A ROLE FOR PROTEIN KINASE DBF2-MOB1 IN MITOTIC EXIT

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Abstract

Exit from mitosis is characterized by precise control of the cyclin-dependent kinase complex (Cdk) activity, breaking down mitotic structures, and completing cytokinesis. In *Saccaromyces cerevisiae*, protein phosphotase Cdc14 is involved in counteracting mitotic-Cdk activity by promoting the degradation of mitotic cyclin Clb2, and stabilizing the Cdc28 inhibitor Sic1. The activity and the cellular localization of Cdc14 are tightly regulated by the cell cycle. Cdc14 is sequestered and inhibited in the nucleolus by forming the RENT (Regulator of Nucleolar Silencing and Teleophase) complex for most of the cell cycle. It is released and distributed into the cytoplasm in anaphase and telophase, and then returns to the nucleolus in G1 phase. Activation of Cdc14 is achieved via multi-site phosphorylation of Net1 leading to the release of Cdc14. Net1 is first phosphorylated by the Fourteen Early Anaphase Release (FEAR) network, and then by the Mitotic Exit Network (MEN).

In this thesis, we show that a MEN component, protein kinase Dbf2-Mob1, plays a role in phosphorylating Net1 in late anaphase. We identified the effective Dbf2-Mob1 phosphorylation sites in the N-terminal of Net1 by in vitro kinase reaction assay. We found that cells that express mutant Net1 show growth defects and chain-like terminal morphology under restrictive temperatures. Genetic interactions suggested that the MEN kinases and Cdc14 are related to the cell cycle defects caused by the phosphosite mutated Net1. Analyzing the phosphosite mutants with Fluorecence-activated cell sorting (FACS)

and immunofluorescence assay, we found that the growth defects and the abnormal cell morphology are due to a defect in releasing Cdc14 in late anaphase, leading to disruption of mitotic exit. This result is further confirmed by western blot assay and the beads releasing assay.

In summary, the regulation of Cdc14 release in late anaphase via phosphorylation of its inhibitor Net1 by Dbf2-Mob1 is demonstrated in this work. This thesis provides a crucial piece of information that furthers our understanding of the mechanism of mitotic exit. It also points to a fascinating mechanism of controlling cytokinesis and meiosis by regulating Net1 phosphorylation by the MEN.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Chapter I

Introduction

Overview of the end of cell cycle

The cell cycle is a series of events that enable a cell to duplicate itself and produce a new generation. It consists of four main phases: (1) Gap 1 (**G1**) phase, where cell size is enlarged and the biosynthesis rate is increased, (2) Synthesis (**S**) phase, where DNA replication occurs, (3) Gap 2 (**G2**) phase, where cell growth continues and (4) Mitosis (**M**) phase, where the replicated DNA is segregated and the cell divides into two daughter cells. Besides these phases, there is a G0 phase, which is when cells exit the cell cycle.

During the M phase, correct partitioning of chromosomes to transmit genetic information to the next generation is very important. The M phase can be further subdivided into four stages: prophase, metaphase, anaphase, and telophase. The M phase is followed by cytokinesis, when nuclei, cytoplasm, and organelles are divided into two almost identical cells when (in budding yeast cells) the cell membrane pinches off at the junction between the mother and daughter cells. Events in the cell cycle, such as spindle disassembly (Li and Cai, 1997), chromosomal condensation (Loidl, 2003), and DNA replication (Piatti, 1997) are tightly regulated by cyclin-dependent kinases (CDKs) (Amon et al., 1994; Holloway et al., 1993; Surana et al., 1993). CDK includes two major components: a cyclin subunit and a protein kinase subunit. In the mammalian system, multiple kinase subunits form complexes with different cyclin subunits in each cell cycle stage. However, in *Saccharomyces cerevisiae*, a single kinase (Cdc28/ Cdk1) regulates the whole cell cycle by forming complexes with different kinds of cyclin subunits.

Various cyclin subunits activate and influence substrate selectivity of the kinase subunit at specific stages of the cell cycle. Cln1, Cln2, and Cln3 bind to Cdc28 to activate

Cdc28 at late G1 phase (Levine et al., 1995). B-type cyclins (Clbs) are involved in regulating activity of Cdc28 in S phase and M phase. Clb5 and Clb6 play a role at the S phase (Kuntzel et al., 1996; Toone et al., 1997), and Clb1, Clb2, Clb3, and Clb4 are involved in the G2/M phase (Fitch et al., 1992). Among all four Clbs, Clb2 is the major mitotic cyclin. The main states of the budding yeast cell cycle can be divided into high or low Clb-Cdk activity. Clb-Cdk activity is low in G1 phase but it is high in S, G2, and M phases (reviewed in Bardin et al., 2001; Deshaies, 1997; Miller and Cross, 2001).

There are at least three means to regulate Cdk complex activity. The first one is to tune the binding of the cyclin to Cdk or to control the degradation of cyclins. The second one is to associate Cdk with its inhibitors (eg. Sic1 and Cdc6). The regulation can also be achieved by phosphorylation of subunits of the Cdk complex (Deshaies, 1999; Mendenhall and Hodge, 1998; Donovan et al., 1994; Calzada et al., 2001). Clb-Cdk activity needs to be turned off so that cells can exit from mitosis and re-enter into G1. In *Saccharomyces cerevisiae*, the activity of Cdc28 is shut down by two distinct pathways: degradation of Clb2, the major mitotic cyclin, and accumulation of Sic1, the Cdc28 inhibitor. It has been shown that Cdc14, a phosphatase, is the key regulator for both pathways. Cdc14 dephosphorylates Hct1/Cdh1, which then binds and activates the ubiquitin ligase Anaphase-Promoting Complex/Cyclosome (APC/C). APC^{Cdh1} ubiquitinates Clb2 and leads to Clb2 degradation by proteasome. Cdc14 also dephosphorylates Sic1 itself and activates the Sic1 transcription factor (Swi5). Dephosphorylation of Sic1 stabilizes it by preventing it from rapid ubiquitination by SCF. Activation of the Swi5 further increases the Sic1 transcription. This creates a positive feedback loop leading to inactivation of Cdc28 (Visintin et al., 1998; Jaspersen et al., 1998).

The phosphatase activity of Cdc14 is inhibited by its nucleolar partner, Net1/Cfi1, which not only inhibits Cdc14 activity but also limits its cellular localization thus abolishing interaction with its substrates (Shou et al., 1999; Visintin et al., 1999). Two signaling networks are responsible for the release of Cdc14 from Net1/Cfi1: Cdc14 Early Anaphase Release (FEAR) and Mitotic Exit Network (MEN). The FEAR network phosphorylates Net1 leading to transient release of Cdc14 in early anaphase. Cdc14 released by this mechanism remains localized to the nucleus. This early release of Cdc14 is important to timely induce mitotic exit but is not sufficient for inactivation of Clb-Cdk. Thus it is insufficient to drive cells to exit from mitosis. To exit mitosis, cells need to activate the MEN, which sustains the release of Cdc14 and enables its disperal throughout the cell (Jaspersen et al., 1998; Pereira et al., 2002; Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999). However, the mechanism by which the MEN sustains Cdc14 release in late anaphase and telophase is unknown. This thesis focuses on revealing the mechanism by which the MEN regulates Cdc14 release from Net1 in late anaphase though the end of mitosis.

FEAR (Cdc Fourteen Early Anaphase Release) Network

Cdc14 activity is tightly regulated throughout the cell cycle stages by control of its cellular localization. Cdc14 is sequestered within the nucleolus by association with Net1/Cfi1. In early anaphase, the FEAR network promotes phosphorylation of Net1/Cfi1 by mitotic Cdks, weakening the binding between Cdc14 and Net1 and thus leading to early Cdc14 release (Azzam et al., 2004). The FEAR network consists of a number of proteins

such as Pds1, Esp1, Slk19, Zds1/2, PP2A, Clb1/2-Cdk1, Cdc5, Fbo1, and Spo12 (Figure I-1, from the review Rock and Amon, 2009).

When cells enter into anaphase, APC/C ubiquitinates the anaphase inhibitor, Pds1/ securin (Cohen-Fix et al., 1996; Cohen-Fix and Koshland, 1997). The destruction of Pds1, which binds to and inhibits Esp1 (known as separase), activates the protease activity of Esp1 to cleave Scc1 (a subunit of cohesion complex) thus promoting separation of sister chromatids (Ciosk et al., 1998). Pds1 also inhibits Esp1's function in the FEAR pathway (Cohen-Fix and Koshland, 1999; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Tinker-Kulberg and Morgan, 1999). Esp1 forms a complex with Slk19 to down-regulate $PP2A^{Cdc55}$ through the Cdc55-interacting protein, Zds1/2 (Sullivan and Uhlmann, 2003; Queralt and Uhlmann F, 2008). Zds1 (zillion different screen 1) and Zds2 are paralogues and involved in divergent cellular processes such as cell cycle, cell polarity, transcription and translation, and stress response (Yu et al., 1996; Schwer et al., 1998; Estruch et al., 2005; Yasutis et al., 2010). By binding to Cdc55, Zds1 and Zds2 manipulate the cellular localization of $PP2A^{Cdc55}$. Without Zds1 and Zds1, PP2ACdc55 accumulates in the nucleus and antagonizes release of Cdc14 from the nucleolus. When Zds1 and Zds2 are activated by Esp1, they promote export of $PP2A^{Cdc55}$ to the cytoplasm (Rossio and Yoshida, 2011). The down-regulation of nuclear PP2ACdc55 activity promotes accumulation of phosphate on Net1 (in a manner that depends on Clb-Cdk), leading to release of Cdc14 from the nucleolus (Azzam et al., 2004).

Other than direct phosphorylation of Net1 to release Cdc14, Clb1-Cdk1 and Clb2-Cdk1 are also responsible for Spo12 phosphorylation (Tomson et al., 2009). Spo12 is believed to be a scaffolding protein in the nucleolus, and is involved in mitosis exit and

meiosis by an unknown mechanism. The protein level of Spo12 appears to be regulated by the cell cycle (Shah et al., 2001). Spo12 forms complex with Fob1, a nucleolar protein required for replication fork blocking, and binds to Net1 through Fob1. Fob1 serves as a negative regulator of FEAR network to maintain the interaction between Net1 and Cdc14. Phosphorylated Spo12 antagonizes Fob1 function leading to Cdc14 early anaphase release (Stegmeier et al., 2004).

Cdc5 is the only known polo-like kinase in *Saccharomyces cerevisiae* and has multiple functions in mitosis and cytokinesis It is also involved in the DNA recombination checkpoint in meiosis (Iacovella et al., 2010). Cdc5 is not only a component in the FEAR network but also in the MEN pathway. It serves as a positive factor for the release of Cdc14 from nucleolus. The role of Cdc5 in the FEAR pathway is still unclear. It is suggested that Cdc5 is downstream of and/or in parallel to separase-Slk19. Cdc5 stimulates the protein degradation of Swe1 (the Cdk inhibitory kinase) through SUMO thus promoting phosphorylation of Cdc14 and Net1 (Liang et al., 2009; Simpson-Lavy, and Brandeis, 2010). Cdc5 also dissociates Cdc14 from the RENT complex by directly phosphorylation of Net1 in vitro (Shou et al., 2002).

