microscopy. The upper panel represents a single cell (of the 5 out of 10 cells monitored) that undergoes lysis while cells are still forming the septum. The lower panel represents a single cell (of the 4 out of 10 cells monitored) that undergoes lysis after cells have completed septum formation and have progressed to separation of the daughter cells.

(C) Graphs indicating quantification of cells (%) undergoing lysis at different phases of the ring constriction (left) and septum formation (right) process as indicated are shown.

Figure 4.2 – Characterization of the timing of cell lysis seen in WT cells expressing the Nak1-Mor2 fusion.



Lysis phenotype observed upon expression of the Nak1-Mor2 fusion is the result of premature cell separation

To test whether the dynamics of ring constriction or septum formation were affected by the expression of the Nak1-Mor2 fusion, we performed time-lapse analyses on these cells while culturing them in media containing the osmotic stabilizer, sorbitol. Addition of sorbitol prevents cell lysis and allows us to monitor the cytokinetic process uninterrupted. To visualize the actomyosin ring, we utilized GFP tagged Lifeact, a 17 amino acid peptide, which binds to F-actin in the cell. In order to follow the timing of the cytokinetic process closely, we monitored the mCherry tagged SPB marker, Pcp1. For this purpose, the time at which the SPB separates was assigned as time zero. Using time-lapse analysis we calculated average time taken for appearance of the actomyosin ring, initiation of ring constriction, completion of ring constriction and initiation of cell separation following SPB duplication by both the control cells expressing the vector as well as cells expressing the Nak1-Mor2 fusion (Figure 4.3A,C). We noticed that initiation of ring constriction was delayed slightly but significantly in cells expressing the Nak1-Mor2 fusion. Duration of ring constriction however, did not show any considerable difference. Most strikingly, the timing at which cell separation occurred was significantly reduced in cells expressing the fusion protein (Figure 4.3C). We concluded that early initiation of separation during late mitosis in cells expressing the Nak1-Mor2 fusion protein indicates premature activation of the cell separation process in these cells. This observation also

offers one explanation for the delay in initiation of ring constriction. Cell wall assembly provides the necessary force for cytokinesis and contributes to ingression of the cleavage furrow (Proctor et al., 2012). Premature cell separation could be explained by untimely recruitment or activation of cell wall degradation enzymes, which could potentially interfere with SIN functions such as formation, and subsequent ingression of the septum as well as initial constriction of the ring. An alternative reason could be that activation of MOR during cytokinesis creates a competition for common cytoskeletal components such as actin, since the cell would now require actin at both the cell tips for MOR induced polarized growth as well as at the cell middle for ring formation. To test this hypothesis, we measured fluorescence ratio of GFP, which is bound to actin, at the actomyosin ring versus the cell ends (Figure 4.4). This measurement was made at a timepoint immediately preceding the initiation of constriction for accuracy. We observed a significant decrease in this ratio in cells expressing the Nak1-Mor2 fusion. However, this effect does not appear to be substantial enough to cause a major defect in ring formation or a slow down ring constriction dynamics in the Nak1-Mor2 expressing cells. Taken together, these results demonstrate that inability to restrain MOR signaling during cytokinesis primarily affects timing of cell separation and has certain minor effects on dynamics of actomyosin ring constriction.

Figure 4.3 – Cells with unregulated MOR signaling separate prematurely

(A) and (B) Cells were grown in medium lacking thiamine for 19h at 25˚C to induce the expression of the empty vector (pREP41) control (A) or the Nak1-Mor2 fusion (B). The medium also contained sorbitol (osmotic stabilizer) to prevent cell lysis. Cells were monitored by fluorescence and DIC time-lapse microscopy for 2-3 h. Images taken every 7.5 minutes are included in the figure. The actomyosin (AM) ring dynamics were followed in WT cells by monitoring GFP tagged lifeact peptide, which binds actin. SPB duplication was monitored using mCherry tagged SPB marker, Pcp1. DIC movies of the cells to observe formation of the division septum were simultaneously filmed and are also included in the panels.

(C) Graphs representing quantification of distance between SPBs, time taken to complete indicated cytokinetic phases and time between different cytokinetic phases (*shown in red*) are shown. Time at which the SPB initiates duplication was assigned as time zero. The cytokinetic process was monitored from start (T_0) to finish in at least 17 cells expressing the vector and 11 cells expressing the Nak1-Mor2 fusion. Statistical analyses using unpaired t tests were done to compare time required for completion of different cytokinetic phases (*in red*). These analyses revealed that time between appearance of ring and initiation of ring constriction varied significantly (**p-value <0.01) between cells expression the vector (left graph) and those expressing the Nak1-Mor2 fusion (right graph). Also, the difference in time between completion of ring constriction and initiation

of cell separation was statistically significant (****p-value ≤0.0001) between cells expressing the vector (left graph) and those expressing the Nak1-Mor2 fusion (right graph).

Figure 4.3 – Cells with unregulated MOR signaling separate prematurely

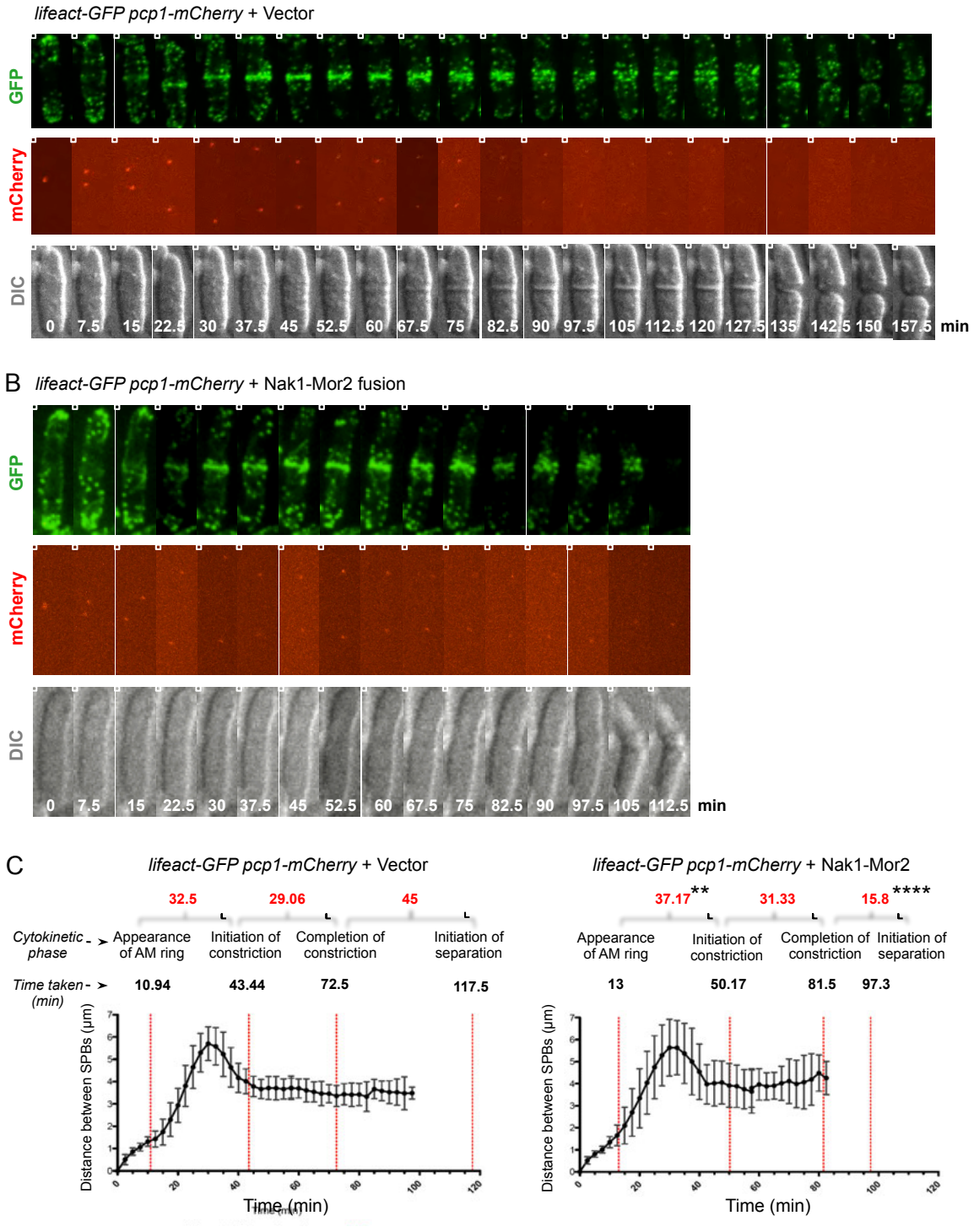
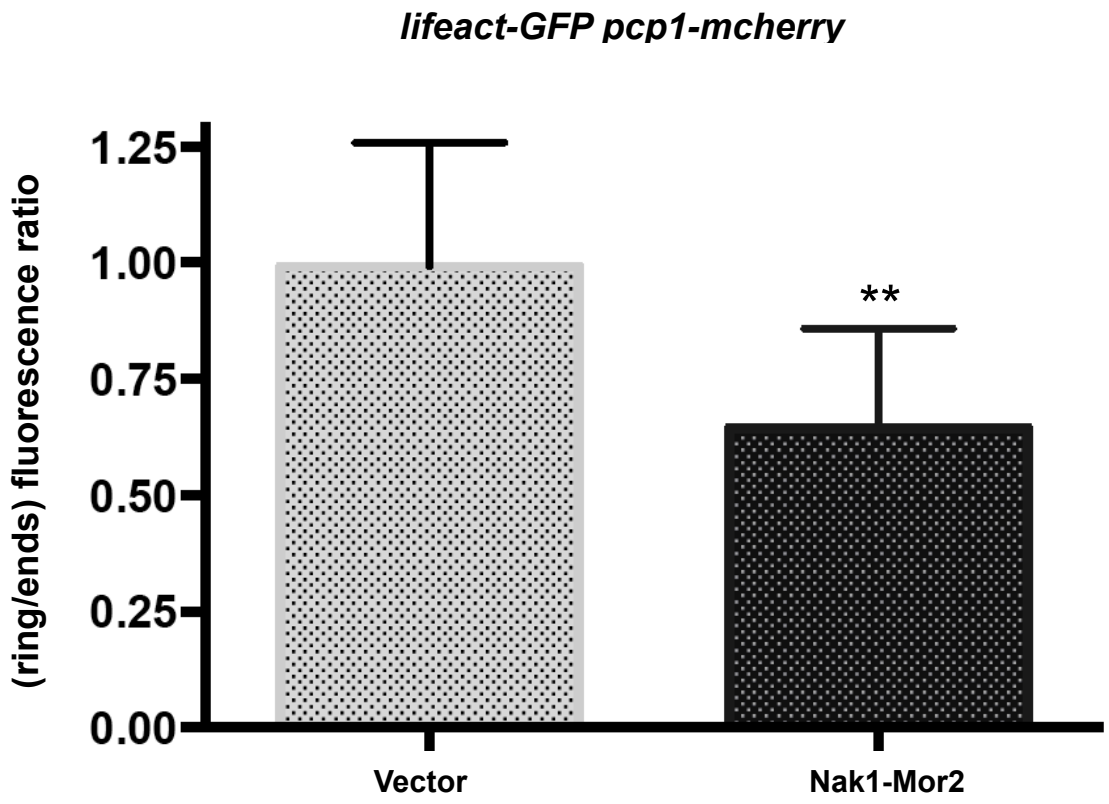


Figure 4.4 – Unregulated MOR reduces "ring/cell ends" actin ratio

Average actin fluorescence intensity was measured at the cell tips and cell middle using Image J software (FIJI). A graph indicating the ratio of fluorescence measured at cell middle (ring) versus cell ends is shown. Statistical analysis done using unpaired t tests shows that the difference in ratios between cells expressing the vector (pREP41; n=12) and those expressing the Nak1-Mor2 fusion (n=13) was statistically significant (**p-value <0.01).

Figure 4.4 – Unregulated MOR reduces "ring/cell ends" actin ratio



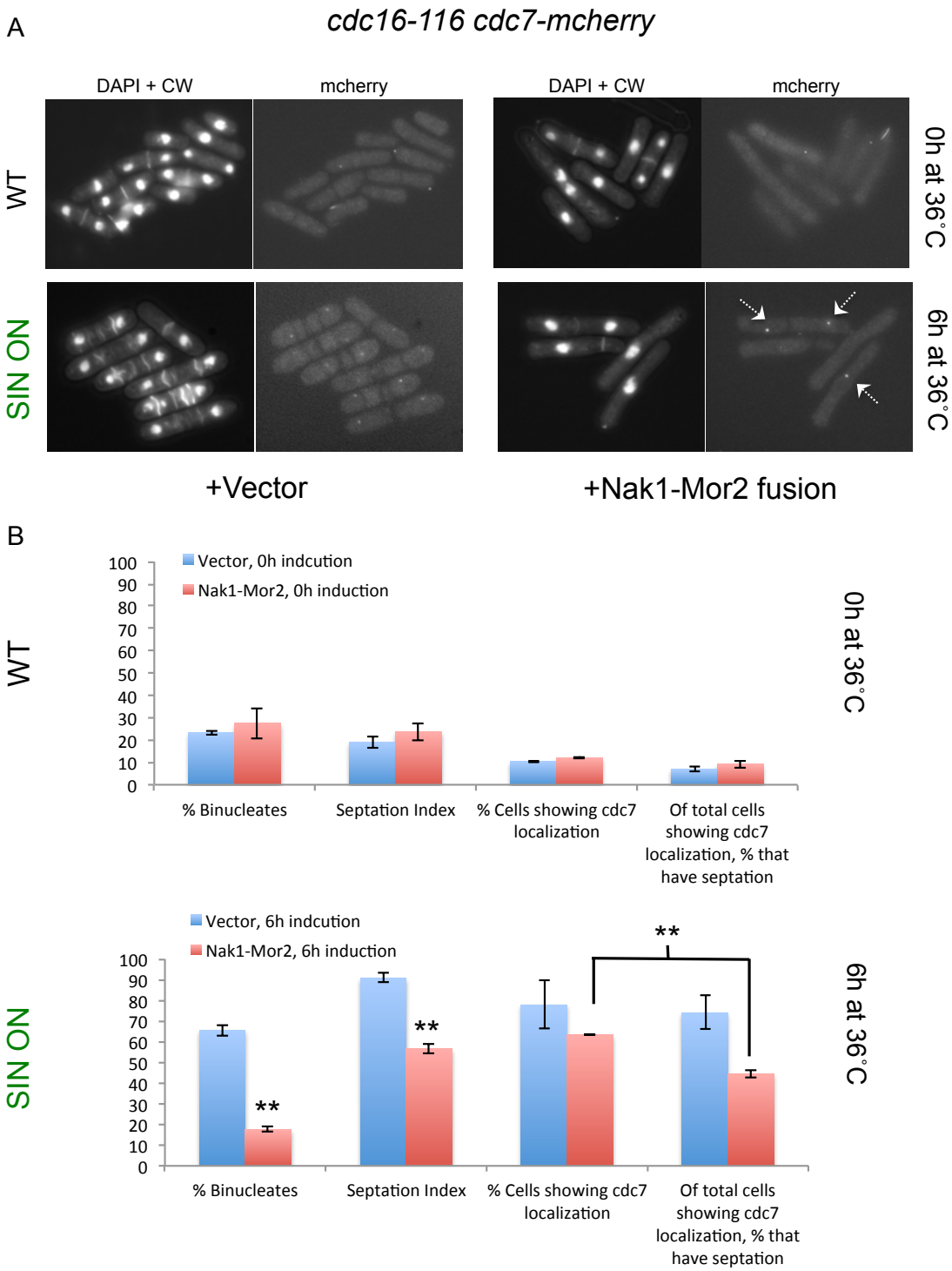
An active MOR may interfere with septum formation

From the time elapse movies, we observed that there was no extensive effect on septum formation upon the expression of the Nak1-Mor2 fusion in WT cells. However, we noticed a significant delay in initiation of ring constriction. Since septum ingression provides a majority of the force required for ring constriction (Proctor et al., 2012) we wanted to test whether MOR signaling could interfere with septum deposition using an alternative approach. We determined the effect of simultaneously active SIN and MOR signaling on SIN mediated septation using *cdc16-116*ts mutants that express the Nak1-Mor2 fusion. Ectopic activation of SIN signaling in *cdc16-116* ts cells normally results in septation during interphase, when MOR is active (mononucleates with one or more septa) as well as during mitosis (binucleates with multiple rounds of septation). Surprisingly, expression of the Nak1-Mor2 fusion protein caused a significant decrease in septation index (Figure 4.5A,B). Cdc7 tagged with GFP was simultaneously expressed to assess number of cells that had an active SIN as measured by Cdc7 localization at the SPB. Nearly all the control cells with an active SIN (displaying Cdc7 localization at the SPB) had one or more septa irrespective of the cell cycle stage. In contrast, in Nak1-Mor2 expressing cells there was a significant percentage of cells that had an active SIN but did not show any septation (Figure 4.5B). Therefore, these results suggest that MOR pathway may suppress downstream functions of the SIN such as septum formation.

Figure 4.5 – Nak1-Mor2 fusion suppresses ectopic septation induced by the SIN

(A) Representative images of *cdc16-116* cells expressing either the vector control or the Nak1-Mor2 fusion plasmid at 0h and 6h after temperature shift to 36˚C are shown. White arrows indicate cells that have an active SIN (as indicated by cdc7 localization at the SPBs) but do not show any ectopic septation. (B) Quantitation of indicated parameters measured from cells shown in A is graphically represented. Asterisks indicate that the difference in the values of these parameters when compared to the controls (or as indicated) is statistically significant as measured by unpaired t-tests (**p-value < 0.01).

Figure 4.5 – Nak1-Mor2 fusion suppresses ectopic septation induced by the SIN



Deletion of cell separation genes can rescue the cell lysis phenotype

Because the MOR is normally required to bring about cell separation through degradation of the primary septum following completion of ring constriction (Nelson et al., 2003; Maerz and Seiler, 2010), we hypothesized that the cell lysis phenotype induced by Nak1-Mor2 expression might be caused by MOR-dependent degradation of the primary septa before new cell wall has been built at the cleavage site. If this was the case, then removal of the enzymes that degrade the primary septa should rescue the cell lysis phenotype. Consistent with this idea, deletion of the Ace2 transcription factor, which is required for cell cycle dependent transcription of genes required for cell separation (Alonso-Nuñez et al., 2005), or deletion of two of its targets involved in cell separation (Agn1 or Mid2) (Dekker et al., 2004, 2006; Tasto et al., 2003) rescues the cell lysis phenotype caused by the Nak1-Mor2 fusion (Figure 4.6A,B). This result is confirmed by the ability of the deletions to rescue growth in cells expressing the fusion protein (Figure 4.6C). Consistent with Ace2 acting downstream or in parallel to the MOR, the cell separation defect in *orb6-25* MOR mutant is rescued by ectopic expression of Ace2 or its target Mid2 as indicated by a decrease in the percentage of binucleate cells with septa (Figure 4.6D). These results suggest that premature initiation of cell separation caused by ectopic MOR signaling pathway results in cell lysis. Therefore, SIN inhibition of the MOR is necessary to prevent cells from undergoing cell separation before the completion of cytokinesis.

Figure 4.6 – MOR functions upstream or in concert with Ace2 transcriptional network to regulate cell separation

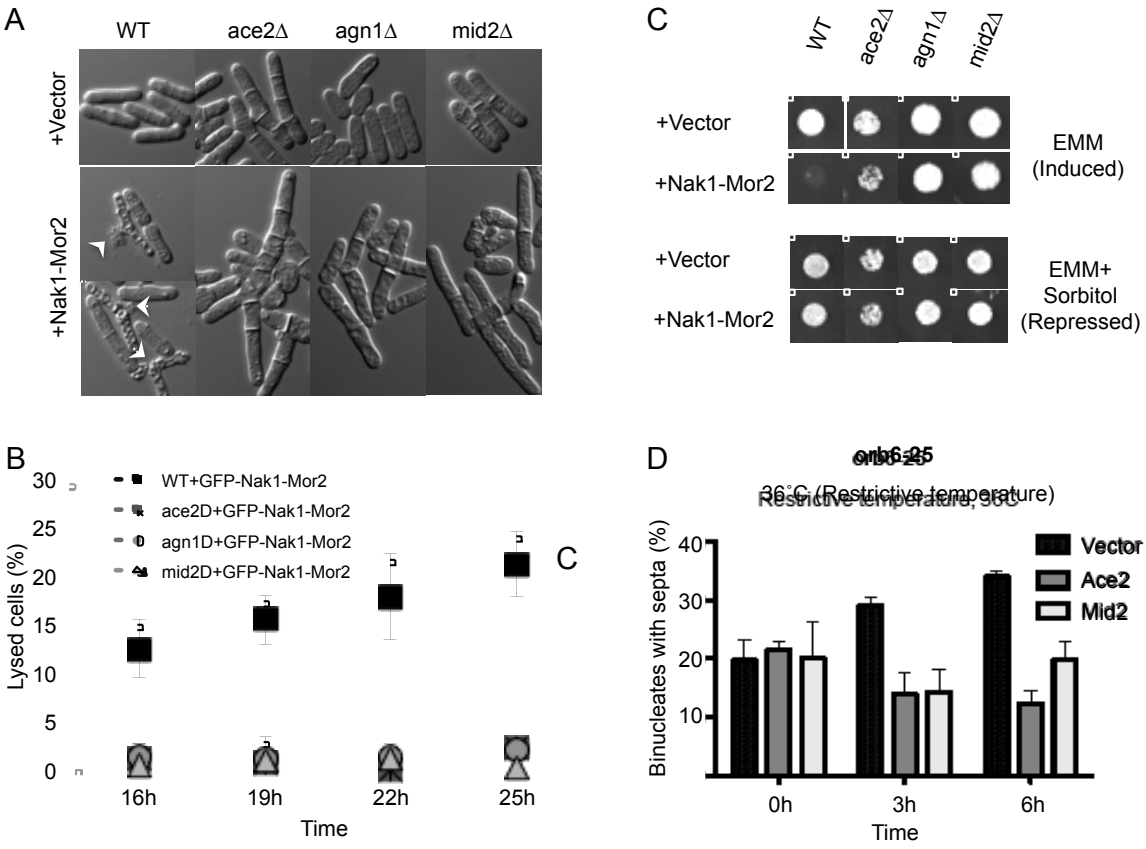
(A) Representative images of WT cells and cells with deletions in ace2 or its targets, agn1 or mid2 expressing either the vector (pREP41) or the Nak1-Mor2 fusion (pREP41-Nak1-Mor2) are shown. The indicated transgenes were induced for 19h at 25˚C in medium lacking thiamine.

(B) Quantitation of the lysis defect in cells, grown as indicated in A, is represented as percentage of lysed cells observed at different times of induction (16h, 19h, 22h, 25h).

(C) Spot tests done with WT, *ace2*Δ*, agn1*Δ and *mid2*Δ cells expressing either the vector (pREP41) or the Nak1-Mor2 fusion (pREP41-Nak1-Mor2) are shown. Tests were performed on medium lacking thiamine to induce expression of vector control or Nak1-Mor2 fusion and on medium containing thiamine as well the osmotic stabilizer sorbitol (as cells showed some lysis defects even under repressed conditions).

(D) Rescue of cell separation defect in *orb6-25* cells by expression of empty vector control, *ace2* and *mid2* (cloned into the pREP41 plasmid) is demonstrated in the graph through measurement of the percentage of binucleate cells with septa. Experiment was performed by shifting the cells to a restrictive temperature of 36˚C. Measurements were taken at the 0h, 3h and 6h time intervals.

Figure 4.6 – MOR function upstream or in concert with Ace2 transcriptional network to regulate cell separation



Discussion

This study provides an explanation for why the SIN inhibits the MOR pathway. Besides establishment and maintenance of interphase polarity, the MOR pathway has an additional role in initiating cell separation. In fission yeast, this role is evident from the phenotype of MOR pathway mutants which not only display a loss of polarity but also show inability to carry out cell separation resulting in G2 arrested, binucleate cells with a septum. G2 arrest occurs because the MOR causes a Wee1 dependent G2 delay (Hirata et al., 2002). Elaborate studies have been done in budding yeast to show that NDR family kinase and Orb6 homolog Cbk1, phosphorylates Ace2, a transcription factor that induces genes that promote cell separation by degrading the septum (Brace et al., 2011; Mazanka et al., 2008; Jansen et al., 2009, 2006). The Ace2 counterpart in fission yeast is also known to have a role in cell separation (Bähler, 2005) through expression of genes such as *mid2*, *agn1* and *eng1* (Alonso-Nuñez et al., 2005) that are required for primary septum breakdown. In this chapter, I have shown that inhibition of the MOR pathway during cytokinesis prevents the MOR from initiating premature septum degradation, which triggers cell lysis. Also, it is noteworthy that even though I have utilized the Nak1-Mor2 fusion to constitutively activate the MOR, our preliminary results shown in Chapter II indicate that co-expression of non-phosphorylatable Nak1-5A and Sog2-5A also cause a similar lysis phenotype. This observation suggests that SIN mediated phosphorylation of

Nak1 and Sog2 probably serve the purpose of inhibiting the interaction of the Nak1/Sog2 complex with the Mor2 scaffold.