The Roles of FEAR Network

The FEAR network is in charge of the regulation of multiple events during early anaphase. It is recognized as a group of proteins that regulates the timely dissociation of Cdc14 from Net1 in early anaphase of mitosis (Stegmeier et al., 2002). Then the released Cdc14 can activate Cdc15, a component of the MEN pathway, and positively regulate

mitotic exit (Jaspersen and Morgan, 2000; Menssen et al., 2001; Stegmeier et al., 2002; Xu et al., 2000). Mutations of the FEAR proteins delay the exit from mitosis but do not abolish the whole process. However, combination of FEAR and MEN mutations leads to synthetic lethality (Stegmeier et al., 2002). In addition to activating the MEN pathway, FEAR is also the gatekeeper, which ensures that timely Cdc14 activation is coordinated to chromosome partitioning. The metaphase-anaphase transition starts when APC/C ubiquitinates and the proteasome degrades Pds1. This activates the separase-Slk19 complex to promote early anaphase Cdc14 release. Released Cdc14 dephosphorylates Pds1, which promotes its degradation and provides a positive feedback loop that enhances chromosome segregation. (reviewed in D'Amours and Amon, 2004; Stegmeier and Amon, 2004; Jeremy and Amon, 2009).

Other than regulating Cdc14 release and chromosome segregation, it has been reported that the FEAR plays a role in controlling the anaphase spindle, meiosis, and proper segregation of rDNA,. Cohesin cleavage is not sufficient for rDNA segregation during anaphase. Although the mechanism of cohesin-independent rDNA segregation is still unclear, it has been reported to be FEAR-activated and Cdc14-dependent. In FEAR mutants, segregation of rDNA but not the rest of the genome is defective. Cdc14 released during early anaphase is responsible for silencing and exclusion of RNA polymerase I from the nucleolus, which silences rDNA transcription. This promotes the recruitment of cohesin-like complexes, condensins, to rDNA. Condensins mediate the condensation of chromosome. It is suggested that the recruitment of condensins to rDNA can facilitate rDNA compaction and segregation (Hwang and Madhani, 2009; clemente-Clanco et al., 2009; Wang et al., 2004).

The FEAR network is important for regulating spindle dynamics, stability, and midzone assembly. Microtubules are highly dynamic structures regulated by Clb-Cdk1. In the beginning of anaphase, the dynamic behavior of microtubules decreases dramatically, which stabilizes the anaphase spindle and allows chromosome segregation. It has been shown that this is affected by FEAR activation and Cdc14 activity. Cdc14 released through FEAR dephosphorylates the microtubule binding proteins, Ask1 and Fin1, which then stabilize the elongating spindle (Higuchi, and Uhlmann, 2005; Woodbury and Morgan, 2007). Furthermore, Cdc14 activation by the FEAR network also regulates assembly of the spindle midzone (the overlaps between the interpolar microtubules). During spindle elongation, numerous proteins are recruited to this area to help stabilize this region. FEAR-dependent release of Cdc14 dephosphorylates a group of proteins, including Ase1 (the microtubule-bundling protein) and Sli15. Dephosphorylation of Ase1 triggers its relocalization to the spindle midzone. Sli15 is the subunit of the chromosomal passenger complex, Ipl1-Sli15-Bir1. This complex is conserved in higher eukaryotes, where is is known as Aurora B-INCENP-Survivin. Dephosphorylation of Sli15 promotes the Ipl1-Sli15-Birl complex to relocate to the spindle midzone. Once Ase1 and Ipl1-Sli15-Birl are localized at the spindle midzone, they recruit additional factors to assist anaphase spindle stabilization (de Gramont et al., 2007; Fu, et al., 2009; Mirchenko and Uhlmann, 2010).

Recently, the FEAR network and Cdc14 have been identified as key players in the regulation of meiosis. Meiosis is a specialized form of cell division that results in the formation of gametes. In meiosis, there is a single round of DNA replication followed by two sequential rounds of chromosome segregation. Meiosis can be divided into two main stages, meiosis I and meiosis II, each of which contains the sequential phases: prophase,

metaphase, anaphase, and telophase. During meiosis I, homologous chromosomes are separated from each other followed by meiosis II, when where sister chromatids are separated. It has been shown that the FEAR network and Cdc14 employ a similar mechanism to regulate chromosomal DNA segregation in both meiosis I and meiosis II via down-regulation of Clb-Cdk1 activity. When the FEAR network or Cdc14 is inactivated, meiotic cells show a severe delay in disassembly of the meiosis I spindle. Furthermore, without the FEAR network and Cdc14, meiotic events become uncoupled, resulting in mixed meiosis I-like and meiosis II-like chromosome segregation patterns. This indicates that the FEAR network and Cdc14 serve as a safeguard to ensure that chromosome segregation occurs in the right sequence in the two stages of meiosis (Kerr et al., 2011; Khmelinskii et al., 2007; Marston et al., 2003).

MEN (Mitotic Exit Network)

The FEAR network regulates transient release of Cdc14 in early anaphase. However, the MEN is required to sustain the release of Cdc14 until the end of mitosis. Cells with defective FEAR delay mitotic exit for 10-20 minutes but eventually are able to complete the process. On the contrary, cells with inactivated MEN are unable to exit mitosis and arrest permanently in late anaphase. Thus, the MEN is the essential network for cells to exit from mitosis.

The MEN is a Ras-like GTPase signaling cascade which consists of four protein kinases (Cdc5, Cdc15, Dbf2, and Dbf20) (Johnston et al., 1990; Kitada et al., 1993; Schweitzer and Philippsen, 1991; Toyn et al., 1991), a two-component GTPase-activating

protein (Bub2 and Bfa1) (Hu et al., 2001; Pereira et al., 2002), a spindle pole body (SPB) scaffold protein (Nud1) (Adams and Kilmartin, 1999), a protein phosphatase (Cdc14) (Wan et al., 1992), a Ras-like GTPase protein (Tem1) (Shirayama et al., 1994a), a GTP/GDP exchange factor (Lte1) (Shirayama et al., 1994b), and a Dbf2 binding protein (Mob1) (Luca and Winey, 1998). Mutation of the proteins of this cascade leads to late anaphase arrest with high Clb2 and high Cdk1 activity (Figure I-1, from the review Rock and Amon, 2009).

Genetic and biochemical evidence indicate that Tem1 is at the top or near the top of the MEN. Tem1 seems to be regulated positively by Lte1, and negatively by Bub2-Bfa1 (Bardin et al., 2000; Fesquet et al., 1999; Geymonat et al., 2002; Pereira et al., 2000). When cells form a mitotic spindle, Tem1 starts to localize to the cytoplasmic face of the spindle pole body, where Bub2-Bfa1 complex is located. When binding with Bub2-Bfa1 complex, Tem1 is in an inactive GDP-bound form. During late anaphase, Tem1 and the Bub2-Bfa1 complex migrate to the daughter cell with one of the spindle pole body following the extended mitotic spindle. Let1 is restricted to the bud cortex. Once Tem1-Bub2-Bfa1 enters the bud and encounters Let1, Tem1 is activated as a substrate for the GEF, Let1 (Bardin et al., 2000; Pereira et al., 2000). The Cdc18-like protein, Nud1, serves as a scaffold for the core of MEN components and recruits the Tem1, Bub2-Bfa1, Cdc15, and Dbf2-Mob1 to the spindle pole body (Gruneberg et al., 2000; Visintin and Amon, 2001). Tem1-GTP is shown to bind to and activate protein kinase Cdc15 at the cytoplasmic face of spindle pole bodies (Asakawa et al., 2001; Lee et al., 2001a). Activated Cdc15 activates the protein kinase Dbf2, which form a complex with Mob1 (Mah et al., 2001). Activated Dbf2-Mob1 phosphorylates the nuclear localization signal of Cdc14 resulting in the retention of Cdc14

in the cytoplasm where it can interact with its substrates, such as Clb-Cdk (Mohl et al., 2009). However, the mechanism resulting in high level of Net1 phosphorylation and the sustain release of Cdc14 by MEN is still unclear.

The other component participating in the MEN pathway is Cdc5. Although Cdc5 is not the core component of the MEN, it activates the MEN in multiple ways. Cdc5 inactivates the GAP activity of Bub2-Bfa1 through phosphorylation and is shown to be involved in Lte1 activation (Lee et al, 2001b; Hu et al., 2001; Geymonat et al., 2003). Furthermore, Cdc5 activates Dbf2-Mob1 in a Bub2-independent mechanism. By promoting FEAR network, early released Cdc14 can serve as positive feedback loop on activating Cdc15 thus promoting Dbf2-Mob1 activation (Jaspersen and Morgan, 2000; Stegmeier et al., 2002; Visintin et al., 2003).

MEN in Cytokinesis

Recently, the MEN was suggested to play an important role in cytokinesis. In the anaphase, Tem1, Cdc5, Cdc15, and Dbf2-Mob1 are recruited to the cytoplasmic face of the spindle pole body. However, at the end of mitosis, Cdc5, Cdc15, and Dbf2-Mob1 relocate to the bud neck where the separation of daughter and the mother cell occurs (Frenz et al., 2000; Hwa Lim et al., 2003; Luca et al., 2001; Song et al., 2000; Xu et al., 2000; Yoshida and Toh-e, 2001, de Bettingnies and Johnston, 2003). This SPB-bud neck translocation of the MEN components suggests a potential link between MEN and cytokinesis. In addition, it has been reported that the MEN components move to the bud neck dependent on each other and along with components of septin ring, Cdc12 and Cdc3 (Frenz et al., 2000; Luca

et al., 2001; Yoshida and Toh-e, 2001). This supports the assertion that the MEN signaling pathway also regulates cytokinesis. The MEN proteins are required for septin ring formation, which is defected when Bub2 is inhibited (Lee et al., 2001a; Lee et al., 2001b; Park et al., 1999). Moreover, some genetic evidence shows that MEN mutants result in defects in cytokinesis. When the lethality phenotype of either *tem1* or *cdc15* deletion is bypassed by the *net1-1* mutant, cells exhibit cytokinetic defects (Lippincott et al., 2001; Shou et al., 1999). *cdc15-lytl* and *mob1* mutant strains also show cytokinesis defects with the chain-like undivided cells (Jimenez et al., 1998; Lippincott et al., 2001; Luca et al., 2001; Shou et al., 1999; Jimenez et al., 2005).

The release and activation of Cdc14 by MEN serves as a guard ensuring mitosis exit to occur before cytokinesis. It has been shown that the bud neck localization of Dbf2-Mob1 is Cdc14-dependent, although the regulation of the Dbf2-Mob1 kinase activity at the bud neck is through Cdc15 (Lee et al., 2001a; Mah et al., 2001). The detailed mechanism about how MEN components regulate cytokinesis is still unclear.

MEN Conservation

In *Schizosaccharomyces pombe*, a pathway known as the septation initiation network (SIN) has been reported as the analogous pathway to the MEN in *Saccharomyces cerevisiae*. This pathway is named SIN because mutating the components in the network leads to defects in septation. Most of the components in the SIN, Plo1, Cdc16-Byr4, Spg1, Cdc7, Sid2-mob1, Cdc11, and Clp1/Flp1, are analogous to Cdc5, Bub2-Bfa1, Tem1, Cdc15, Dbf2-Mob1, Nud1, and Cdc14 in MEN. The orthologue of Let1 in the MEN

remains exclusive in the fission yeast. On the other hand, the orthologues of the scaffolding protein, Sid4, and the protein kinase complex, Sid1-Cdc14, which plays a role between Cdc7 and Sid2-Mob1, has not been found in the budding yeast. The major difference between the MEN and SIN is that SIN is not essential for mitosis exit but it plays a role in regulating cytokinesis (reviewed in Bardin and Amon, 2001; Krapp et al., 2004; Stegmeier and Amon, 2004; McCollum and Gould, 2001).