Our results offer mechanistic insight into how SIN inhibition of the MOR pathway is required for orderly execution of cytokinetic events to prevent cell lysis during late mitosis in *S. pombe*. In a normal cell cycle, SIN signaling drives actomyosin ring constriction and septum formation, while inhibiting the MOR mediated cell separation and polarized growth. Completion of normal cytokinetic events triggers SIN inactivation (García-Cortés and McCollum, 2009), allowing the MOR to become active and cause cell separation and initiation of interphase polarity. Presumably the lysis phenotype in SIN mutants at a semi-permissive temperature is due to the incomplete or weakened septa in SIN mutants being sensitive to premature septum degradation caused due to a compromised inhibition of the MOR. Further, it is possible that, as seen in budding yeast, the MOR may have a direct role in regulation of the Ace2 transcriptional network in order to carry out cell separation. It has been shown that deletion of Ace2 and its targets involved in cell separation can rescue the lysis phenotype observed in SIN mutants at low restrictive temperatures (Jin et al., 2006). Therefore, the lysis phenotype observed in these cells could be a result of MOR mediated premature activation of the Ace2 and/or its targets such as, the cell wall degradation enzymes Agn1 and Eng1 or Mid2, which are required for proper targeting of the cell wall degradation enzymes at the cell middle (Dekker et al., 2004; García et

al., 2005; Martín-Cuadrado et al., 2003; Tasto et al., 2003). However, further investigations will have to be performed in order to explore whether MOR acts upstream of Ace2 or whether the MOR and the Ace2 network function independently to control the cell separation process. Taken together, these results expand our knowledge of the cross-regulation between NDR kinase pathways observed in fission yeast by elaborating on the significance of this interaction. It would be interesting to see if similar crosstalk exists between homologous mammalian pathways.

CHAPTER V

General Discussion

Over the last two decades, the fission yeast *S. pombe* has been studied extensively to advance our knowledge of genes involved in the processes of cell morphogenesis, cell size control and cytokinesis. Genes that function in the SIN and MOR pathways and regulate cytokinesis and polarized growth respectively were initially described in findings reported in the late 90's (Balasubramanian et al., 1998; Verde et al., 1995). Chapters II, III and IV present results that advance our understanding of how cells coordinate cytoskeletal rearrangements that accompany transitions between cell cycle stages. Particularly, we have focused on understanding regulatory mechanisms that allow the SIN signaling network to maintain the cytoskeleton in the cell middle and form the cell division apparatus while simultaneously inhibiting localization of the cytoskeletal components at the cell tips as well as to prevent cell separation until successful completion of cytokinesis.

The SIN inhibits the MOR by blocking interaction between Nak1 kinase and the Mor2 scaffold thereby preventing Orb6 activation

The first step in this process was expanding our limited understanding of SIN functions through identification of new SIN targets. We identified and validated several targets that provided us with clues to potential roles of SIN in processes such as interphase polarity, septum assembly, spindle checkpoint, actomyosin

ring assembly and nuclear positioning. We especially focused on two top hits in our screen, Nak1 and Sog2, both of which are essential proteins that function in the Morphogenesis Orb6 Network (MOR), which controls cell separation following cytokinesis as well as polarized growth during interphase. We determined that the two proteins are part of a complex and display very similar cellular localization throughout the cell cycle. Both Nak1 and Sog2 mutants display a spherical phenotype indicating loss of polarity and are required for maintaining Orb6 kinase activity (Chapter II and Kume et al., 2013). Further, we identified Sid2 phosphorylation sites on Nak1 as well as Sog2 in vivo. Site directed mutagenesis of identified and putative sites allowed us to create phosphorylation site mutants. Detailed experiments performed in *cdc3-124 cdc16-116* cells (genetic background with activated SIN) using the Nak1-5A non-phosphorylatable mutant revealed that Sid2 phosphorylation of Nak1 is an important post-translational modification required for inhibition of polarized growth by the SIN through a decrease in the activity of the downstream MOR kinase, Orb6. How Nak1 phosphorylation by Sid2 executes a reduction in Orb6 kinase activity remained unexplained.

Since Nak1 kinase is essential for activation of Orb6, it was possible that Nak1 mediated activation of Orb6 was being targeted. This query was addressed by creating a fusion of the two kinases to artificially enhance interaction between them and thereby promoting signaling through the MOR kinase cascade. The

ability of this fusion to bypass SIN inhibition, as assessed by the cell elongation phenotype, was quite comparable to the Nak-5A non-phosphorylatable mutant, which was consistent with our initial hypothesis. However, expression of either of these proteins did not display any observable defects in cycling WT cells, suggesting that either SIN inhibition of the MOR is not crucial under normal growth conditions, or the SIN has additional mechanisms by which it inhibits the MOR. Since, Nak1 interacting protein Sog2 was also identified as a SIN target we tested whether bypassing SIN regulation of both proteins simultaneously could address this issue. Although expressing the Sog-5A non-phosphorylatable protein alone was insufficient for circumventing SIN inhibition of the MOR, co-expressing Nak1-5A and Sog2-5A did display a stronger bypass of growth inhibition than expression of Nak1-5A alone. More interestingly, we observed that simultaneous expression displayed a lysis defect in the WT cells consistent with cytokinetic failure (Jin et al., 2006). Finally, we tested whether binding of Nak1 kinase to the scaffold Mor2 was a critical step in the activation of Orb6. This was achieved by fusing the Nak1 kinase to the Mor2 scaffold and creating a functional Nak1-Mor2 fusion protein. To our surprise, this modification resulted in a massive increase in Orb6 activity in cells with an active SIN (in comparison to the increase seen using Nak1-5A phosphorylation defective mutant). This observation was also reflected in a very strong cell elongation phenotype seen in these cells indicating a strong bypass of SIN inhibition of bipolar growth. We think this is a critical result that highlights the importance of the Nak1-Mor2 interaction in MOR

signaling. Intriguingly, mitotic kinase Cdk1 might also be targeting this interaction as the Nak1-Mor2 fusion can induce cell elongation in metaphase arrested cells (Figure 3.7) Therefore, interaction between Ste20 like/GCK family member Nak1 and the Mor2 scaffold could be a significant regulatory step in preventing signal transduction through the MOR pathway during mitosis.

Ste20-related/GC family kinases, such as the *S.pombe* Nak1 act as upstream regulators of Mitogen activated protein kinase cascades, which regulate gene expression to control a diverse array of cellular processes including cell division proliferation, cell morphology, survival and apoptosis (Dan et al., 2001; Qi and Elion, 2005). Role of scaffolds in these signaling networks (ERK1/2-, JNK- and p38) has been clearly demonstrated in studies using *S. cerevisiae* and also being studied extensively in mammals. Scaffolds represent a growing family of regulatory proteins that not only allow organization of various signaling components but may also be involved in allosteric activation of associated kinases, controlling subcellular localization and regulating specificity of signal transduction by creating a functional signaling module. Despite the fact that the purpose of the Mor2 scaffold is to facilitate interaction between the two kinases (also observed by the ability of the Nak1-Orb6 fusion to rescue growth defects in *mor2-786* ts mutant) and promote signal transduction through the kinase cascade, the phenotypic effect of simply fusing the two kinases (Nak1-Orb6 fusion) was significantly poorer that the Nak1-Mor2 fusion. This suggests that

Mor2 might not only bridge the interaction between Nak1 and Mor2 but might also affect Nak1 and/or Orb6 activity, or the ability of Orb6 to be activated by Nak1. Further, Orb6 but not Nak1 activity is compromised in *mor2* mutant, suggesting that Mor2 may not enhance Nak1 activity itself but simply promote Nak1 mediated Orb6 activation (Kanai et al., 2005). Mor2 may also have a role in determining subcellular localization of MOR pathway constituents. Notably, our attempt to show *in vivo* regulation of interaction between Nak1 and Mor2 by the SIN indicated that while Nak1 and Mor2 showed association *in vivo* by co-immunoprecipitation, upon ectopic activation of the SIN, Mor2 was consistently degraded (Figure 3.4). This observation raises the interesting possibility that targeting Mor2 for degradation could be an auxiliary mechanism of MOR inhibition by the SIN further highlighting it as an important regulatory target. Several studies have been published recently that target kinase-scaffold interactions as viable approaches to cancer therapy emphasizing that information on how scaffolds regulate signaling networks could be of potential importance for therapeutic applications (Stuart and Sellers, 2013; Jameson et al., 2013; Nie et al., 2013; Wu et al., 2013). It would be of interest to determine precise interaction domains needed for Nak1 association with the scaffold in the future.

SIN inhibition of the MOR during cytokinesis is crucial

Our study determined that Nak1 and Sog2 proteins in the MOR pathway are key targets of the SIN during cytokinesis. We also determined that SIN phosphorylates Nak1 (and possibly Sog2) to inhibit interaction of the Nak1 kinase with the protein scaffold Mor2. This inhibition shuts down signaling through the MOR pathway thereby preventing tip elongation during cytokinesis. However, the physiologically relevance of this regulation was not clear. Using the Nak1-Orb6 fusion, which is able to bypass SIN inhibition to some extent, we were able to show that MOR inhibition is of particular importance when the SIN is not fully functional (Figure 4.1A). Expressing the Nak1-Orb6 fusion in cells in which the cytokinetic apparatus was compromised by using the drug Latrunculin A (that normally causes a SIN dependent arrest until cytokinesis is complete), also aggravated cytokinetic failure (Ray et al., 2010). Together these results led us to conclude that MOR inhibition, while important under conditions of cytokinetic duress, was not an essential function. Contrary to this initial deduction, ectopic activation of the MOR by utilizing the Nak1-Mor2 fusion was more successful in disrupting SIN inhibition of the MOR and resulted in lysis as cells underwent division. This result was intriguing since the phenotype resembled that of a SIN mutant at semi-permissive temperature indicating that partial SIN activity might be the reason for this phenotype (Jin et al., 2006). However, ectopic activation of the MOR by expressing the fusion proteins did not appear to affect strength of SIN signaling (Figure 3.8). Since these Nak1-Mor2 expressing cells had normal

levels of SIN signaling, it was possible that decrease in ability of normal SIN activity to inhibit a hyperactivated MOR during cytokinesis caused the observed undesirable effects on cytokinesis. Furthermore, partial SIN mutants have a similar lysis phenotype suggesting that the Nak1-Mor2 lysis phenotype is not just due to a hyperactive Orb6 (as measured in our kinase assay, Figure 2.4C) but may also be due to the fact that the equilibrium between SIN and MOR activity is disturbed and favors MOR function. Overall, our results indicate that the delicate balance between SIN and MOR activities must be maintained to prevent these pathways from interfering with each other's functions.

How increase in MOR activity during cytokinesis interferes with the downstream functions of the SIN is unclear. Our analysis of ring and septum dynamics clearly indicated that increased MOR activity causes premature separation in cells resulting in lysis. Lysis occurs because initiation of primary septum degradation before the secondary septum is complete creates weaknesses or gaps in the cell wall. Hence, we argued that MOR activity was suppressed during mitosis to prevent premature targeting of enzymes to the cell middle that could result in cell lysis. In *S. pombe*, Ace2 (a transcription factor) and its targets (*agn1, eng1, mid2*) help promote cell wall degradation to initiate the cell separation process (Figure 5.1). Studies from budding yeast have clearly demonstrated that that the Ace2 transcriptional network is under direct control of the Orb6 homolog, Cbk1 (Jansen et al., 2006; Bidlingmaier et al., 2001; Mazanka et al., 2008). While no

direct link between Orb6 and Ace2 has been established in *S. pombe*, our work shows that the two pathways could be working in tandem to regulate cell separation. Additionally, a previous study from our lab determined, that deletion of Ace2 or its targets could rescue that cell lysis phenotype observed in SIN mutants at semi-permissive temperatures. To summarize, while our results in conjunction with previous studies provide useful clues to the possible molecular outcomes of MOR enhancement during late mitosis, further work needs to be done to delineate downstream mechanisms by which SIN inhibition of the MOR pathway co-ordinates cell separation and cytokinesis.