The budding yeast Cdc14 and its orthologue Clp1 in the fission yeast are very similar; both are localized in the nucleolus during G1 and S phase. However, they are different in several aspects. For example, during G1 and S phase, Clp1 is also present on spindle pole bodies. Moreover, unlike Cdc14, Clp1 is released during early mitosis while Cdc14 is released during the anaphase. When Cdc14 is released from the nucleolus it enters the nucleus or even into cytoplasm, whereas when Clp1 is released it localizes to the mitotic spindle and medial ring instead. Additionally, SIN is not responsible for releasing Clp1 though it maintains its release status, whereas the MEN both regulates the release of Cdc14 and maintains its release status (Cueille et al., 2001; Trautmann et al., 2001). Furthermore, budding yeast Cdc14 and fission yeast Clp1 use different mechanisms to inhibit mitotic Cdks. Cdc14 promotes accumulation of the Cdc28 inhibitor, Sic1, and the degradation of Clb2, the active subunit of mitotic Cdks. Clp1 is not responsible for the accumulation of the inhibitor of the fission yeast Cdc28 orthologue Cdk1/Cdc2, Rum1. It is also not required for promoting the degradation of the fission yeast B-type cyclin, Cdc13 or activation of the APC/C specificity factor Ste9. It is suggested that Clp1 inhibits Cdk activity by controlling the phosphorylation status of Cdc2 (Esteban et al., 2004; Guertin et al., 2002; Cueille et al., 2001; Trautmann et al., 2001; Wolfe and Gould, 2004).

Numerous components of the MEN have been identified in species other than yeast. The orthologues of the budding yeast Cdc14 was found in *Caenorhabditis elegans*, *Xenopus laevis*, and humans (Gruneberg et al., 2002; Kaiser et al., 2004; Li et al., 1997). Interestingly, these Cdc14 orthologues do not appear to be required for mitotic exit but they are essential for regulation of cytokinesis (reviewed in Trautmann and McCollum, 2002). In addition, their cellular locations at the cell cycle stages are somewhat different from the budding yeast Cdc14. CeCdc14 localizes to the central spindle in the anaphase and is essential for embryonic division and cytokinesis (Gruneberg et al., 2002; Saito et al., 2004). In *Xenopus*, there are two orthologues of Cdc14, namely XCdc14α and XCdc14β, both of which are localized in nucleolus and centrosome and play a critical role in regulating cytokinesis (Kaiser et al., 2004; Piel et al., 2001). There are also two Cdc14 orthologues in human, hCdc14A and hCdc14B. The cellular location of these proteins is cell cycle dependent (Kaiser et al., 2002; Mailand et al., 2002). Human Cdc14A locates at centrosomes in the interphase, after which it down-regulates Cdc25 activity to ensure timely activation of Cdk1-Cyclin B1 complexes at the G2/M phase transition and mitosis entry (Sacristan et al., 2011). Human Cdc14B localizes to the nucleolus in the interphase and it is recently reported to promote correct chromosome segregation and bipolar spindle formation through mitosis. (Tumurbaatar, et al., 2011)

Conservation of Dbf2-Related Kinases

The Dbf2 of *Saccharomyces cerevisiae* belongs to a highly conserved serine/threonine kinase family, namely NDR (the nuclear Dbf2-related), which is a subclass

of the AGC (protein kinases A, G, and C) group of kinases. The NDR kinases can be further classified into two families based on their structures and functions: the ndr family and the Wts/Lats family. Cbk1, Dbf2, and Dbf20 in *Saccharomyces cerevisiae*, Orb6 and Sid2 in *Schizosaccharomyces pombe*, Cot1 in *Neurospora crassa*, sax-1 in *C. elegans*, and LATS (Large tumor suppressor) kinases of *Drosophila* and humans belong to the Ndr family, while LATS in *C. elegans*, and the Lats1 and Lats2 of *Drosophila* and humans belong to the Wts family (Table I-1, from the review Hergovich et al., 2006). The unique conserved feature of the NDR family kinases are 30–60 amino acids located at the catalytic subdomains VIII, which is a hydrophobic motif located at the C terminus. The phosphorylation sites in this motif are essential for catalytic activity. The function of these kinases is conserved and they control morphological changes, mitotic exit, cytokinesis, cell proliferation and apoptosis (reviewed in Hergovich et al., 2006). By analyzing different model systems it is further suggested that Ndr signaling and Wts signaling are involved in centrosome duplication (Hergovich et al., 2007; Toji et al., 2004) and chromosome alignment (Chiba et al., 2009). This also implies that the kinases in the NDR family may be activated by a conserved mechanism. They are controlled by the kinases from the Ste20 family through phosphorylation. In addition, activation of their functions requires the formation of the complex with a broadly conserved protein Mob (Mps1-one binder, a protein binds to the N-terminal of the NDR kinases) (reviewed in Tamaskovic et al., 2003; Hergovich et al., 2006). Recently, the NDR kinases have been found to play a role in neural fate specification, neurite outgrowth and branching, and receptive field determination (reviewed in Emoto, 2011).

It has been reported that the kinases of the NDR family in different species seem to

possess similar functions. Cbk1 is another member of NDR family in *Saccharomyces cerevisiae*, and similarly to Dbf2, it requires Mob family protein (Mob2) to activate its activity and it may also be regulated by a network of proteins (Colman-Lerner et al., 2001; Nelson et al., 2003; Weiss et al., 2002). Cbk1 localizes to sites of polarized growth and cell wall remodeling, for example, bud necks, through its associating partners, Hym1 and Mob2. It has been reported that Cbk1 regulates multiple cellular morphogenesis pathways: the Ace2-independent pathway controlling polarized apical growth and mating projection formation, and the Ace2-dependent pathway regulating efficient cell separation following cytokinesis. Cbk1 is also involved in the "cytokinesis checkpoint" when forming a complex with Mob2, (Bidlingmaier et al., 2001; Colman-Lerner et al., 2001). The NDR kinase, Orb6, is the Cbk1 orthologue in *Schizosaccharomyces pombe*. It has been shown that Orb6 binds to its Mob2 counterpart and regulates cell polarity (Hou et al., 2003). In *C elegans*, the NDR kinase, Sax-1, has 66% identity and 74% similarity to human NDR1 and it regulates neuronal cell shape and polarity (Zallen et al., 2000). The Drosophila NDR kinase, Trc (tricornered), displays 70% identity and 79% similarity to the human NDR1 and it is involved in controlling actin polarization and it plays a role in the morphogenetic checkpoint (Geng et al., 2000). In addition, Trc is regulated by the same conserved phosphorylation sites as in Dbf2 and Ndr and forms complex with Mob protein (He et al., 2005; Mah et al., 2001; Millward et al.,1999)

The best studied NDR kinases are WARTS (Wts) and LATS1 (large tumor suppressor), which both belong to the Wts family in *Drosophila* and humans. The LATS1 in *Drosophila* was first identified in mosaic fly screens for tumor suppressors and negative regulators of cell proliferation (Xu et al., 1995). Wts negatively regulates cell proliferation

and promotes apoptosis by inhibiting the transcription of Cyclin E and by promoting the loss of DIAP1 (reviewed in Hay and Guo, 2003). Wts is phosphorylated by a Ste20-like kinase, Hpo, similar to Cdc15 in yeast (Mah et al., 2001; Wu et al., 2003) and it binds to a Mob protein, Mats, which simulates Wts kinase activity (Lai et al., 2005). Human LATS1 rescues *lats* alleles in flies. Cellular localization and phosphorylation of hLATS1 is cell cycle dependent. hLATS1 localizes to the centrosomes in interphase and anaphase, and then to the midbody in the telophase. It also directly binds to hCDK1 and acts as hCDK1 negative regulator. Through regulating hCDK1 activity, hLATS1 is suggested as a tumor suppressor that negatively regulates cell proliferation and modulating cell survival. hLATS1 also regulates cytokinesis through LIMK1 inhibition (Xia et al, 2002; Nishiyama et al., 1999; Yang et al., 2004).

Two other NDR kinases in human are NDR1 and NDR2, which belong to the Ndr family. It has been shown that the hNDR may perform similar functions as its relatives in other species. hNDR interacts with hMob1, homologue of Mob1/2 in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and is tightly cell cycle regulated. hNDR1/2 plays a role in regulating cell proliferation and tumor development through controlling centrosome duplication and mitotic chromosome alignment. hNDR1/2 also play a role in regulating the G1/S phase transition by stabilizing c-myc and preventing p21 accumulation (Coulombe et al., 2003).

Thesis Overview

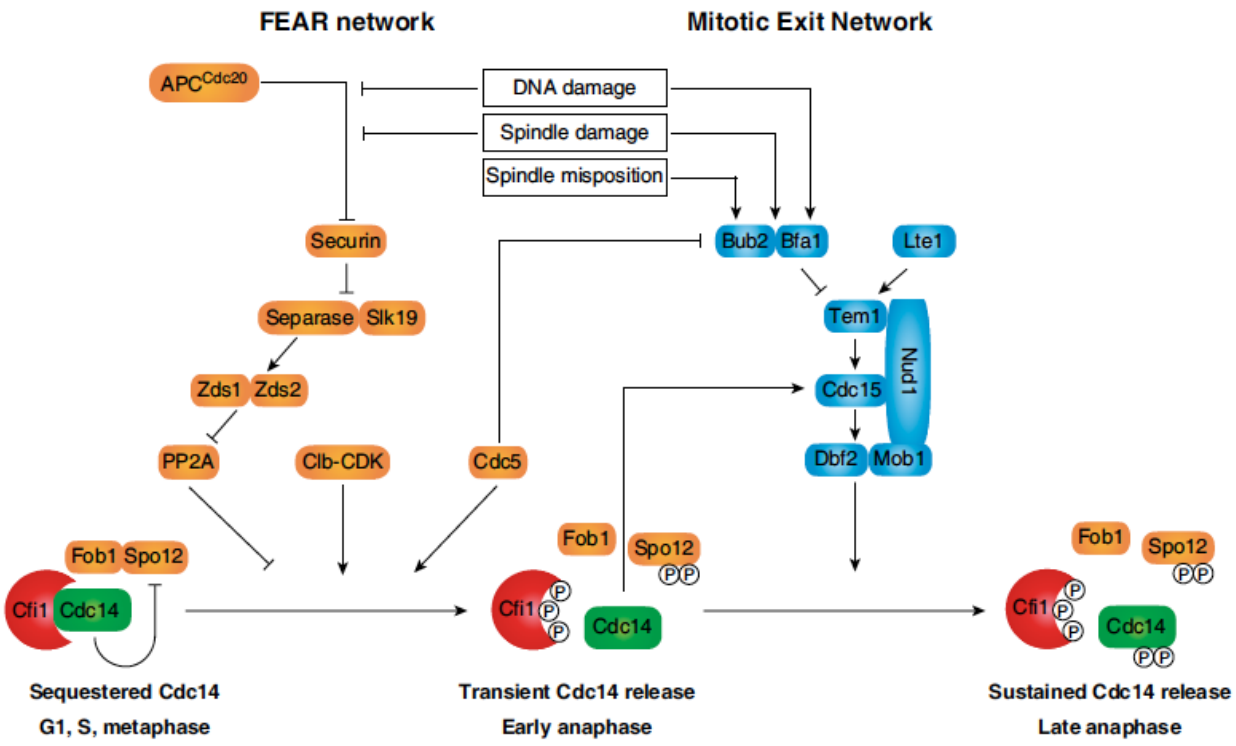
Net1 is first phosphorylated by the Fourteen Early Anaphase Release (FEAR)

network, and then by the Mitotic Exit Network (MEN) through an unknown mechanism. In this thesis, we showed that a MEN component, protein kinase Dbf2-Mob1, played a role in phosphorylating Net1 in the late anaphase. In Chapter II, We identified the effective Dbf2-Mob1 phosphorylation sites in the N-terminal of Net1 by *in vitro* kinase reaction assay and by comparing site conservation between different *Ascomyces*. In Chapter III, we demonstrated that the phosphosite-mutated Net1 cells exhibit growth defects and chain-like terminal morphology under restrictive temperature. Genetic interactions suggested that the MEN kinases and Cdc14 are related to the same pathway that causes defects in the phosphosite-mutated Net1. In Chapter IV, we analyzed the phosphosite mutants with Fluorecence-activated cell sorting (FACS) and immunofluorescence assays, and we found that the growth defects and the abnormal morphologies are due to defects in releasing Cdc14 in the late anaphase leading to disruption of the mitotic exit. Western blot assays and the complex beads releasing assays further support our hypothesis. This thesis provided evidence for the hypothesis that the regulation of Cdc14 release in the late anaphase is via phosphorylating its inhibitor Net1 by Dbf2-Mob1.