Since SIN and MOR pathways both regulate the actin cytoskeleton to carry out their respective functions of medial division and tip growth, it is presumably very important that only one of the two pathways be active at a time. SIN mediated inhibition of MOR pathway kinase Orb6 is consistent with this notion. While SIN inhibition of the MOR is crucial to regulate timing of cell separation, we also wanted to uncover possible effects of unregulated MOR signaling on SIN-dependent maintenance and constriction of the actomyosin ring as well as on formation of the consequent primary septum in the cell middle. From time-lapse experiments performed to monitor effect of ectopic MOR activation on the aforementioned SIN functions, we were able to determine a slight delay in initiation of ring constriction. This observation was attributed to possible alteration in the dynamics of septum ingression, required for providing the mechanical force

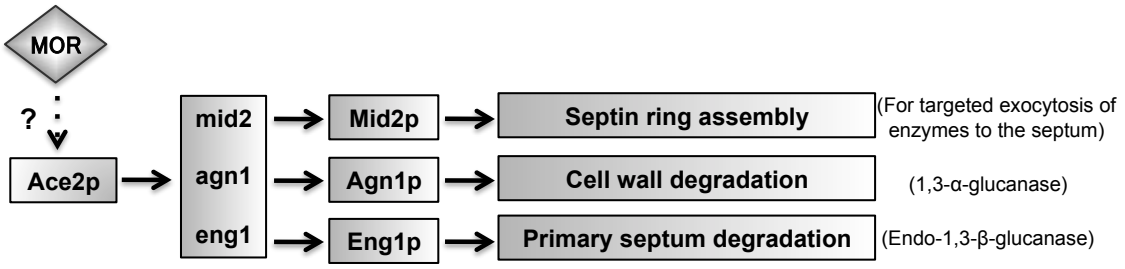
needed to initiate ring constriction (Proctor et al., 2012), due to simultaneous activation of SIN and MOR functions of septum formation and degradation, respectively. In addition, since both pathways compete for common cytoskeletal components such as actin, we also determined that ratio of actin in the middle was decreased compared to tips of the cells that have unregulated MOR activity (Figure 4.4). Our experiments to test whether MOR signaling could interfere with septation (Figure 4.5) showed that MOR pathway may suppress downstream functions of the SIN such as septum formation. This may be occurring due to competition for cytoskeletal components needed to execute ring formation and constriction. For instance, Cdc42 and its Guanine Exchange Factor, Gef1, are common regulatory proteins involved in cytoskeletal rearrangements during cell cycle transitions. Notably, Gef1 localizes mainly to the cell middle during actomyosin ring constriction and plays a role in cytokinesis. Gef1 along with Scd1 form a ring that depends on the actomyosin ring (Hirota et al., 2003). Shrinking of the Gef1 ring coincides with Cdc42 recruitment to the division site. Since Cdc42 has a suggested role in cytokinesis (Hirota et al., 2003; Rincon et al., 2007), it is possible that Gef1 localization at the cell middle serves to activate Cdc42 for it presumed role in cytokinesis. On the other hand, studies also show that MOR maintains active Cdc42 at the cell tips by inhibiting medial Gef1 localization in interphase (Das et al., 2009). Therefore, MOR inhibition during cytokinesis may be necessary to direct Cdc42 to the cell middle for carrying out cytokinesis. However, this is merely speculation and further investigation will be needed to

identify common cytoskeletal proteins controlled by the MOR and SIN and to determine whether simultaneous activation of the two contrasting polarity programs affects the functions of these proteins. It is possible that our inability to observe the full impact of MOR misregulation on cytokinetic steps such as ring and septum formation under WT conditions could be due technical limitations of our present approach.

Figure 5.1 – Ace2 signaling cascade controls transcription of genes involved in cell separation

This figure illustrates the regulation of genes by the Ace2 transcriptional network and summarizes their function in cell separation.

Figure 5.1 – Ace2 signaling cascade controls transcription of genes involved in cell separation



Ace2 signaling cascade

SIN phosphorylation of MOR proteins has additional role in regulating SIN onset

Mitotic progression is accompanied by the most significant change in the fission yeast cytoskeleton. It requires shutdown of tip extension and reassembly of cell growth components at the cell middle needed for impending cytoplasmic division. MOR proteins are required for polarization of the interphase cytoskeleton. Consistent with this, MOR proteins, including the Nak1 kinase, assemble at the cell tips during interphase. Monitoring the localization of Nak1 and its binding partner Sog2 revealed that both proteins appear at the SPB upon mitotic entry and are displaced from the SPB at the start of anaphase B. We performed experiments to test whether SPB localization of MOR protein had a role in mitosis and determined that Nak1 had a role in inhibiting premature septation in cells thereby regulating the timing of SIN activation (Figure 3.6). How Nak1 is recruited to the SPB in mitosis is however unknown. Since Nak1 contains putative and identified Cdk1 phosphorylation sites (unpublished work of the Gould Lab, Cipak et al., 2013), it is possible that phosphorylation by Cdk1 might be involved in determining Nak1 localization in early mitosis. As mentioned earlier, Nak1 phosphorylation by Cdk1 might be an important regulatory step required for normal execution of early mitotic functions. Further, how Nak1/Sog2 localization at the SPB inhibits SIN onset is unclear. One hypothesis could be that since both Sid1 and Nak1 are Ste20 related/GCK family members, they are recognized by a common docking motif at the SPB and removal of Nak1 is

necessary for recruitment of Sid1 and full activation of the SIN. However, this suggestion needs experimental verification. Taken together, Sid2 mediated phosphorylation of Nak1 serves two purposes in regulating SIN function (Figure 5.2). Firstly, this modification is required to shut down MOR signaling in late mitosis in order to prevent a switch to polarized growth as well as coordinate the process of cell separation. Secondly, Nak1 phosphorylation by the Sid2 kinase also promotes displacement of Nak1 from the SPB, which regulates onset of the SIN in late mitosis.

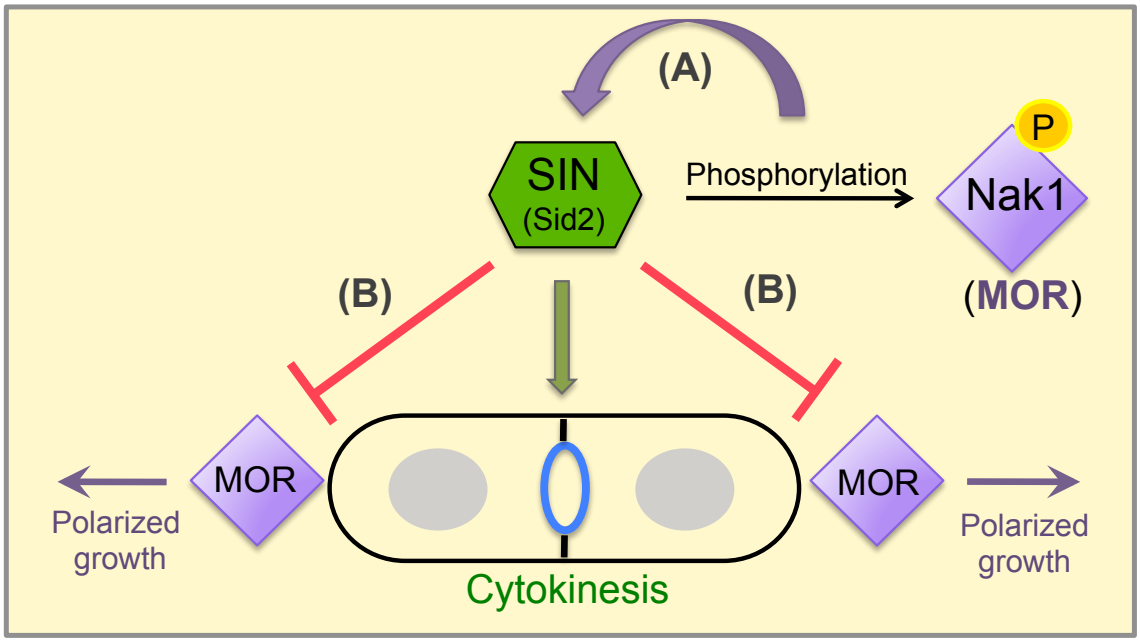
Figure 5.2 - Sid2 mediated phosphorylation of Nak1 serves two functions during late mitosis

Graphical representation of the functions of Sid2 phosphorylation of Nak1

(A) Sid2 kinase phosphorylates Nak1 to promote its own activity through displacement of Nak1 from the SPB.

(B) Sid2 kinase phosphorylates Nak1 to inhibit polarized growth by interfering with the interaction between Nak1 and the Mor2 scaffold.

Figure 5.2 - Sid2 mediated phosphorylation of Nak1 serves two functions during late mitosis



Crosstalk observed between conserved NDR kinase pathways in other eukaryotic systems

Our study in *S. pombe* confirms that opposing effects of the SIN and MOR networks on regulation of cell growth and division necessitates the presence of an antagonistic interaction between the two pathways. From work in other model systems it is now evident that homologous NDR kinase pathways have contrasting functions in various cellular processes. For instance, recent observations in *Drosophila melanogaster* indicates that the NDR kinases, Trc (Orb6 homolog) and Wts (Sid2 homolog) have opposing roles in regulation of cell shape and timing of hair morphogenesis in wing cells (Fang and Adler, 2010). Furthermore, various studies in mammalian systems have shown that their SIN and MOR counterparts, namely, the MST1/2-LATS1/2 (Hippo) and MST3-NDR1/2 signaling pathways have contradictory effects on cell proliferation (Cornils et al., 2011b; Visser and Yang; Hergovich et al., 2008). These observations suggest the possibility that an antagonistic crosstalk similar to the one observed in fission yeast may exist between homologous NDR kinase pathways in higher organisms.

Alternatively, there are several examples where the two NDR pathways work in concert to promote common cellular functions. In the budding yeast *S. cerevisiae,* the MEN (Mitosis Exit Network) and RAM (Regulation of Ace2p and morphogenesis) signaling networks correspond to the *S. pombe* pathways SIN

and MOR respectively (McCollum and Gould, 2001; Maerz and Seiler, 2010). It has been reported that MEN and RAM function together to regulate the function of the Ace2p transcription factor in daughter cell separation (Weiss et al., 2002). In *Drosophila*, the two NDR kinases Trc and Wts share the same upstream regulator Hippo (Hpo), which may help coordinate their roles in the establishment and maintenance of dendritic tiling in neuronal cells (Emoto et al., 2006; Emoto, 2011). While the MST1/2-LATS1/2 pathway in mammals plays a role in tumor suppression, several recent reports now implicate MST1/2 in the additional regulation of NDR1/2 kinases to control various cellular processes like centrosome duplication, mitotic chromosome alignment, and apoptotic signaling (Vichalkovski et al., 2008; Hergovich et al., 2009; Chiba et al., 2009). In contrast, Mst3 kinase appears to be important for the growth promoting functions of Ndr1/2 (Cornils et al., 2011a). Therefore, in animal systems, the regulation of NDR kinases functioning in separate pathways by a common upstream kinase of the STE20-like kinase family appears to be a conserved characteristic (Figure 5.3). Intriguingly, a similar regulation has been proposed by a study in *S. pombe*, which indicates that the SIN kinase Sid1 not only regulates cytokinesis through activation of Sid2 but also functions in enhancement of MOR activity during the subsequent interphase (Kanai et al., 2005). It is possible that SIN imparts both activating and inhibitory modifications on its MOR targets. While the inhibitory regulation dominates during mitosis, its removal in the subsequent interphase could result in MOR activation. Taken together with our findings, it reveals a dual

role for SIN in regulation of the MOR pathway that enables it to modulate MOR activity in accordance with the cell cycle stage (Figure 5.4).

Figure 5.3 – Conservation of NDR kinase pathways across species

Members of the Ste-20 like kinase family (Sid1, Nak1, Hpo, Mst1/2, Mst3) and the Nuclear Dbf2-related kinase family (Sid2, Orb6, Wts, Trc, Lats1/2, Ndr1/2) constitute the core of the NDR kinase signaling pathways in *S. pombe*, *D. melanogaster* (Fly), and mammals. This figure illustrates the cellular functions performed by these pathways in their respective organisms and crosstalk (red arrows) that occurs between pathway components.

Figure 5.3 – Conservation of NDR kinase pathways across species

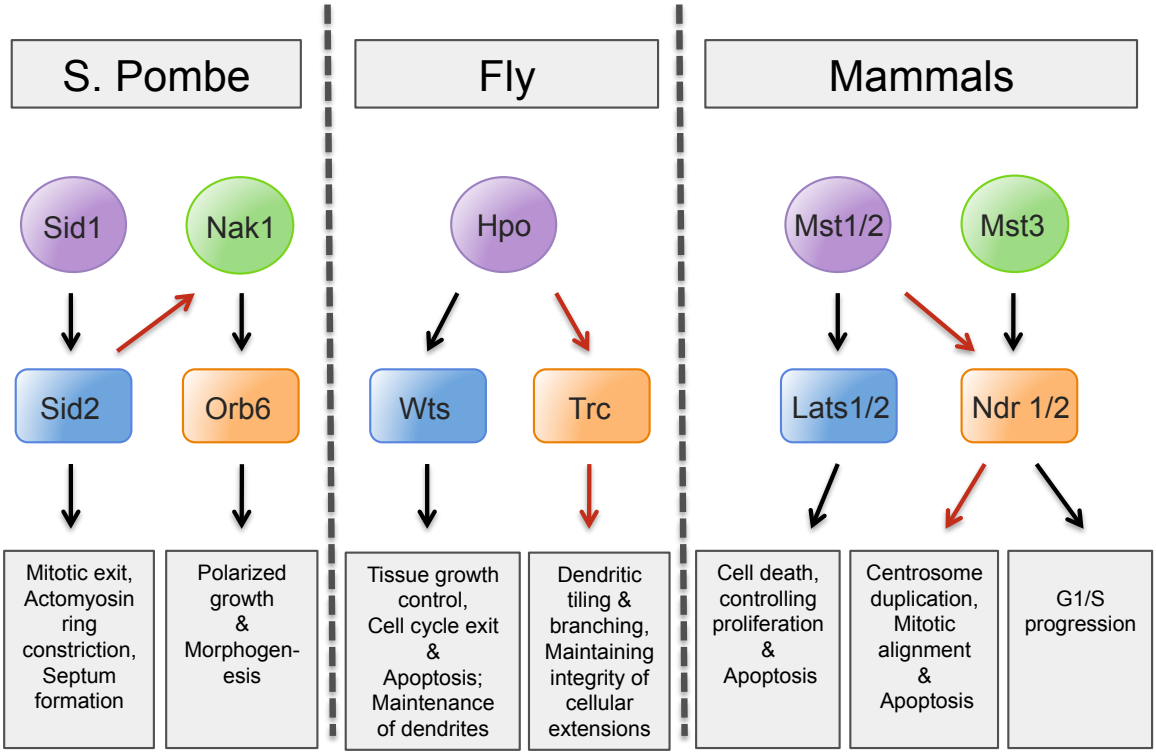
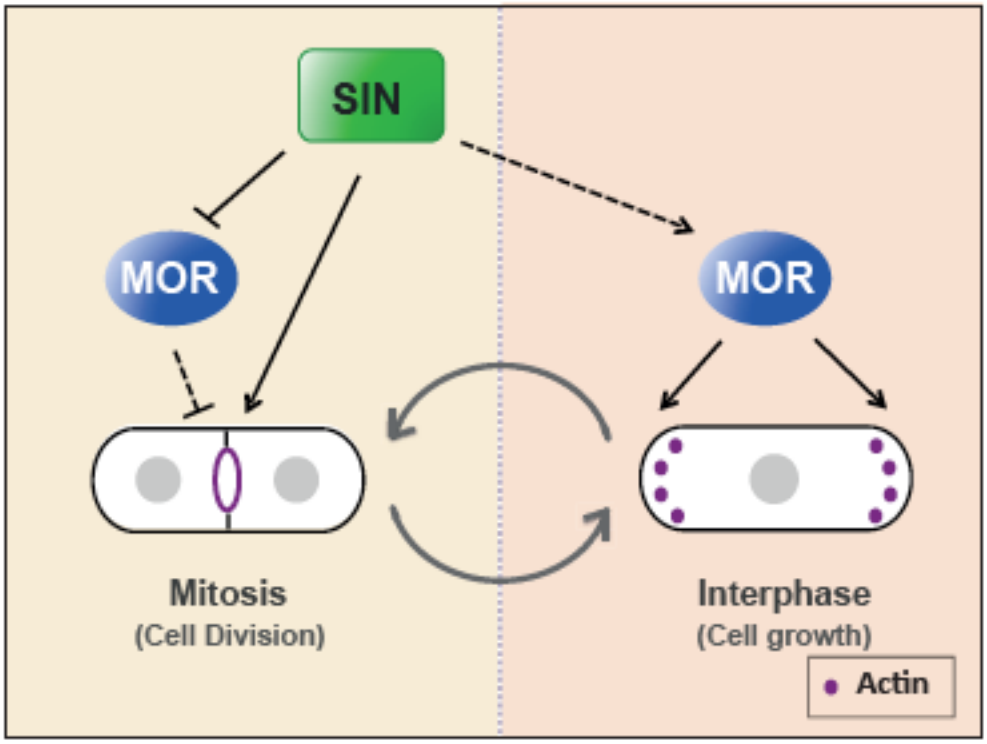


Figure 5.4 – A dual role for the SIN in MOR regulation during the cell cycle

The SIN mediated regulation of MOR during mitosis appears to have two distinct cell cycle dependent effects on MOR function. While the SIN inhibits MOR activity during mitosis to keep MOR from interfering with cytokinesis, it may also play a role in enhancing MOR activity during the following interphase in order promote polarized growth.

Figure 5.4 – A dual role for the SIN in MOR regulation during the cell cycle



Conclusions

In fission yeast, NDR kinases constitute central effectors of the MOR and SIN pathways that regulate cell polarity and cytokinesis, respectively. Our studies have uncovered mechanisms by which these pathways communicate in order to achieve sequential reorganization of the actin cytoskeleton during the cell cycle. In particular, we have determined that downstream SIN kinase Sid2 phosphorylates the upstream MOR kinase Nak1 and suppresses its interaction with the Mor2 scaffold (Figure 5.5). Since this interaction is required to promote Nak1 mediated activation of the effector kinase Orb6, our result is the first to emphasize that association of the scaffold with the upstream kinase can form a key regulatory node in NDR kinase signaling pathways. We were also able to determine that this regulation is important to coordinate cytokinesis with MOR mediated cell separation. Furthermore, inability to execute this cross-regulation may also affect integrity of the cytoskeletal structures during mitosis.

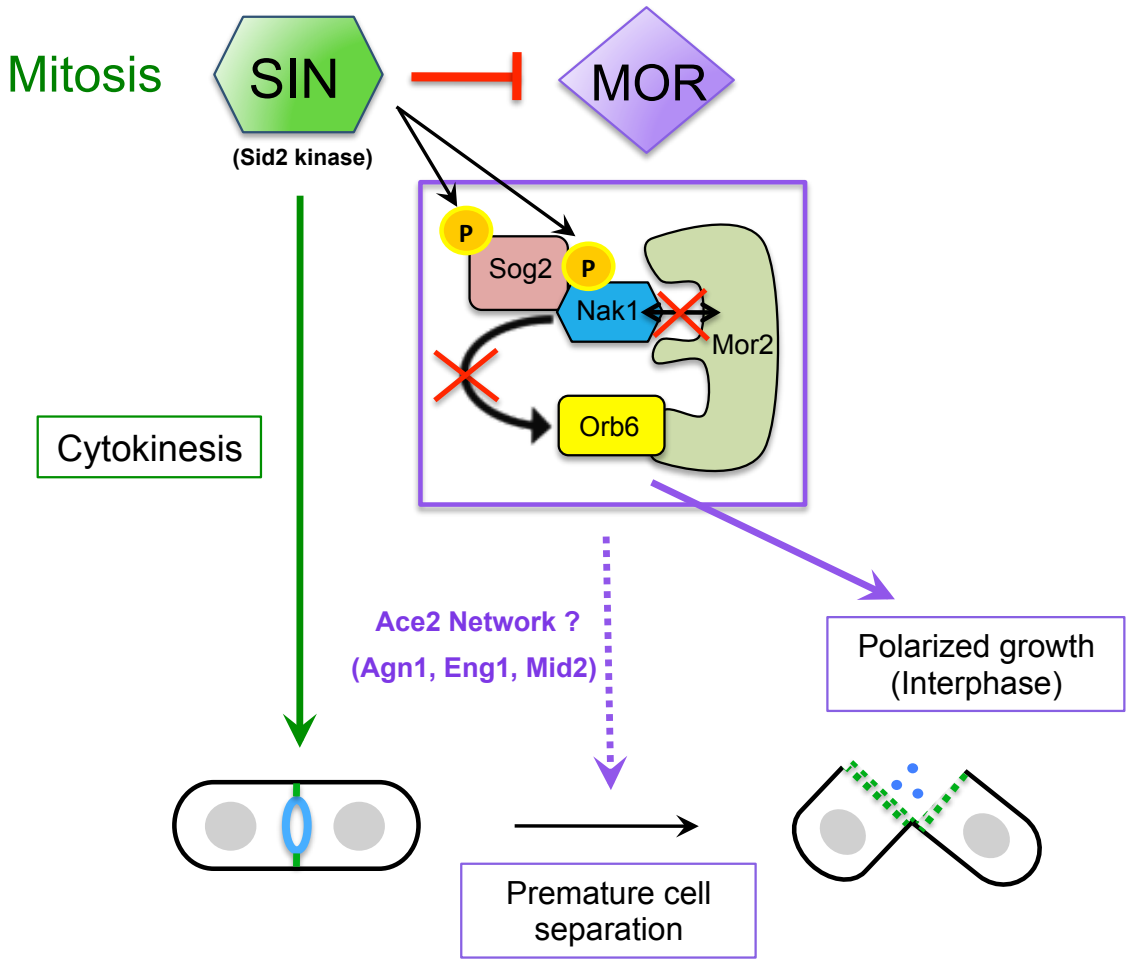
Both the NDR kinase signaling pathways in *S. pombe* are conserved in higher eukaryotes, where several studies have provided a flurry of information emphasizing their biological relevance. For instance, in *Drosophila* and mammalian systems, NDR kinase homologs Wts/LATS1/2 and Trc/NDR1/2 act as tumor suppressors and proto-oncogenes in addition to having conserved cell cycle functions in mitotic exit and morphogenesis. Regulation of targets like p21 and c-Myc by NDR1/2 makes them highly appealing candidates for cancer

therapy (Cornils et al., 2011b). However, the presence of multiple isoforms of core components of the pathway like the Ste-20 like kinases, scaffold proteins, and MOB activators adds to the diversity of their biological functions (See Table 1.1), making it difficult to dissect these complex signaling networks in mammals. Therefore, examination of these pathways in relatively simple model systems like *S. pombe* can provide highly useful cues enabling us to pursue a more clear understanding of their functions in humans.

Figure 5.5 – Model proposed for mechanism of SIN inhibition of the MOR during late mitosis

SIN inhibition of MOR during cytokinesis is executed by phosphorylation of Nak1 kinase and its associated protein Sog2 by the Sid2 kinase. This modification inhibits Nak1 association with the Mor2 scaffold thereby shutting down signaling through the MOR kinase cascade. Inhibition of the MOR during late mitosis prevents untimely activation of the cell separation process that is mediated through (or in parallel with) the Ace2 regulatory network. Therefore, in the absence of this SIN-MOR crosstalk, cell separation is triggered prematurely causing cell lysis. This lysis could be a result of recruitment of hydrolytic enzymes to the incomplete septum.

Figure 5.5 – Model proposed for mechanism of SIN inhibition of the MOR during late mitosis



APPENDIX A

Examination of MOR pathway protein interactions at the Spindle Pole Body (SPB)

Introduction

The Septation Initiation Network (SIN) is a signaling pathway that initiates cytokinesis at the end of mitosis and consists of a kinase cascade regulated by an upstream GTPase, Spg1. Proteins involved in the SIN pathway assemble at the Spindle Pole Body (SPB), the centrosome equivalent in *Schizosaccharomyces pombe*. Anchoring of SIN proteins to the SPB requires scaffold proteins namely, Sid4 and Cdc11 (Krapp et al., 2001). It has been shown that Cdc11 acts as bridge that localizes most SIN components to the SPB through its association with the Sid4 scaffold (Morrell et al., 2004). Following chromosomal segregation, Cdk1 activity declines and cells enter anaphase. This transition is accompanied by SIN activation, which occurs through disengagement of the GAP protein complex (Cdc16-Byr4) from the GTPase Spg1 at the SPBs (Krapp et al., 2008; Furge et al., 1998). An active Spg1 then recruits the Cdc7 kinase to both SPBs at first. Cdc7 in turn recruits Sid1 kinase (Ste20 related/GC kinase family), which is required for activation of Sid2 kinase (NDR kinase) at the SPB. Sid2 kinase activation in late anaphase results in complete activation of the SIN along with loss of SIN activity at one of the SPBs as indicated by asymmetric localization of Cdc7 in anaphase B. Of all the SIN proteins, only Sid2 kinase localizes at the septum indicating that it is the only component involved in relaying SIN signaling from the SPB to the cell middle essential for carrying out downstream functions of the SIN (Sparks et al., 1999).