Table I-1. Conservation of the NDR kinase signaling pathway

Organism	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Caenorhabditis elegans*	*Drosophila melanogaster*	*Homo sapiens*
NDR kinase	Dbf2p, Cbk1p	Sid2p, Orb6p	LATS‡, SAX-1	Lats/Warts, Trc	LATS1/2, NDR1/2
Upstream kinase	Cdc15p[8,52,53,65,107,109,130], Kic1p[54]	Sid1p, Nak1p[79]/ Cdc7p[5,36,132]	NP_508749*, NP_ 505309*	Hippo[13,16,17,19–21], AAN71385*	MST1/2 (REF. 23), MST3 (REF. 29)
Co-activator	Mob1p[7–9,52,65,130], Mob2p[2,10,54]	Mob1p[4,5,55], Mob2p[4,6]	NP_501179, NP_ 502248, NP_741916*	dMob1 (REFS 14,18), dMob1-4 (REF. 14)	hMOB1 (REF. 51), hMOB1/2 (REFS 22,24,26,51)
Scaffold protein	Nud1p[108,130,131], Tao3p[3,54]	Cdc11p/Sid4p[5,36,132,133], Mor2p[56,79]	CAE58896*, SAX-2 (REF. 12)	Salvador[13,16,17,19–21,68], Furry[11,15,98]	hWW45 (REFS 23,68), hFURRY1‖, hFURRY2‖
GTPase/interactor	Tem1p[8,53,65,109], Hym1p[32,54]	Spg1p[5,36,132], MO25[79]	Unknown, NP_496092*	Unknown, dMO25§	Unknown, MO25α,β*

Figure I-1. The FEAR network and the Mitotic Exit Network



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Chapter II

Analysis of Net1 Phosphorylation by Protein Kinase Dbf2-Mob1 in vitro

Introduction

In *Saccharomyces cerevisiae*, mitotic exit is tightly regulated by the MEN, Mitosis Exit Network. The MEN is a Ras-like GTPase signaling cascade network containing a Ras-like GTPase protein (Tem1) (Shirayama et al., 1994a), a GTP/GDP exchange factor (Lte1) (Shirayama et al., 1994b), a two-component GTPase-activating protein (Bub2 and Bfa1) (Hu et al., 2001; Pereira et al., 2002), a spindle pole body (SPB) scaffold protein (Nud1) (Adams and Kilmartin, 1999), a protein phosphatase (Cdc14) (Wan et al., 1992), a Dbf2 binding protein (Mob1) (Luca and Winey, 1998), and four kinase proteins (Cdc5, Cdc15, Dbf2, and Dbf20) (Johnston et al., 1990; Kitada et al., 1993; Schweitzer and Philippsen, 1991; Toyn et al., 1991). One of the major functions of the MEN is to control mitotic exit through regulation of the release of Cdc14. It has been found that at the end of mitosis, Cdc14 is released from the nucleolus to the cytoplasms, and both Cdc14 and the Cdc14 nucleolus inhibitor Net1 are highly phosphorylated. When the MEN is defective, the phosphorylation status of both Cdc14 and Net1 drops. When the FEAR network is defective, mitotic exit is delayed but not terminated. However, when components in the MEN are mutated, cells are arrested at mitosis. In addition, when the MEN is defective, cells cannot sustain the cytoplasmic Cdc14 release and Cdc14 is sequestered in the nucleolus. This information indicates that the MEN plays a key role in regulating mitotic exit through regulating Cdc14 localization. However the detailed mechanism of how the MEN controls the mitotic exit is still unclear. The MEN is localized at the spindle pole body which is far from the nucleolus where both Net1 and Cdc14 are located.

A fraction of Mob1 and Dbf2 colocalizes with Cdc14 in the nucleus and all three proteins function at kinetochores and other nuclear structures during anaphase. Dbf2-Mob1 is also involved in maintaining chromosomal passenger protein on the mitotic spindle (Stoepel et al., 2005). This implies that Dbf2-Mob1 can directly interact with Cdc14 and even Net1. In order to locate Dbf2-Mob1 substrates, the first step is to identify the Dbf2-Mob1 kinase recognition sites and the consensus domains in the protein. It has been shown that Dbf2-Mob1 prefers phosphorylating serine rather than threonine. In addition, it recognizes and phosphorylates the serine or threonine with a specific sequence motif, R/KXXS/T, where the two amino acids in the middle of the motif are not selective. Through proteome chip assays, several possible substrate candidates such as Net1 (Mah et al., 2005) were found. Two Dbf2-Mob1 substrates, Cdc14 in the mitosis and Hof1 in the cytokinesis, were reported recently. Cdc14 is phosphorylated by Dbf2-Mob1 at its nuclear localization signal, leading to the loss of nuclear localization function, which then results in cytoplasmic localization of Cdc14 (Mohl et al., 2009). Hof1 is first phosphorylated by Cdc5, and then by Dbf2-Mob1. This phosphorylation promotes Hof1 to release from the septin ring and to bind with medial actomyosin ring (AMR), where it promotes AMR contraction and membrane ingression (Meitinger et al., 2011).

Net1 is a phosphoprotein that is responsible for inhibition and sequestration of Cdc14 (Shou et al., 1999; Visintin et al., 1999; Traverso et al., 2001). In the early anaphase, Net1 is phosphorylated by Clb1-Cdk1 and Clb2-Cdk1, and possible by Cdc5, leading to early anaphase Cdc14 release (Azzam et al., 2004; Shou et al., 2002). Net1 is highly phosphorylated at the end of mitosis, when FEAR is no longer involved, and it is also one of the top substrate candidates of Dbf2-Mob1 from the proteome chip assay. Therefore, it

is interesting to verify whether Dbf2-Mob1 is responsible for phosphorylating Net1 during the end of mitosis. Traverso et al. have found that the first 600 amino acids of Net1 act like full-length Net1 to Cdc14. It binds with Cdc14, which results in Cdc14 localization in the nucleolus, and it acts as a competitive inhibitor by blocking the active site of Cdc14 (Traverso et al., 2001). Because the first 600 amino acids of Net1 are the functional domain for Cdc14 regulation, we cloned and expressed the first 600 amino acids of Net1 tagged with 6x His and evaluated the potential of being a Dbf2-Mob1 substrate *in vitro*.

Here we use kinase reaction systems to locate the putative Dbf2-Mob1 sites in a truncated Net1 *in vitro*. Net1 WT is phosphorylated by purified and preactivated Dbf2-Mob1 rather than kinase-dead Dbf2 (N305A)-Mob1. The phosphosite-mutated Net1s showed defective phosphorylation by Dbf2-Mob1. We suggest that Net1 is a specific substrate for Dbf2-Mob1 and the first ten putative phosphorylation sites are crucial in the *in vitro* system.

Methods

Multi site-directed mutagenesis

1800 bp of the NET1 ORF, starting with the initiation codon, was PCR amplified, digested with XmaI and KpnI, and ligated into PET21b. Multi site-directed mutagenesis was performed with QuikChange® Multi Site-Directed Mutagenesis Kit (strategene) with primer sets: KJC33 for RSA204T→A (5'-AACAACCATTCGTAGTGCCGCTAATGGATCCATGAGGG-3'), KJC34 for KIV228S→A (5'-ACACTTCTTCGAAGATCGTCGCCAACAACTCAGAT GACGAA

G-3'), KJC35 for KIK269S→A (5'-GACGCAGGTAAAAAAATAAAAGCA AGCATCGTCGAGGAAGA-3'), KJC36 for RSA282T→A (5'-TCGTGTCCAGATCA GCAGCCGTGGATCCAGATAAAAC-3'), KJC37 for RIT362S→A (5'-ACCACTCCA AGAATAACAGCAGGAATGTTGAAAATCCCCG-3'), KJC38 for RSQ439S→A (5'-CCTTGCAACGTAGTCAAGCCGCCATCGCAGATAATAATGG-3'), KJC39 for RKS497S→A (5'-CAACCACCAAGAAAGGCTGCACTGGAAACTATAGTGGAAA AG-3'), and KJC40 for KVS574S→A (5'-CACTAGCTGGGAAAGTTGCTGCAAACA ACAACGCTTCAAAG-3'). PS5M was generated by Dane Mohl.

Protein expression and purification

Variety of 6His-600aa NET1 proteins were expressed in Rossetta bacteria cells and purified by nickel affinity with MagneHis particles (Promega). Plasmid carried WT and variety of mutant Net1 was transformed into competent Rossetta cells and plated on the LB/Ampicillin plate. Pick a single colony and incubate in 5 ml of LB/Ampicillin at 37 °C overnight. Transfer 2 ml of overnight culture into 1L of LB/Ampicillin and incubate at 37 °C for about 4-5 hours until it reaches 0.5 $O.D._{600}$. Add 0.2 mg (final concentraion) of IPTG and shift the culture to 30 °C for another 5 hours. Cells were harvested with 4000 g for 20 min at 4 °C. Resuspend the cell pellets in 10 ml of wash buffer (50 mM Tris-HCl pH7.5 and 150 mM NaCl), spin down in SS34 rotor and frozen in liquid N_2. Resuspend cell pellet in 10 ml lysis buffer (50 mM Tris-Cl pH7.5, 150 mM NaCl, 2% Triton X-100 and 20 mM Imidazole). Sonicate cell suspension for 40 sec, with power at 60% output power for 4 times with 60 sec cooling on ice in between. Spin the extract for 40 min in SS34 at 15K, 4

°C. Wash MagneHis resin for 3 times with lysis buffer. Collect the supernatant and incubate with 200ul washed MagneHis resin for 45 minutes in cold room on the rotator. Wash beads 2 times with 10 ml of Ni-NTA wash buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.2% Triton X-100, and 20 mM Imidazole) for 10 minutes incubation on the rotator in cold room. Resuspend resin pellet in 1 ml Wash buffer and transfer to microfuge tube. Wash beads 2 times by incubating with 1 ml wash buffer for 2 minutes on ice. After final wash, do a 400ul elution with elution buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.2% Triton X-100, and 300 mM Imidazole) for 4-5 times and save each elution individually. Perform a Bradford assay with each elutions and combine the peak fractions for dialysis into storage buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 1mM DTT and 10% Glycerol).