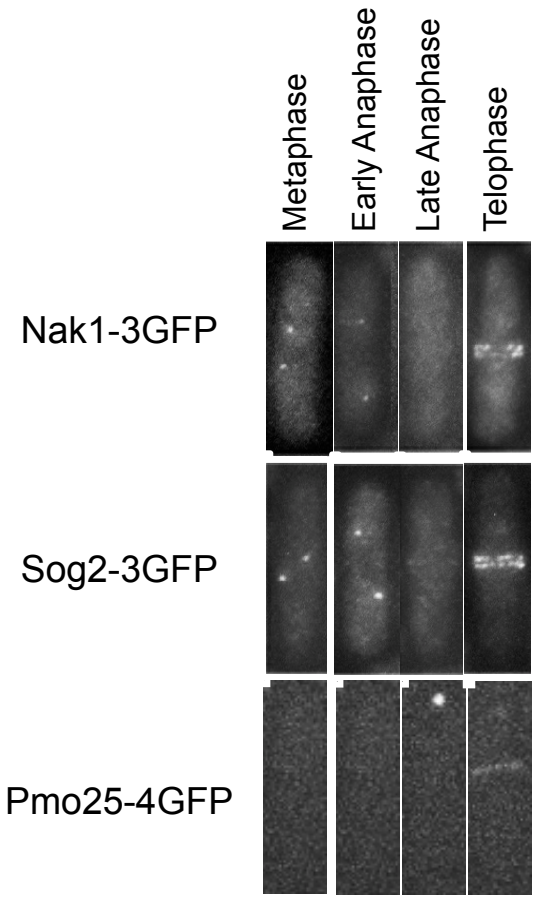
Knowing that the SIN targets proteins in the MOR (Morphogenesis Orb6 Network) signaling network that is involved in cell separation following cytokinesis as well as subsequent polarized growth during interphase, we were interested in examining SPB localizations of MOR pathway components. Like the SIN, the MOR is also an NDR kinase signaling network with Orb6 (an NDR kinase bound to Mob1 protein) acting downstream in its signaling cascade. Other proteins in the MOR pathway include the upstream kinase, Nak1 (Ste20 related/GC kinase family) and its associated proteins, Pmo25 and the more recently identified Sog2. Lastly, Mor2 is a large scaffold that binds to and promotes interaction between the two kinases, Nak1 and Orb6. Of all the MOR proteins, Nak1 and its binding partners, Pmo25 and Sog2 are the only ones that localize to the SPB. Co-localization of these MOR proteins at the SPB along with the SIN components is consistent with our identification of upstream MOR proteins, Nak1 and Sog2 as targets for Sid2 phosphorylation (see Chapter II). Additionally, the localization pattern of these proteins was quite intriguing. Although Nak1 and Sog2 localized to the SPB during metaphase and anaphase A, as cells entered Anaphase B, these proteins were displaced from the SPB along with simultaneous appearance of Pmo25 that persisted at the SPB until the end of cytokinesis (Figure A.1). Therefore, Nak1/Sog2 displacement from the SPB and Pmo25 recruitment to the SPB coincided with SIN activation. Finally, in telophase, all three proteins appear at the division septum. For sometime now,

our lab has been interested in understanding this curious localization pattern demonstrated by MOR proteins.

Figure A.1 – Localization of MOR pathway proteins, Nak1, Sog2 and Pmo25 at the SPBs during mitosis

Normal localization patterns of Nak1-3GFP, Sog2-3GFP and Pmo25-4GFP at the SPB are shown during mitosis. SPB is seen as one or two dots (as illuminated by GFP signal) within the cell.

Figure A.1 – Localization of MOR pathway proteins, Nak1, Sog2 and Pmo25 at the SPBs during mitosis



Results and Discussion

To uncover whether regulated localization of the MOR proteins to the SPB was important, we constitutively targeted them to the SPBs by creating fusions with the C-terminal portion of the protein Ppc89. Ppc89 is required for SIN activity and associates with Sid4 to anchor SIN proteins to the SPB (Rosenberg et al., 2006). Pmo25 SPB targeting experiments were preformed earlier (Samriddha Ray, unpublished results) which surprisingly resulted in ectopic septation indicating activation of the SIN pathway. Upon further analysis, we found that this phenotype was dependent on downstream SIN proteins, Sid1 and Sid2 but not on Spg1 or Cdc7. Consistent with this observation, Sid1 (as well as Cdc7) was recruited to the SPB in cells with ectopic septa. While the purpose of Pmo25 localization to the SPB is still unclear, this approach was an interesting technique to analyze interactions between SIN and MOR components. Since Nak1 localizes to the SPB in early mitosis, we created a Nak1-Ppc89 fusion protein (Figure A.2.1) to try and test its role at the SPB in early mitosis. It is of note that we have already demonstrated the role of Nak1 localization at the SPB in preventing premature initiation of septation (see Chapter III). Therefore, it was particularly surprising that targeting Nak1 to the SPB, like Pmo25, resulted in ectopic septation (Figure A.2.3). According to a previous study, Pmo25 might form a complex with both Nak1 and Sid1 kinase (Kume et al., 2007). If this were the case, our observations could be due to indirect targeting of Sid1 kinase to the SPB along with Nak-Ppc89(C-t) or Pmo25-Ppc89(C-t). We monitored localization

of GFP tagged Sid1 as well as Cdc7 in cells expressing the Nak1-Ppc89 (C-t) fusion. Both proteins were found to localize to the SPB (Figure A.3.1). Expression of the Nak1-Ppc89 (C-t) fusion in *sid1* and *sid2* ts mutants, got rid of ectopic septation (*results not shown*) indicating that this phenotype was Sid1 dependent. Additionally, targeting of a kinase dead allele of Nak1 fused to Ppc89(C-t) (Nak1-KD-Ppc89) did not cause the ectopic septation phenotype (*results not shown*). This suggested that kinase activity of Nak1 might be required to maintain its association with, or activate the Sid1 kinase.

Further, we also determined whether Nak1 and Pmo25 required each other to ectopically activate the SIN when either protein was targeted to the SPB.

Targeting of either Nak1 or Pmo25 to the SPB could induce septation without requiring the other (Figure A.4). This would indicate that either protein was capable of independently recruiting Sid1 to the SPB. Finally, we also determined which MOR proteins were recruited upon Nak1 targeting to the SPB. Both Pmo25 and Sog2, known Nak1 interacting proteins localized to the SPB (Figure A.5). However, Mor2, which is not observed at the SPBs normally, did not localize to the SPB in cells expressing the Nak1-Ppc89(C-t). It is possible that ectopic activation of the SIN triggers a MOR inhibition program, which would in turn inhibit Nak1 and Mor2 interaction. However, this would need to be tested further to confirm.

These experiments demonstrate various associations between SIN and MOR proteins. Targeting of proteins to the SPB can be used as a technique to confirm putative interactions between proteins and also assess the composition and organization of protein complexes. In addition, mutation analysis can be performed to determine potential interaction domains between associated proteins.

Figure A.2 – SPB targeting of Nak1 results in ectopic septation

1) Schematic of the Nak1-Ppc89(C-t) and GFP-Nak1-Ppc89(C-t) fusions.

2) Localization of the GFP-Ppc89 (c-t) and GFP-Nak1-Ppc89(C-t) fusions at the SPB (indicated by white arrows). Expression of the transgenes was induced for 6h in medium lacking thiamine followed by fixation, staining and imaging.

3) Representative images of cells expressing the indicated transgenes after 25h of induction in medium without thiamine are shown. Cells with ectopic septation are indicated with white arrows.

Figure A.2 – SPB targeting of Nak1 results in ectopic septation

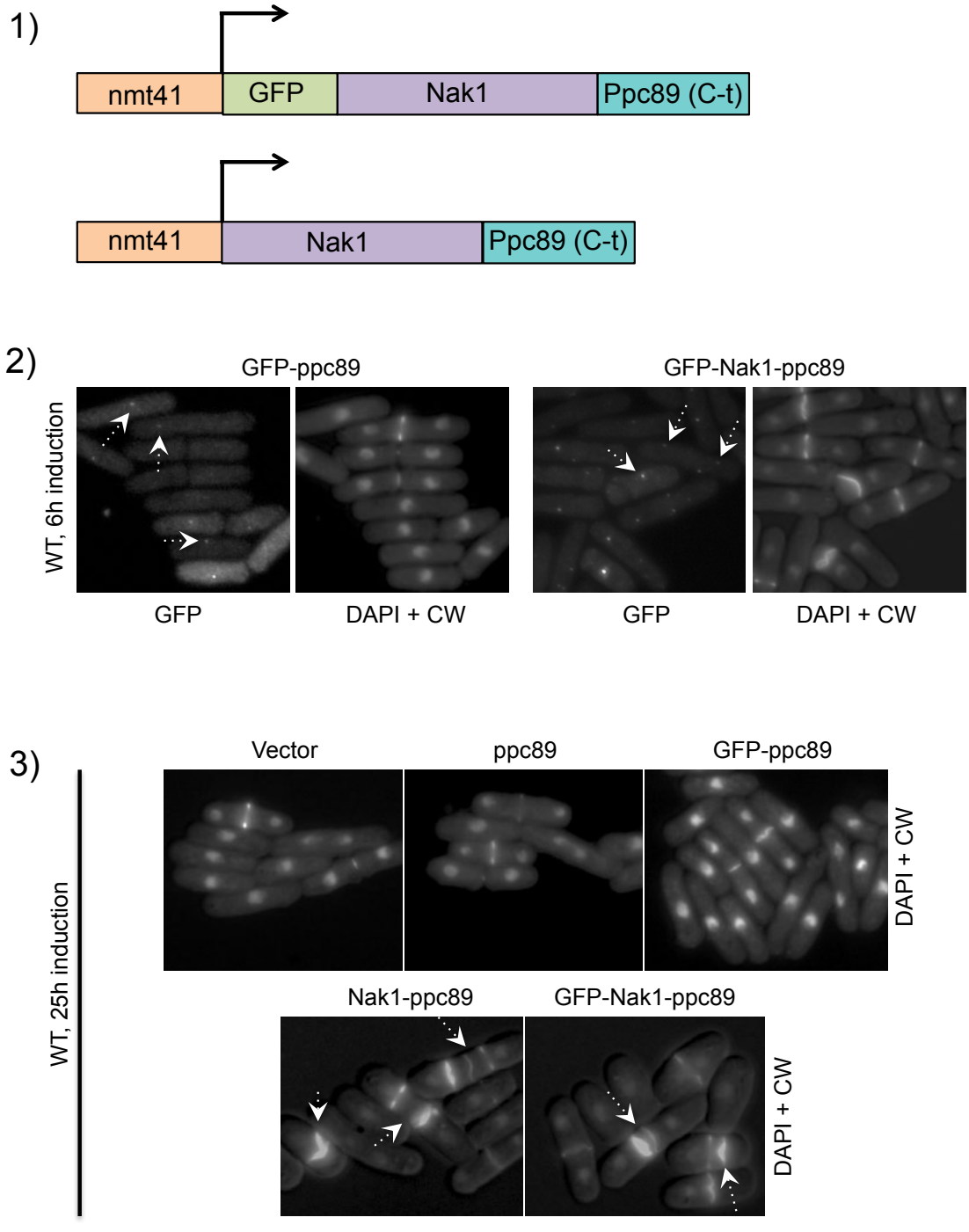


Figure A.3 – SPB targeting of Nak1 recruits Cdc7 and Sid1

Representative images of cells expressing the specified transgenes after 19h of induction in medium lacking thiamine are shown. White arrows indicate cells with ectopic septa showing GFP tagged proteins (Cdc7 or Sid1) at the SPB.

Figure A.3 – SPB targeting of Nak1 recruits Cdc7 and Sid1

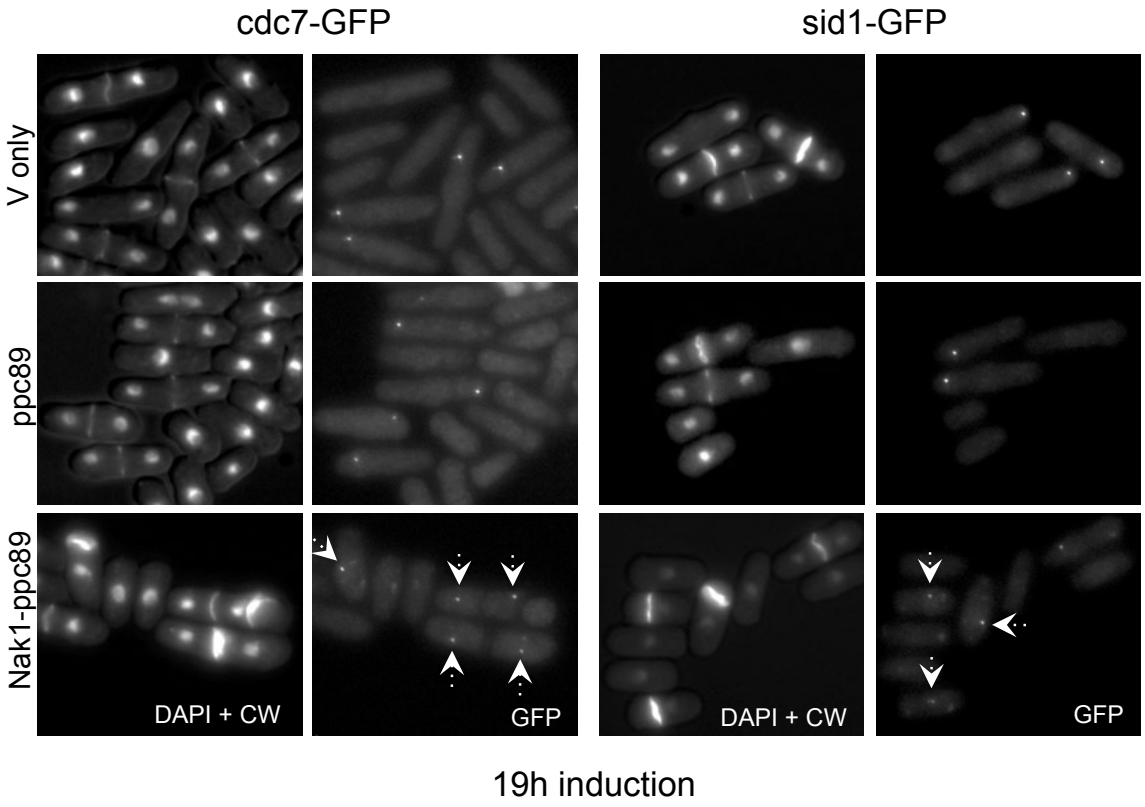


Figure A.4 – Nak1 or Pmo25 targeting to the SPB causes ectopic septation independent of the other

(1) Representative images of *pmo25-35* ts cells expressing the indicated transgenes after 25 of induction in medium without thiamine. Cells were maintained at 25˚C or shifted to 36˚C for 6h following which they were fixed, stained and imaged.

(2) Percentage of normal/ectopic septation (see diagram for phenotypes scored) in *nak1-167* ts cells expressing the indicated transgenes after 23h of induction in medium lacking thiamine is indicated in graphs. Cells were maintained at 25˚C or shifted to 36˚C for 6h.

Figure A.4 –Nak1 or Pmo25 targeting to the SPB causes ectopic septation independent of the other

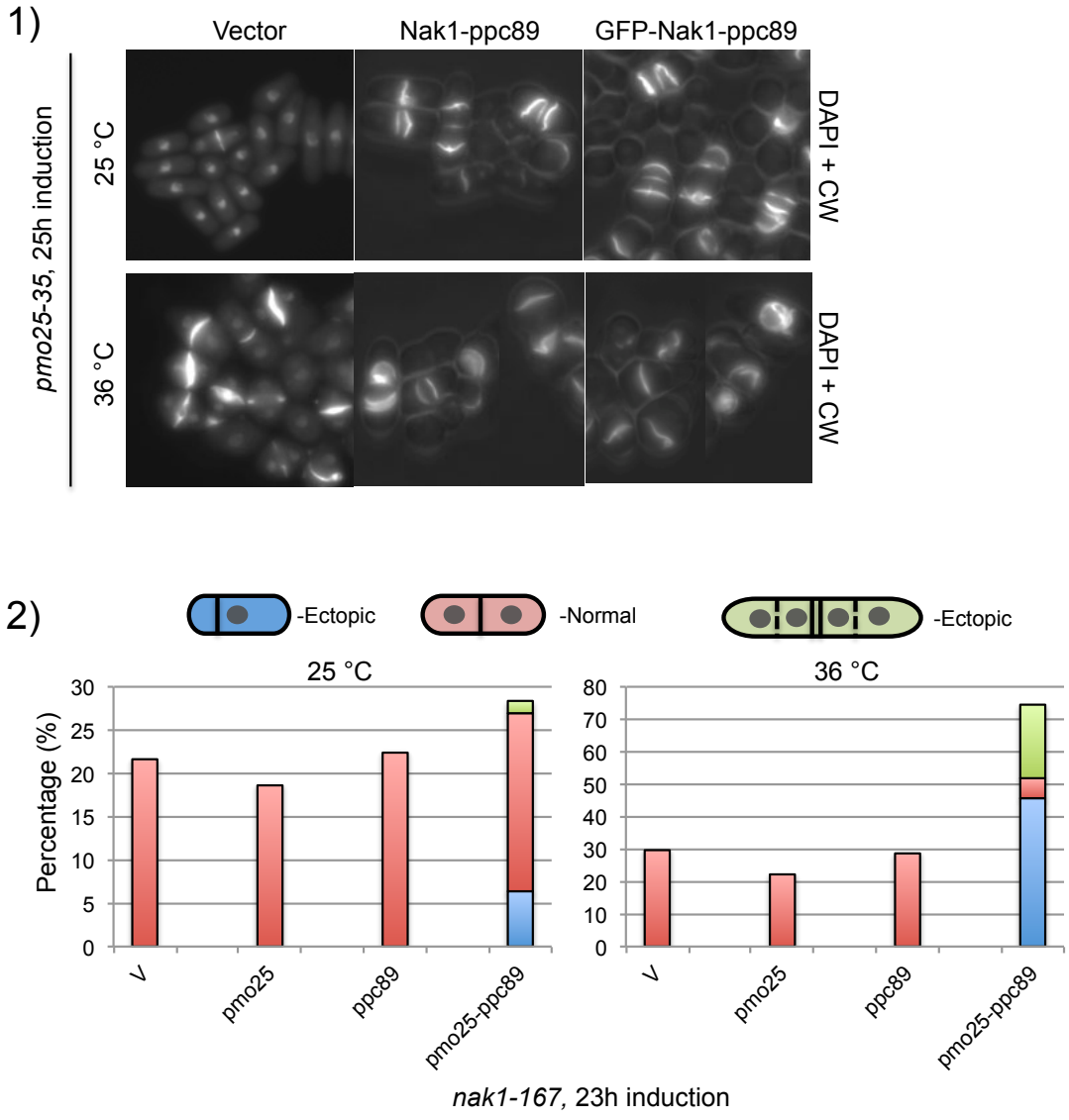
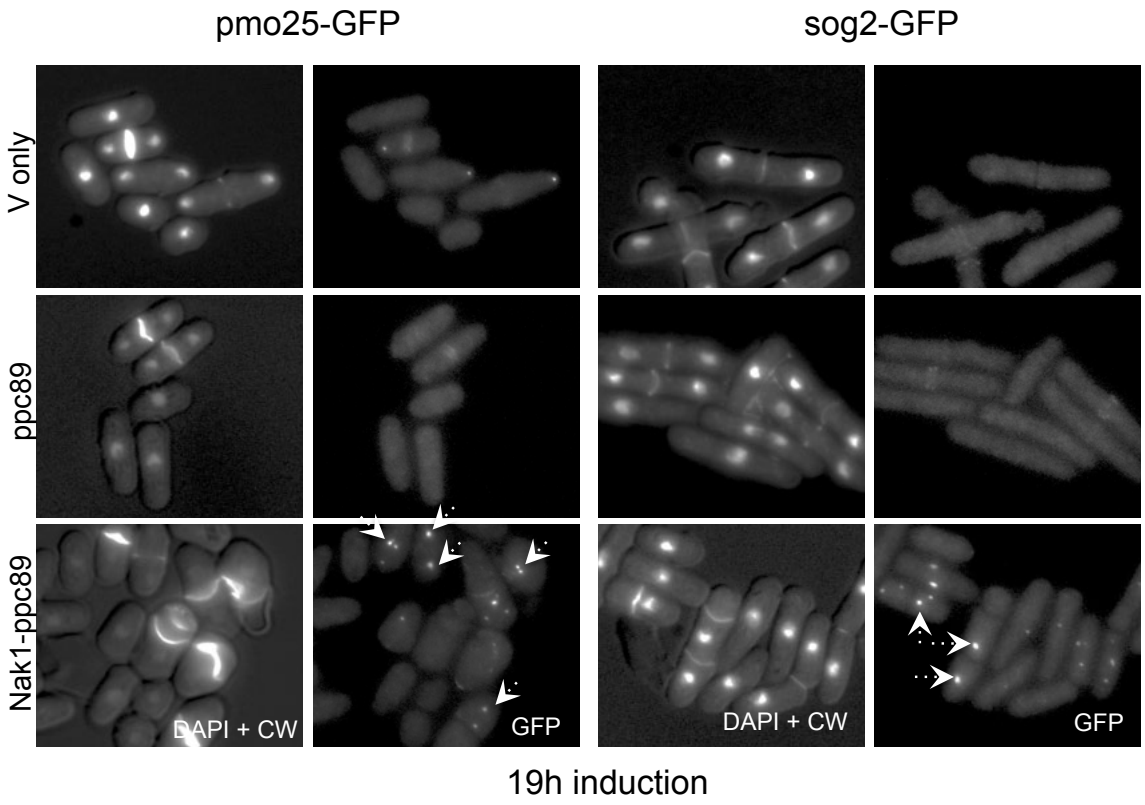


Figure A.5 – SPB targeting of Nak1 recruits MOR proteins, Pmo25 and Sog2

Representative images of cells expressing the specified transgenes after 19h of induction in medium lacking thiamine are shown. White arrows indicate cells with ectopic septa showing GFP tagged proteins (Pmo25 or Sog2) at the SPB.

Figure A.5 – SPB targeting of Nak1 recruits MOR proteins, Pmo25 and Sog2



APPENDIX B

Materials and Methods

Chapter II

Yeast strains and growth conditions

General yeast techniques and manipulations were carried out as previously described (Moreno et al., 1991). Cells were grown in either YE (Yeast Extract) medium or in EMM (Edinburgh Minimal Medium) with appropriate supplements. All strains were cultured at 25°C (unless indicated otherwise) except for temperature-sensitive strains, which were grown either at the permissive temperature of 25°C or restrictive temperature of 36°C. *S. pombe* transformations were carried out using either a lithium acetate method (Keeney and Boeke, 1994) or electroporation (Prentice, 1992). The 3HA-TAP tag was inserted at the carboxy-terminus of the *rad24* locus to create a strain expressing Rad24-3HA-TAP. DNA was prepared from bacteria and isolated from agarose gels using Qiagen kits. DNA sequencing was performed by Genewiz Inc. Oligonucleotide primers were obtained from Integrated DNA Technologies.

Microscopy

All imaging was done using a Nikon Eclipse E600 fluorescence microscope coupled to a cooled charge-coupled device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ). Image processing and analysis were carried out with IPLab Spectrum software (Signal Analytics, Vienna, VA) and ImageJ software (National Institutes of Health). Cells were either fixed in methanol or visualized live. DNA and cell wall material were visualized by staining with DAPI (Sigma-Aldrich) at 2 µg/ml and calcofluor white (CW; Sigma-Aldrich) at 50 µg/ml, respectively.