6His-Cdc15 was expressed in Sf9 cells and purified as previously described (Mah et al., 2001). Flag-6His-HA-DBF2/ 6His-Mob1 complex was expressed together in Sf9 cells and purified as previously described (Mohl et al., 2009).

Kinase reaction

1 μg Dbf2-Mob1 or Dbf2 (N305A)-Mob1was activated by 0.2 μg Cdc15 kinase for 20 min at 25 °C in Cdc15 kinase buffer (50 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM beta-glycerophosphate, 1 mM DTT, and 20 μM ATP). Activated Dbf2-Mob1 was diluted with Dbf2 kinase buffer (50 mM Tris-HCl, pH 7.4, 60 mM potassium acetate, 10 mM magnesium chloride, 1 mM DTT, and 10 μM ATP) for 1:1 volumn ratio. 3μ Ci of γ-[^{32} P]ATP and 5 μg N-600 aa 6His-Net1 either wild-type, or variety of

phosphorylation sites mutants are used for each reaction. Kinase reactions were incubated at 25 °C for 20 min and stopped with the addition of 2x SDS sample buffer. Phosphorylated proteins were separated by either 10% SDS-PAGE or 10% SDS-PAGE with 12 uM Phos-tag^{TM} (AAL-107). Gels were stained with commassive blue to detect for Net1 protein condition and mobility. After staining with commassive blue, gels were dried with heated platform with vacuum and ^{32}P signals were detected by phosphoimager (Storm 860). Gel images were further quantified with imageQuant software.

Results

Mapping the first 600 amino acid sequence

It has been shown that Dbf2-Mob1 phosphorylates a specific sequence motif, R/KXXS/T (Mah et al, 2005). By analyzing the amino acid sequence of Net1, we found 15 potential Dbf2-Mob1 recognition sites (bold-faced amino acids in Figure. II-1). We used sequence alignment to compare the Net1 protein sequence in *S. cerevisiae* to the Net1 orthologs in other ascomycetes, *S. bayanus*, *S. mikatae*, *S. paradoxus*, and *S. kudriavzevii*, of *Saccharomyces* sensu stricto species, *S. castellii* of *Saccaromyces* sensu lato species, and *Kluyveromyces latis*, *Candida glabrata*, *Lachancea thermotolerans*, *Zygosaccharomyces rouxii*, and *Vanderwaltozyma polyspora*, that are loosely related to *Sacchromyces* but still under *Sacchromycetaceae* (Oda et al., 1997; Naumove et al., 2000). The first 12 potential Dbf2-Mob1 phosphorylation sites are conserved in at least five out of the six *Saccharomyces*, except RSQ439S which is only conserved in five out of the six *Saccharomyces*. Most of the first 12 sites are even conserved in more than three out of the

five loosely related fungi, except KRPP192T, RSA204T, KIV228S, and RKS497S (Table II-1). KRPP192T and RKS497S are only conserved in two out of the five loosely related fungi. RSA204T is conserved in only one out of the five loosely related fungi and KIV228S is the only putative site that is in the first 500 amino acids but is not found out of the *Saccharomyces*.

Some sites are highly conserved in the 11 fungi. For example, RVS212T and RIS259S are conserved in all 11 fungi, RSA282T, RLL295S, RIT362S are conserved in ten out of the 11 fungi, and the KIKS269S, RVV317S, RSQ439S are conserved in nine out of the 11 fungi. Most of the conserved sites are located in the first 500 amino acids. The last three putative Dbf2-Mob1 phosphorylation sites are poorly conserved even in the six *Saccharomyces* (Table II-1).

Dbf2-Mob1 phosphorylates potential phosphorylation sites in Net1 in vitro

In order to identify which putative sites are phosphorylated by Dbf2-Mob1, we generated three multi-site mutations of the potential phosphorylation sites in a plasmid construct that contains the first 1800 bp of the *NET1* ORF followed by a 6x His tag. For simplicity, we refer to these constructs as phosphosite mutants (PSxM) with a number in place of x to indicate how many sites were mutated (e.g. PS5M). We made three multi-site mutants: PS5M, PS10M, and PS13M. In PS5M, five conserved sites (RPP192T, RVS212T, RIS259S, RLL295S, and RVV317S) were mutated. This was constructed by Dane Mohl, a former member of our group. In PS10M, not only the previous five mutation sites but also

the remaining sites (RSA204T, KIV228S, KIK269S, RSA282T, and RIT362S) of the first 10 conserved sites were mutated. With the exception of KRM520T and KED534T, all of the other potential phosphorylation sites were mutated in PS13M (Figure. II-1).

Plasmids encoding these different mutants were transformed into the bacterial expression strain. Then we induced protein expression by IPTG, and purified the expressed proteins on a Ni-NTA column. His6-tagged Dbf2-Mob1, His6-tagged Dbf2 (N305A)-Mob1 (kinase dead), and GST-tagged Cdc15 were purified by previous lab members (Mah et al., 2005; Mohl et al., 2009). When His6-tagged Dbf2-Mob1 was pre-activated with purified protein kinase Cdc15 and incubated with the wild type Net1 fragment containing the first 600 amino acids (WT) in the presence of γ-[^{32}P]ATP for 20 min at room temperature, we observed incorporation of isotope label into the purified Net1. There was no incorporation of isotope label when the purified Net1 was incubated with His6-tagged Dbf2(N305A)-Mob1 (kinase dead) (Figure II-2a). This indicated that Net1 is a substrate of Dbf2-Mob1 in vitro. In addition, the efficiency of label incorporation was decreased when more phosphorylation sites were mutated to alanine. This indicates that the mutated phosphorylation sites of Net1 were the primary targets of Dbf2-Mob1 (Figure II-2a). Analyzing the intensities of the incorporated isotope labels, we found that PS5M still had 70% of the isotope incorporation when compared to WT, whereas both PS10M and PS13M exhibited strong decreases in labeling. The relatively similar labeling observed for PS10M and PS13M indicates that the first ten conserved potential Dbf2-Mob1 phosphorylation sites were the major targets for Dbf2-Mob1 (Figure II-2b).

To analyze the different phosphoisoforms of the mutant Net1 proteins, we used Phos-tag^{TM} (AAL-107) gel to separate proteins by their modification levels. WT Net1

exhibited multiple phosphoisoforms, but PS10M and PS13M exhibited only background signal. Although PS5M still incorporated substantial [^{32}P], the slowest migrating phosphoisoform migrated faster than the major WT phosphoisoform. This indicates that the mutated sites in PS5M did cause a specific defect in phosphorylation. The commassie staining also shows consistent results. WT and PS5M were barely detectable possibly because the proteins were distributed over different phosphorylation states whereas protein staining of the PS10M and PS13M were similar to WT treated with kinase-dead Dbf2 (Figure II-2c). The distribution and strength of the incorporated [^{32}P] signals in the various Net1 mutants were quantified and are displayed in Figure II-2d.

Discussion

Although it was previously shown that Dbf2-Mob1can phosphorylate Net1, the phosphorylation sites were not characterized (Mah et al., 2005). In this study, we screened for putative Dbf2-Mob1 phosphorylation sites in Net1 by using a combination of mutagenesis and *in vitro* kinase assays. By comparing candidates from the screen with published mass spectrometry experiments, we found that some of the sites that we identified are phosphorylated in a regulated manner during the cell cycle (Chen et al., 2002; Chi et al., 2007; Holt et al., 2009; Dephoure and Gygi, 2011). However, no correlation between the level of the conservation and the possibility of being phosphorylated was found (Table. II-2). For example, although phosphorylation of KIV228S is regulated during cell cycle, it is one of the less conserved Dbf2-Mob1 phosphorylation sites.

We further showed that there are multiple phosphorylation sites in the first 600

amino acids of Net1 using the quantitative kinase assays and the Phos-tag^{TM} (AAL-107) gel system. The idea that Net1 is multi-phosphorylated by Dbf2-Mob1 could account for the observation that Net1 is heavily phosphorylated at the end of mitosis. Comparing the phosphorylation status of PS10M and PS13M to the conservation of these sites in a variety of fungi suggested that the first 10 putative Dbf2-Mob1 recognition sites may be particularly relevant for Net1 regulation.

Several important questions remain unanswered. What is the stoichiometry of phosphorylation of the various sites? Are all sites phosphorylated simultaneously or consecutively? Understanding the detailed of the regulation mechanism of these phosphorylation sites is an interesting and urgent topic.

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Figure Legends

Figure II-1. Potential Dbf2-Mob1 phosphorylation sites are highly conserved in Net1 among Saccharomyces species

Potential Dbf2-Mob1 sites, RXXT, RXXS, KXXT, or KXXS, are marked with bold face. The sites mutated in the *net1-PS5M* and *net1-PS10M* are labeled with blue and red underlines, respectively. The green underlines indicate sites mutated in *net1-PS13M*.

Figure II-2. Purified Dbf2-Mob1 phosphorylated purified Net1 WT but not phosphosite mutants

(a) Incorporation of [^{32}P] into purified Net1 shows an inverse correlation with the number of phosphosite mutated. Equal amounts of WT and mutant proteins were incubated with γ- [^{32}P]ATP and with either pre-activated Dbf2-Mob1 (wild-type) or pre-activated Dbf2 (N305A)-Mob1 (kinase dead) at room temperature for 20 min. Dbf2-Mob1 was activated in vitro by prior incubation with protein kinase Cdc15 and ATP. Following the kinase reaction, samples were fractionated by 7% SDS-PAGE. The gel was stained with coomassie blue and subjected to autoradiography. “WT” refers to wild-type Net1 protein. “5M”, “10M”, or “13M” refer to mutation of 5, 10 or 13 potential Dbf2-Mob1 phosphorylation sites.

(b) Quantification of the [^{32}P] signal intensities from panel (a). [^{32}P] signal was captured by phosphoimager and analyzed with ImageQuant. [^{32}P] intensity of WT Net1 was set to 1. [^{32}P] intensities from all the other reactions were normalized to the signal from WT Net1. 5M shows about 30% decrease of the [^{32}P] intensity. 10M and 13M exhibited a reduction of almost 90% in the [^{32}P] signal. [^{32}P] signals from WT or mutant Net1 incubated with Dbf2 (N305A)-Mob1 (kinase dead) were barely detectable.

(c) Loss of hyperphosphorylated species in Net1 phosphosite mutants. Equal amounts of purified WT and mutant Net1 proteins were incubated with γ-[^{32}P]ATP and with either pre-activated Dbf2-Mob1 (wild-type) or pre-activated Dbf2 (N305A)-Mob1 (kinase dead) at room temperature for 20 min. Equal amounts of reactions were fractionated on a 10% SDS-polyacrylamide gels that contained 12 uM Phos-tag^{TM} (AAL-107). Following SDS-PAGE, the gel was stained with coomassie blue and subjected to autoradiography. "WT" refers to wild-type Net1 protein. "5M", "10M", or "13M" refer to mutation of 5, 10 or 13 potential Dbf2-Mob1 phosphorylation sites.

(d) Quantification of the pattern of [^{32}P] signal distribution from panel (c). [^{32}P] signal was captured by phosphoimager and analyzed with ImageQuant. [^{32}P] intensities along each lane were normalized to background signal and plotted to reveal the intensity distribution. Proteins with different levels of [^{32}P] modification exhibited different mobility. Multiple [^{32}P]-labeled species were observed in WT Net1 incubated with Dbf2-Mob1. The majority of the [^{32}P] signal for 5M shows faster mobility than that for WT Net1, consistent with loss of phosphorylation sites. 10M and 13M are nearly indistinguishable from the background.

Table II-1. Potential Dbf2-Mob1 phosphorylation sites are highly conserved among *Saccharomyces* species.

YJL076W/ NET1 of *Saccharomyces cerevisiae* ORF sequence was used to compare with fungi using online webpage of Saccharomyces genome database (http://www.yeastgenome.org/cgi-bin/FUNGI/showAlign).

S. cerevisiae	KRPP192T	RSA204T	RVS212T	KIV228S	RIS259S	KIKS269S	RSA282T	RLL295S
Lachancea thermotolerans	-	-	RVST	-	RISS	-	-	RLPS
Zygosaccharomyces rouxii	RNPS	-	RVST	-	RVSS	KIKS	RSET	RLLS
Candida glabrata	KRGST	KGQS	RIST	-	RISS	KITS	RSAT	RLLS
Vanderwaltozyma polyspora	-	-	RIST	-	RIRS	RIIT	KSET	RFLS
Kluyveromyces lactis	-	-	RIST	-	RISS	-	KSET	RLPS
S. bayanus	KRPPT	RSAT	RIST	KMMS	RISS	KIKS	RSET	RLLS
S. castellii	KRPST	RNPT	RIST	-	RISS	RIRS	RSGT	-
S. kudriavzevii	KRPPT	-	RIST	KIMS	RISS	KIKS	RSET	RLLS
S. mikatae	KRPPT	RSAT	RIST	KIIS	RISS	KIKS	RSET	RLLS
S. paradoxus	KRPPT	RSGT	RIST	KIVS	RISS	KIKS	RSAT	RLLS

S. cerevisiae	RVV317S	RIT362S	RSQ439S	RKS497S	KRM520T	KED534T	KVS574S
Lachancea thermotolerans	-	RITS	RQTS	-	-	-	-
Zygosaccharomyces rouxii	RVVS	RITS	RQTS	RKTS	-	KSAS	-
Candida glabrata	RVIS	RITS	RQTS	-	-	-	-
Vanderwaltozyma polyspora	RVIS	RITS	-	RISS	-	-	-
Kluyveromyces lactis	-	-	RLPS	-	RAPS	-	-
S. bayanus	RVVS	RITS	RSQS	RKNS	-	-	-
S. castellii	RVLS	RITS	-	-	-	-	-
S. kudriavzevii	RVVS	RITS	RSQS	RKSS	RRMT	-	-
S. mikatae	RVVS	RITS	RSQS	RKSS	-	KEES	-
S. paradoxus	RVVS	RITS	RSQS	RKSS	KRMT	KEDS	KVST

S. cerevisiae	% Identities	% Positives	% Gap
Lachancea thermotolerans	33	37	24
Zygosaccharomyces rouxii	39	41	37
Candida glabrata	36	50	12
Vanderwaltozyma polyspora	33	43	22
Kluyveromyces lactis	31	44	19
S. bayanus	56	63	4
S. castellii	29	42	20
S. kudriavzevii	60	65	2
S. mikatae	60	66	2
S. paradoxus	66	69	1

Table II-2. Summary of phosphorylated sites in Net1 first 600 amino acid

- **Lower-case letters in the boxes represent the phosphorylation site.**
- **+ or – represent increasing or decreasing phosphorylation level in log L/H.**

	RPP192T	RSA204T	RVS212T	KIV228S	RIS259S	KIK269S	RSA282T
Mohl's Mass-spec					✔	✔	
Azzam (2004)			✔		✔		
Holt (2009)							
Gygi(asynchronous)				+KIVSNNs			
Gygi(nocodazole)				+KIVSNNs			
Gygi(Δclb2)				+KIVSNNs			

	RLL295S	RVV317S	RIT362S	RSQ439S	RKS497S	KRM520T KED534T	KVS574S
Mohl's Mass-spec	✔	✔			✔		
Azzam (2004)					✔		
Holt (2009)				✔	✔		
Gygi(asynchronous)					+RKSs		
Gygi(nocodazole)				-RSQs			
Gygi(Δclb2)	-RLLsGt			-RsQSs	+RKSs		

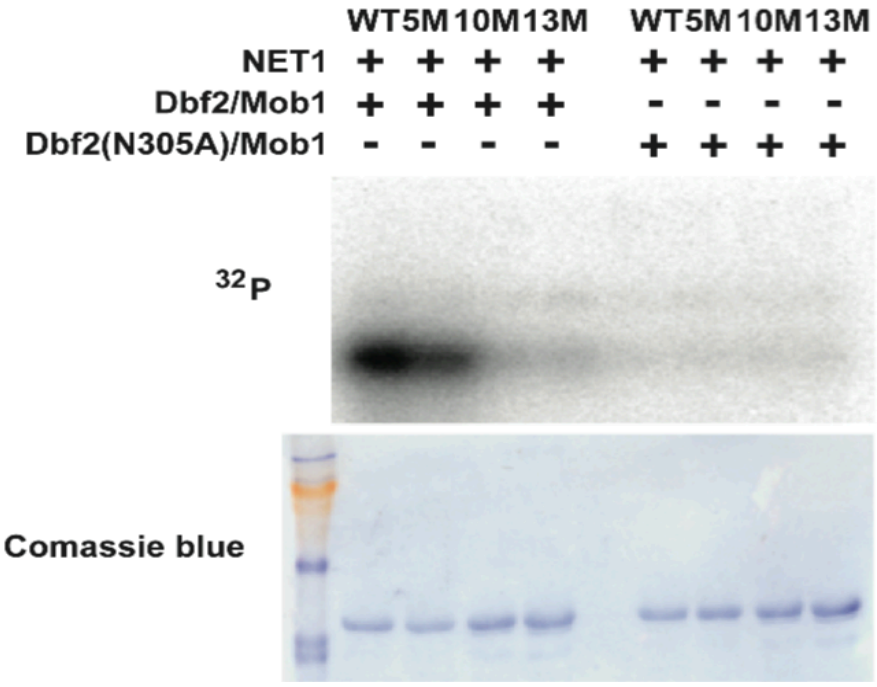
Figure II-1. Potential phosphorylation sites in the first 600 amino aci

***Saccharomyces cerevisiae* Net1**

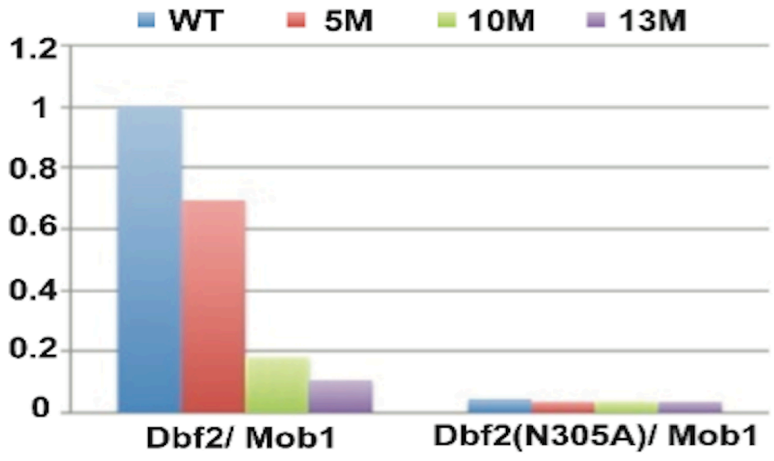
```
  1  MYKLQVVLVP PSLQATMPIQ FGYGPTIAES SQLLPNRTNM AQSAGD
 51  YANLRSANVS FTPSYFNQSR FRKFLLFTKP TNTLLNLSDE IIDKCE
101  SLQEDIEILS LQDNSGCDLD PDFLVKDVFN VNNIVRVILK NEIDLD
151  VSLYKSVKRS KLNNGSPQSV QPQQQIPSSS GVLRIAKKRP PTGTTT
201  RSATNGSMRV STPLARQIYP PPSSKIVSNN SDDEDEDIGE RSFLPP
251  QSPPIRISSG IDAGKKIKSS IVEEDIVSRS ATVDPDKTKQ QRLLSG
301  STMTPNRVTL TGQRVVSEHA HKNELVFSAS ASSSSFANGG TAAVTA
351  RKPPVTTPRI TSGMLKTPEP RISEIEKELK EGPSSPASIL PAKAAK
401  KPYLENGENY ESDDSSSSEN QETPETEPHS KASLQRSQSS IADNNG
451  NSPLGDAMPH NVHLAELPKA SNTSITKSSN GESWGKQQEH QPPRKS
501  IVEKKSQAEP SGIVEPKRMT NFLDDNQVRE KEDTNDKLLE KEILPT
551  DQPILASSDK SNGTLKSLAG KVSSNNNASK EDGTIINGTI EDDGND
```

Figure II-2. Net1 was phosphorylated on multiple sites by Dbf2-Mob1 in vitro

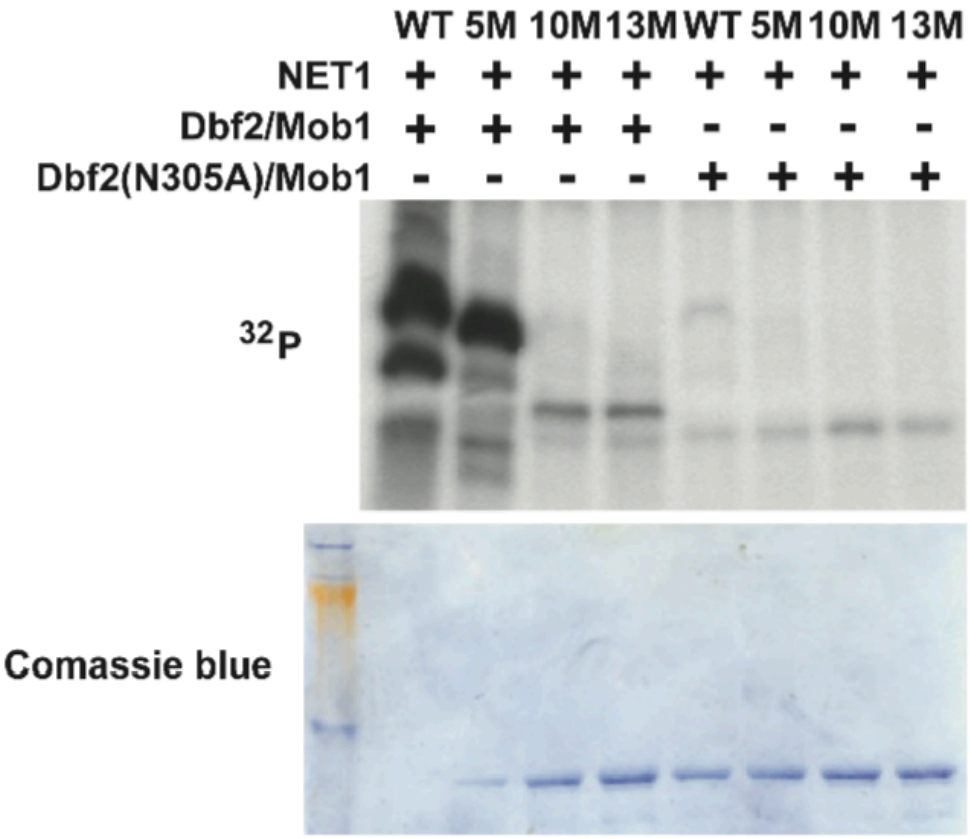
(a)