Plasmid Construction

To create the pREP41-GFP-Nak1-5A and pREP41-GFP-Nak1-5E constructs, phosphorylation site mutations were created in the pREP41-GFP-Nak1 construct using a QuikChange Site-Directed Mutagenesis kit (Agilent Technologies). To create the pDUAL-GFP-Sog2-5A construct, phosphorylation site mutations were created in the pDUAL-GFP-Sog2 construct using a QuikChange Site-Directed Mutagenesis kit (Agilent Technologies). pDUAL-HFG1c-Sog2 and pDUAL-HFG1c-Sog2-5A vectors were integrated into the *leu1*$^{+}$ chromosomal locus (Matsuyama et al., 2004, Invitrogen). For co-expression studies, integrated Sog2/Sog2-5A (leu+) were expressed from the full strength nmt1 promoter where as Nak1-5A was expressed ectopically (ura+) from the pDUAL-HFG41c-Nak1-5A vector (both Nak1-5A-GFP and Nak1-GFP were cloned into the pDUAL vector) using the medium strength nmt41 promoter. All mutant plasmid vectors were

sequenced. For localization studies, pDUAL-Nak1-5A-GFP and pDUAL-Nak1-GFP were integrated into the *leu1*$^{+}$ chromosomal locus (Matsuyama et al., 2004).

Immunoprecipitation and Western blot Analysis

Protein lysates were prepared from 20 OD cells, which were collected by centrifugation and flash frozen in liquid nitrogen. Cells were lysed by bead disruption in a multi-vortexer for 1 min at 4°C. Cell lysis was performed in NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.15mg/ml PMSF, PIC (1:200, Sigma), 50 mM NaF, 0.1 mM Na_3VO_4). Relative protein concentrations were determined using a BCA protein assay kit (Thermo Scientific). Immunoprecipitation experiments were performed as previously described (Sparks et al., 1999). For detection of GFP-tagged Nak1 in immunoprecipitated samples, cell lysates were separated by SDS-PAGE (6%), and transferred to Immobilon P PVDF membrane (Millipore Corp.) using a wet protein transfer apparatus (BioRad). Blots were probed with the anti-GFP mouse monoclonal IgG (Santa Cruz Biotechnology) at a 1:1,000 dilution or anti-Myc antibody (Santa Cruz Biotechnology) at a 1:500 dilution respectively, and developed using Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate for horseradish peroxidase (HRP) or using the Odyssey Infrared Imaging System (LI-COR Biosciences). For detection of Sid2 phosphorylation in immunoprecipitated Nak1, blots were probed with the anti-phospho Akt substrate specific antibody that specifically detects the RXXS (phosphoserine) motif

(110B7E, Cell Signaling Technology). For phosphatase treatments, immunoprecipitated protein was incubated with λ -phosphatase (New England Biolabs Inc.) for 20 min at 30°C. In samples treated with λ -phosphatase, phosphatase inhibitors (50 mM NaF and 0.1 mM Na_3VO_4) were washed 3 times with 1 ml of NP-40 buffer lacking the inhibitors prior to incubation with λ -phosphatase.

Rad24-TAP purification

Rad24-3HA-TAP was purified from wild-type cells, cells with active SIN (*cdc3-123 cdc16-116*), and cells with inactive SIN signaling (*cdc11-123* or *sid1-239*) as described previously (Gould et al., 2004). Cells were grown in 1.5 liter cultures of 4x YE media at 25°C to 1.6 OD, shifted to 35.5 °C for 2 hr., collected by centrifugation at 3000 RPM, and frozen in liquid nitrogen. Cells pellets were thawed on ice and washed with NP-40 Buffer (1% NP-40, 150 mM NaCl, 2 mM EDTA, 6 mM Na_2HPO_4, and 4 mM NaH_2 PO_4) supplemented with yeast protease inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF, and phosphatase inhibitors (50 mM NaF, and 100 uM $NaVO_4$). Cells were lysed on ice using a bead beater (Biospec) filled with approximately 250 ml of glass beads (425–600 µm G8772 Sigma). The bead beater chamber was immersed in an ice-water mix and run for 8 cycles of 30 s. with 30 s cooling periods in between each cycle. Beads were then extracted three times with 50 ml of NP-40 buffer with inhibitors (see above).

Cells extracts were then cleared by centrifugation at 3000 RPM for 5 min at 4°C. Subsequent TAP purification was performed as described (Gould et al., 2004).

LC-MS/MS and MS data analysis

S*id1-239*, *cdc11-123*, and *cdc16-116* Rad24-3HA-TAP purifications (in duplicate) were denatured, reduced with Tris 2-carboxyethyl phosphine, alkylated with iodoacetamide, and digested overnight at 37°C with Trypsin Gold (Promega) after diluting to 2M urea with 50 mM Tris pH 8.5. The resulting peptides were subjected to 2D LC-MS/MS (MudPIT) on a Thermo LTQ as previously detailed (McDonald et al., 2002). Thermo RAW files were converted to DTA and MZML files using Scansifter 2.0.13 (Ma et al., 2011) and spectra with fewer than 20 peaks were excluded from analysis. Protein identification was performed with the Myrimatch algorithm v1.6.33 (Tabb et al., 2007), on a high performance computing cluster (ACCRE at Vanderbilt University) using the *S. pombe* genome (May 2011, Pombase.org). Contaminant proteins were added and all sequences were reversed and concatenated to allow estimation of false discovery rates (10352 entries). Myrimatch parameters were as follows: strict tryptic cleavage; modification of methionine (oxidation, dynamic modification, +16 Da), S/T/Y (phosphorylation, dynamic modification, +80 Da) and cysteine (carboxamidomethylation, static modification, +57 Da) were allowed; precursor ions were required to be within 0.6 *m/z* of the peptide monoisotopic mass; fragment ions were required to fall within 0.5 *m/z* of the expected monoisotopic

mass; any number of missed cleavages were allowed. IDPicker v2.6.165 (Ma et al., 2009) was used to filter peptide matches with the following parameters: max. FDR per result 0.05, max. ambiguous IDs per result 2, min. peptide length per result 5, min. distinct peptides per protein 2, min. additional peptides per protein group 2, minimum number of spectra per protein 5, indistinct modifications M 15.994 Da, C 57.05 Da and distinct modifications S/T/Y 80 Da. Spectral counts for all proteins in each run were normalized (divided by the spectral counts for Rad24 and multiplied by 1000) and averaged between duplicates in Excel (Microsoft). To identify SIN targets, SIN "ON" (*cdc16-116*) values were divided by SIN "OFF" (*sid1-239* and *cdc11-123*) values. Phosphorylation of candidate Sid2 targets was identified using the Sequest (TurboSequest v2.7 rev12) algorithm (allowing carbamidomethylation, oxidation of the methionine, phosphorylation of serine, threonine, and tyrosine, peptide mass tolerance was set to 2.5 m/z), and Scaffold PTM (3.0 Proteome software). All reported RXXS sites were manually inspected and had Ascores greater than 7.

In vitro kinase assays

Substrates for the Sid2 kinase assay were cloned in pDEST15 (Invitrogen) and purified as GST fusions following the manufacturer directions. *cdc16-116 cdc3-124* cells with or without (control) *sid2-13Myc* were grown to 0.4 OD at 25°C, and then shifted to 36°C for 2 h (for Sid2 kinase assay). For Orb6 kinase assays, *mob2-13Myc* cells either expressing the transgenes in WT or *cdc3-124 cdc16-*

116 background (as indicated) were induced for 21h at 25˚C followed by shift to 36˚C for 3h. 50 pellets were collected and frozen in liquid nitrogen. Cells were disrupted by bead beating (Fast-prep), cleared by centrifugation, and immunoprecipitated with mouse anti-Myc antibody (Santa Cruz) and protein G beads. Beads were then washed three times with NP40 buffer (see above), and once with kinase buffer (10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$). Kinase assays were performed as described previously (Sparks et al., 1999; Ray et al., 2010). Briefly, beads were incubated in kinase buffer supplemented with yeast protease inhibitor cocktail (Sigma-Aldrich) along with 1 mM PMSF, 2uM ATP, 5 uCi γ-^{32}P-ATP, and 0.5 to 1 ug of purified protein substrate for Sid2 kinase assays and with 25uM ATP, 5 uCi γ-^{32}P-ATP and 10ug of Myelin basic protein (Sigma-Aldrich) for Orb6 kinase assays at 30°C for 30 min. Reactions were stopped by the addition of 20 µl of 2x SDS sample buffer followed by boiling. Samples were subjected to SDS-PAGE, gels were dried, and the signal was quantified using a Phosphorimager (GE).

Chapter III

Yeast strains and growth conditions

See Materials and Methods for Chapter II

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 DAPI (Sigma-Aldrich) and Calcofluor White (CW; Sigma-Aldrich), respectively. Cell staining with DAPI and Alexa Fluor 488–conjugated phalloidin was performed as described previously (Balasubramanian et al.,1997). All imaging was done at room temperature using a Nikon Eclipse E600 fluorescence microscope coupled to a cooled charge-coupled device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ). Image processing and analysis were carried out with IPLab Spectrum software (Signal Analytics, Vienna, VA) and ImageJ software (National Institutes of Health). Z-series of images were captured with a 100x oil (NA 1.3) objective lens and 3D stacked view was obtained using IP Laboratory software.

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 (Nak1-Orb6; Nak1*-Orb6) were sequenced to ensure there were no frame shift mutations. The kinasedead 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 QuikChange Site-Directed Mutagenesis kit. The resulting constructs were sequenced to confirm the changes. The Nak1-Mor2 fusion construct was created by cloning Nak1 and Mor2, in that order into the pREP41 and pREP41-GFP plasmids (craven et al). The final constructs were

sequenced to ensure that no unwanted mutations were introduced.

Chapter IV

Yeast strains and growth conditions

See Materials and Methods for Chapter II for details. In case of cells displaying a lethal cell lysis phenotype, 1.2M Sorbitol was added to the medium as needed.

Microscopy/Time-lapse Analysis

Cells were either fixed in methanol or visualized live. Time-lapse analysis to visualize lysis in *myo2-YFP*+Nak1-Mor2 cells was done using a Nikon Eclipse E600 fluorescence microscope coupled to a cooled charge-coupled device camera (ORCA-ER; Hamamatsu, Bridgewater, NJ). Image processing and analysis were carried out with IPLab Spectrum software (Signal Analytics, Vienna, VA) and ImageJ software (National Institutes of Health). 2µl of a log-phase cell suspension was placed on a microscope slide between a 2% YE agar slab and a cover slip, sealed with VALAP and imaged for 30min to 2hr. To follow the dynamics of the actomyosin ring and septum, time-lapse movies were produced from frames taken once every two minutes with a 50x oil objective (NA 0.9; Nikon). Time-lapse movies to visualize ring dynamics in *lifeact-GFP pcp1-mCherry* cells growing in medium containing sorbitol were acquired using an inverted microscope (TE 2000-E2; Nikon) equipped with a spinning disk confocal system (CSU10B; Solamere Technology Group) utilizing the MetaMorph

software. Time-lapse Z-series of images were captured using a 60x Plan Apo oil objective (NA 1.4; Nikon) using a camera (MGi EMCCD; Rolera). Cellular actin (lifeact-GFP), SPBs (pcp1-mCherry), and cell septum (DIC) were simultaneously monitored by collecting a Z-stack of nine images (0.5μM wide) every 2.5 minutes for about 180-250 minutes. Time-lapse images were processed using Volocity 3D image analysis software (PerkinElmer) and ImageJ software (National Institutes of Health). Fluorescence intensity measurements (average intensity) for GFP at the actomyosin ring and in the cytoplasm were performed using ImageJ software (National Institutes of Health) and Microsoft Excel.

Plasmid Construction

For construction of the Nak1-Mor2 fusion see Materials and methods from Chapter III.

Statistical Analysis

All experiments were performed in triplicates. Time-lapse data represented in this chapter comes from cells analyzed from three movies recorded at room temperature. The data are represented as mean ± S.D. P-values were calculated using an unpaired Student's t -test. $P < 0.05$ was considered as statistically significant.

Appendix A

Yeast strains and growth conditions

See Materials and Methods for Chapter II

Plasmid construction

To create the pREP41-ppc89, pREP41-GFP-ppc89, pREP41-Nak1-ppc89, pREP41-GFP-Nak1-ppc89 constructs, we cloned the constructs into the pREP41 or pREP41-GFP vector system (Craven et al). All proteins were expressed ectopically (leu+) from the plasmids using the medium strength nmt41 promoter.

Microscopy

GFP fusion proteins were observed in cells after fixation with -20˚C methanol. Cells were stained with DAPI (Sigma-Aldrich) and Calcofluor White (CW; Sigma-Aldrich) to visualize DNA and septum material, respectively. Images were acquired at room temperature using a microscope (Eclipse E600; Nikon) equipped with a cooled charge-coupled device camera (Orca-ER; Hamamatsu Photonics) and IPlab Spectrum software (Signal Analytics, Vienna, VA).

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