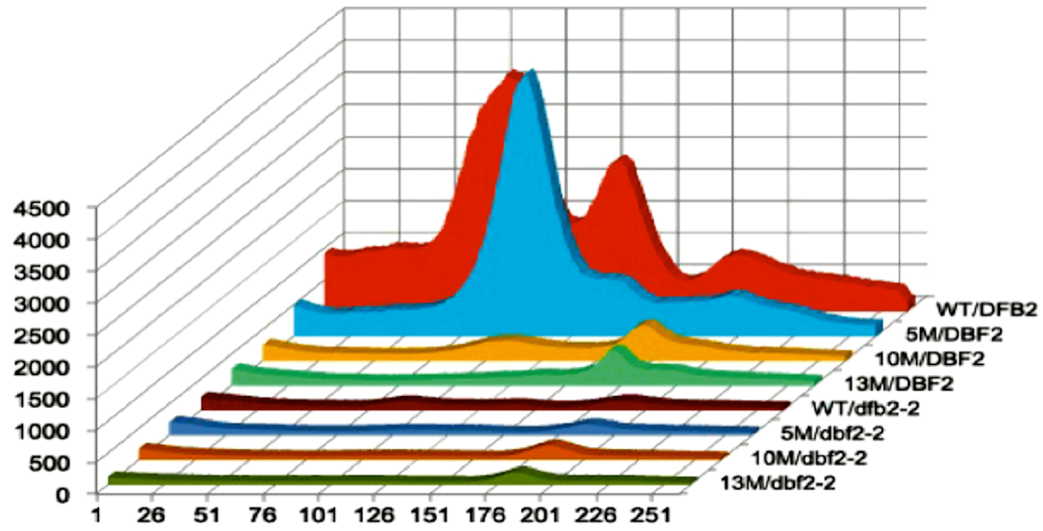
(b)



(c)


WT 5M 10M 13M WT 5M 10M 13M
NET1 + + + + + + + +
Dbf2/Mob1 + + + + - - - -
Dbf2(N305A)/Mob1 - - - - + + + +
32P
Comassie blue

(d)


4500
4000
3500
3000
2500
2000
1500
1000
500
0
1
26
51
76
101
126
151
176
201
226
251
WT/DFB2
5M/DBF2
10M/DBF2
13M/DBF2
WT/dfb2-2
5M/dbf2-2
10M/dbf2-2
13M/dbf2-2

Chapter III

net1 Phosphosite Mutant Cells Exhibited Defects in Mitotic Exit

Introduction

The MEN is responsible for heavy phosphorylation of Net1 and for releasing and maintaining Cdc14 in the cytoplasm. This regulation is thought to proceed through a signaling cascade where Ras-like GTPase Tem1 activates kinase Cdc15, which then activates the downstream kinase Dbf2 (Asakawa et al., 2001; Lee et al, 2001a). Mutations of the components in the MEN lead to cell arrest in the late mitosis or cytokinesis. Two kinase mutants, *cdc15-2* and *dbf2-2*, lead to temperature-sensitive phenotypes and the mutations cause apoptosis at 34 °C and 37 °C, respectively (Grandin et al., 1998). In addition, mutations of the MEN components (Tem1, Cdc15, and Dbf2) or Cdc14 result in abnormal cell morphology. At the restrictive temperature, *tem1-1*, *cdc15-2*, *dbf2-2*, and *Cdc14-1* cells form chain-like terminal morphology (Grandin et al., 1998; Jimenez et al., 2005; Jimenez et al., 1998). If exit regulation from mitosis though the MEN is by phosphorylation of Net1 with Dbf2-Mob1, it would be interesting to verify whether the phosphosite-mutated *net1* (PS11M and PS13M) cells lead to growth defects or abnormal morphologies *in vivo*, since PS11M and PS13M terminates the phosphorylation by Dbf2-Mob1. In addition, it would be interesting to test if *net1* phosphosite mutant cells exhibit defects in the cell cycle.

Here we analyze the growth phenotype and the morphology of both WT and mutant *net1* cells. We found that phosphosite mutant net1 shows both cold- and temperature-sensitive phenotypes. *net1* mutant cells also show chain-like morphology, similar to that in the MEN mutants. This indicated that *net1* mutants cause defects in mitosis exit and/ or cytokinesis. We also looked at genetic interactions by combining WT *NET1* or

phosphosite-mutated *net1* with the MEN mutants. The phosphosite-mutated *net1* cells exhibited a synthetic lethal phenotype in *cdc15-2* background. We also study the mitotic deficiency with *net1* phosphosite mutant cells by Fluorescence-activated cell sorting (FACS) and western blot assay, and we found that Net1 phosphosite mutant exhibits defects in mitotic exit. We suggested that Dbf2-Mob1 phosphorylates Net1 and promotes the exit from mitosis.

Methods

Strain construction, materials, and Net1 mutagenesis

All strains used are in the W303 background (can1-100, leu2-3, his3-11, trp1-1, ura3-1, ade2-1).

A strain, RJD5605, with base pair of 45-1008 of *NET1* replaced with "CORE cassette", KIURA3-KanMAX4, and with pGAL1-I-SCEI integrated in genomic DNA is a gift from Dr. Christopher M. Yellman of professor Shirleen Roeder's lab in Yale University. The PCR fragment of full length *NET1* ORF with 38 bp before start codon and 31 bp after stop codon containing either WT or 13 phosphosite mutant (192T, 204T, 212T, 228S, 259S, 269S, 282T, 295S, 317S, 362S, 439S, 497S, and 574S) was amplified with primer set, KJC52/ KJC85, and used for yeast trasformation to generate WT or mutant untagged *NET1* in its original chromosome locus. Dilute overnight growth of RJD5605 from YP-2% dextrose into YP-2% raffinose with starting concentration of 0.25 $O.D._{600}$ at 30 °C. When cells reach approximate 0.6-0.8 $O.D._{600}$, add galactose to a 2% of final concentration to induce expression of I-SceI for 1 hour prior to yeast trasformation. Yeast

trasformation is followed the "high-efficiency lithium acetate/PEG" method generated from Gietz's lab (http://home.cc.umanitoba.ca/~gietz/method.html). Transformants were plated onto YPD plates and replicated to FOA plates after ~2 days of growth at 30 °C. Chromosome DNA from the colonies grew on FOA plates were extracted and the *NET1* sequences were amplified by PCR and sequenced. Because of partial recombination, we end up with *net1-PS11M* (192T, 204T, 212T, 228S, 259S, 269S, 282T, 295S, 317S, 362S, 439S) and *net1-PS13M* (192T, 204T, 212T, 228S, 259S, 269S, 282T, 295S, 317S, 362S, 439S, 497S, and 574S) with no tag or any other genetic modification to the strain.

Double mutation strains that with *cdc15-2* were first transformed with PCR fragment to tag NET1 with 3xFlag then crossed to either WT or net1-PS13M and select spores with proper markers on different selecting medium.

Growth assay

For plating assays, strains were grown overnight in YP-2% dextrose and diluted to an $O.D._{600}$ of 0.25 in YP-2% dextrose. Cultures were grown at permissive temperature for strains with MEN mutants and 30 °C the rest of the strains for about 2 doubling cycles to reach stationary growth. Dilute cultures to an $O.D._{600}$ of 0.3 in water and perform a serial five-fold dilutions in water and were spotted onto YPD plates. Plates were incubated at a variety of temperatures as indicated in the text for 2–3 days before the photos were taken.

Morphology observation with microscope

Overnight yeast cultures were diluted in YPD to 0.25 $O.D._{600}$ and grow for 2-3

generation at indicated temperature. Cells are fixed with formaldehyde (HCHO) at a final concentration of 4% for 15 minutes at the original growth condition with constant shaking. Cells were collected by centrifugation and wash with 500 µl of Wash Buffer (1% β-mercaptoethanol in 1x PBS). After the wash, cells were digested with 100 µl of lyticase solution to make spheroblast at 30°C. Cells condition were checked under microscope before proceed further staining. Spheroplastes were diluted with 10X volume PBS and spot 30 µl of cell suspension to polyethyleneimine (Sigma P-3143) coated slides and leave in a moisture chamber for 30 min at RT. Slides were then washed with 1xPBS and fixed with MeOH and Acetone. After fixation, slides were washed with 0.1% Triton X-100 and blocked with blocking buffer [2% BSA, 0.1% Tween20 in DPBS] and labeled with DAPI. Images of cells were collected on a Zeiss LSM510 META inverted confocal microscope with 2-photon laser. Cell images were performed using ImageJ software. More than 100 cells are counted for budding index quantification.

Fluorescence Activated Cell Sorting (FACS)

The 0.25 $O.D._{600}$ diluted wild-type and phosphosite mutants from overnight culture in YP-2% dextrose were grown for about 1 doubling cycle. Cells were treated with 0.3 µg/ml of alpha-factor for 2.5 hours at permissive temperature. For cells treated with nacodazole at either permissive or restrictive temperature were harvested after 2.5 hours of treatment without going through time course. Cells that were going to test under restrictive temperature, we treated cells with alpha factor for 2 hours at permissive temperature then switched to restrictive temperature with additional added alpha factor for another 2 hours.

Alpha factor was released by washing cells through 0.2 μm filter with pre-warmed YP-2% dextrose. For each time point, we collected 1 $O.D._{600}$ unit of cells at each time points as indicated. Collect cells by spinning down with 8000 rpm at room temperature. Cells were fixed by resuspending cell pellets with 300 μl of ice-cold water and immediately add 700 μl of -20 °C ethanol on vortex. Cells were left in 70% ethanol for at least 3 hours at 4 °C. Spin down the fixed cells at top speed for 5 minutes at 4 °C, discard the supernatant, and wash once with 1 ml of 50 mM, pH 7.4 sodium citrate. RNA was removed by treating cells with 4 mg/ ml of RNAse in 50 mM sodium citrate overnight at 37 °C. RNAse was removed by adding 50 μl of 20 mg/ml of Proteinase K and incubated for another 2.5 hours at 37 °C. After RNAse and Proteinase K treatment, cells were washed once with 50 mM sodium citrate buffer. DNA was stained with 1 ml of 16 μg/ml of propidium iodide with brief vortex and incubated for at least 30 min at room temperature in dark. Cell clumpy or aggregates was resuspended and separated by brief sonication for 10sec at settings of 30% with 1 sec On and 1 sec Off right before analyzing by cytometer.

Cell extract preparation and Western Blotting

The growth and synchronize condition was performed as previously described in method for FACS. 8 $O.D._{600}$ units of cells was collected at indicated time points, washed with 2 ml of ice cold sodium azide buffer, and frozen in liquid N_2. Cell pellets were resuspended in 300 μl of 1x SDS loading buffer [70 μl of 100 mM Tris-HCl (pH 7.5), 20% glycerol, 4% SDS, 200 mM DTT, 1% of β- mercaptoethanol]. Cells were first boiled on dry base for 3 minutes with 100 μl of acid-washed glass beads (500 μm). Then, we lyses

the cells by vortexing on the Bio 101 multi-bead vortexer with top speed for 90 sec followed by boiled on dry base for another 5 minutes. After 1-minute spin at top speed, 10 µl lysates were fractionated on a 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane in wet chamber for 2 hours. Western blot analysis was performed with the primary antibodies at the indicated dilutions: polyclonal anti-Net1 (Santa cruz sc-27758, 1:500), monoclonal anti-Flag (Sigma F1804, 1:1000), polyclonal anti-Clb2 (Santa cruz sc-9071, 1:1000), polyclonal anti-Sic1 (Santa cruz sc-50441, 1:1000), monoclonal anti-Tubulin (Santa cruz sc-53030, 1:3000), polyclonal anti-Cdc28 (Santa cruz sc-6709, 1:2000).

Results

net1 phosphosite mutants showed temperature sensitive phenotypes and abnormal terminal morphologies

Net1 antagonizes Cdc14 by restricting its localization to the nucleolus and inhibiting its activity. When Net1 is phosphorylated by the FEAR network in early anaphase, it loses its binding to Cdc14 and thus releases Cdc14 into the nucleus. At the end of mitosis, Net1 is heavily phosphorylated and Cdc14 is completely released into both nucleus and cytoplasm. However, the mechanism is still unclear. Our previous efforts identified Net1 phosphosite mutations that greatly reduced Net1 phosphorylation by Dbf2-Mob1 in vitro. We next sought to study these mutations in vivo to see if they have an effect on exit from mitosis.

We used gene replacement technology to integrate Net1 phosphosite mutants (*net1-PS11M* and *net1-PS13M*) at the natural chromosomal locus. To evaluate the effect of these mutations on growth, cells were spotted at five-fold serial dilutions on YP-2% dextrose and grown for 2-3 days at different temperatures. At 25 °C or 30 °C, WT cells and the phosphosite mutants grew equivalently. However, *net1* phosphosite mutants exhibited growth defects at 16 °C and 38 °C whereas WT cells grew normally (Figure III-1).

In addition to their growth defect, MEN mutants at the non-permissive temperature also show abnormal morphology. We therefore sought to test whether *net1* phosphosite mutants also exhibited abnormal morphology. Cells incubated under either permissive (30 °C) or restrictive (16 °C and 38 °C) conditions for more than 3 generations were fixed with formaldehyde and stained with DAPI to indicate the position of the nucleus. At the permissive temperature, WT cells and phosphosite mutants exhibited similar morphology, although a small portion of the *net1* mutants exhibited slightly elongated buds. Under the restrictive temperature the morphology of WT cells was normal, whereas the phosphosite mutants exhibited formation of chains of cells that failed to separate. In addition, a fraction of the phosphosite mutant cells exhibited elongated, rod-shaped buds (Figure III-2a). The unusual morphology of the phosphosite mutant cells suggested that mitosis and/or cytokinesis was defective when Net1 could not be phorphorylated by Dbf2-Mob1.

net1 phosphosite mutant cells were synthetic-lethal with *cdc15-2*

If phosphorylation of Net1 contributes to the role of the MEN in promoting exit from mitosis, one predicts that the phosphosite mutants would display synthetic lethality

with MEN mutants. To address this hypothesis, we performed a genetic interaction assay by incorporating *net1-PS13M* into *cdc15* strains. The MEN mutant, *cdc15-2* (the $Ala^{206}\rightarrow Asp^{206}$ mutant) that arrest in late mitosis at 38 °C were selected. Single and double mutant cells were spotted in five-fold serial dilutions on YP-2% dextrose, and grown for 2-3 days at different temperatures. We found that the *net1-PS13M cdc15-2* double mutant displayed synthetic-lethality. Double mutant cells showed less tolerance to the intermediate temperature of 28 °C than cells with either single mutation alone (Figure III-3). Taken together, these observations are consistent with the idea that MEN-dependent phosphorylation of Net1 regulates the exit from mitosis.

net1 phosphosite mutants cells were defective in exit from mitosis

Net1 sequesters Cdc14 in the nucleolus, and this antagonistic function of Net1 is counteracted by the MEN. Given our observation that the terminal MEN signaling component Dbf2–Mob1 directly phosphorylates Net1 in vitro, we hypothesized that this phosphorylation might disrupt Cdc14 binding, thereby enabling Cdc14 to escape to the nucleus and cytoplasm, where it promotes the exit from mitosis. If this is the case, a mutant of Net1 that cannot be phosphorylated should exhibit defects in both the release of Cdc14 from the nucleolus and the exit from mitosis.

Our previous data suggested that at both low and high temperatures, *net1* phosphosite mutants exhibited abnormal morphology similar to what is observed in cells with MEN mutations. This suggests that the *net1* phosphosite mutant cells may have defects in exit from mitosis. To evaluate this idea, we performed Fluorescence Activated

Cell Sorting (FACS) to measure chromosomal DNA in WT and phosphosite mutant cells. We arrested cells in G1 phase of the cell cycle with alpha-factor at the permissive or restrictive temperature. Then we removed alpha-factor to allow resumption of cell cycle progression and collected cells at 30 minute intervals for analysis by FACS. Seventy-five minutes after release, alpha factor was added back to trap in the subsequent G1 phase any cells that had successfully divided.

At 30°C, WT, *net1-PS11M*, and *net1-PS13M* cells showed similar DNA distribution patterns throughout the time course. At restrictive temperature (38°C), WT cells completed replication by 60' after release from alpha-factor arrest and had largely returned to the subsequent G1 phase by 90'. On the other hand, *net* phosphosite mutant cells showed similar kinetics of DNA replication but stayed at 2N and failed to divide for at least 180'. This indicates that phosphorylation of Net1 by Dbf2-Mob1 was crucial for cells to complete mitosis and cell division (Figure. III-4d-f).

To further understand the cell cycle regulation in the phosphosite mutant cells, we used western blot assay. Two proteins, Sic1 and Clb2, are normally used as indicators for cell cycle stages. Clb2 is a B-type cyclin involved in regulating cells proceeding through the G2 and M phases of the cell cycle (Enserink and Kolodner, 2010). Clb2 is expressed during the G2-M phase and activates Cdc28 to promote the transition from G2 to M. Clb2 is ubiquitinated by APC^{cdh1} and degraded by the proteasome, which allows cells to exit M phase and enter into G1 phase. Failure to degrade Clb2 leads to cell cycle arrest at mitosis exit (Surana et al., 1993). Taken together, Clb2 is a marker protein for indicating the G2-M stages of the cell cycle. By contrast, Sic1 is usually used for indicating G1 phase. Sic1 is a cyclin-dependent kinase inhibitor specific for the B-type cyclin-CDK complex and

regulates the cell cycle at the G1 to S phase transition. Sic1 is highly expressed during late mitosis and the G1 phase, and then quickly degraded by the combined actions of SCF ubiquitin ligase and the proteasome when cell enters into S phase.

For the western blot assay, we grew WT and phosphosite mutant cells at permissive or restrictive temperatures, and collected cells at different time points after release from the alpha factor arrest (alpha factor was added back after 90' to trap in G1 phase cells that completed mitosis). Cell lysates were fractionated by SDS-PAGE and immunoblotted to detect Sic1 and Clb2 protein levels in each sample. Cdc28 was monitored as the loading control. WT and phosphosite mutant cells showed similar patterns for both Sic1 and Clb2 at 30°C. As expected, Sic1 was expressed at time 0 (G1) and disappeared at later time points, while cells started to accumulate Clb2. Clb2 levels declined and Sic1 reaccumulated at 120', as cells completed mitosis and were arrested in the subsequent G1 phase. On the other hand, WT and phosphosite mutant cells showed different Sic1 and Clb2 expression patterns at the restrictive temperature. WT cells showed similar patterns for both Sic1 and Clb2 at 30°C and 38 °C, except that exit from mitosis occurred one time point earlier at the high temperature. By contrast, Clb2 levels never declined and Sic1 levels never exhibited a second peak in *net1-PS13M* cells grown at 38 °C This indicates that phosphosite mutant cells exhibited defects in exit from mitosis at 38 °C (Figure. III-4g).

Discussion

In this work, we showed low and high temperature-sensitive phenotypes of the cells with Dbf2-Mob1 phosphorylation site mutations in Net1. We also showed that *net1-PS11M* and *net1-PS13M* cells exhibit abnormal morphology, which is similar to the morphology of the MEN kinase mutant. Double mutants with *cdc15-2* and *net1-PS13M* show synthetic-lethal phenotype. Furthermore, we showed that phosphosite mutant cells exhibit defects in mitotic exit under restrictive temperature with both western blot assay and FACS analysis. This suggests that the MEN is involved in regulating Net1 phosphorylation.

Permissive and restrictive temperature experiments allow us to analyze abnormal morphology, and these assays showed that cells with phosphosite mutant Net1 are defective and exit mitosis abnormally. Moreover, some cells showed defects in exit from cytokinesis. The majority of Net1 phosphosite mutant cells were in budded form, but a portion of the cells exhibited chain-like undivided budded form at both 16 °C and 38 °C (Figure III-2a and -2b). The large percentage of the cells arrested at the budded stage indicated that cells exhibited defects in mitotic exit. However, the multibudded/ chain-like morphology indicated that cells suffer from defects in premature mitotic exit before closing of the bud neck. On the other hand, we also found a very small percentage these populations of cells were unbudded. This suggested that some cells are able to leak through the mitosis or the cytokinesis defects, which matches the growth assay results that colonies showed up in the first 5 fold diluted spot. The chain-like morphology has been reported in the *tem1-1*, *cdc15-2*, *dbf2-2*, and *cdc14-1* cells (Grandin et al., 1998; Javier et al., 2004). Together with our morphology data, it further indicated the mechanism for regulating the exit from mitosis is through phosphorylation of Net1.

To understand the relationship between the MEN and Net1, we performed the genetic interaction assay. We found that double mutation with *cdc15-2* and *net1-PS13M* showed a synthetic-lethal phenotype, which indicated that Cdc15 and Net1 are in the same pathway. It has been shown that the kinase activity and the cellular location of Dbf2 are both regulated by Cdc15. In the *cdc15-2* cells, Dbf2 not only fails to be activated but it also fails to localize at the bud neck and spindle pole body. Dbf2 travels on the spindle pole body and seems to meet with Net1 when the spindle pole body encounters with the daughter bound nucleolus (Visintin and Amon, 2001; Stoepel et al., 2005).

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Figure Legends

Figure III-1. Net1 phosphosite mutants were low and high temperature-sensitive

Five-fold serial dilution of cells were plated onto YP-2% dextrose. WT indicated wild-type cells. *net1* phosphosite mutant cells are mutated in its original chromosome DNA locus by DNA recombination. *net1-PS11M* (11M) cells exhibited one more site, S439→A439, than *PS10M* used in *in vitro* kinase reaction, while *net1-PS13M* (13M) cells are the same as the *PS13M* in *in vitro* kinase reaction. Temperatures used to test the growth phenotype are as indicated.

Figure III-2. Microsopic terminal morphology of *net1* phosphosite mutant cells

(a) *net1* phosphosite mutant cells showed abnormal morphology under restrictive temperature. *Net1-WT*, *PS11M*, and *PS13M* cells grew in YP-2% dextrose under indicated

temperature for more than 3 doubling times were formaldehyde fixed and stained with DAPI to indicate DNA localization. DAPI staining is presented in bright white dot.

(b) Quantification of the DAPI staining results in each strains under 16 °C, 30 °C, and 38 °C. Cells were grouped with the form of the budding stage and labeled as "unbudded", "budded", and "multibudded". A total number of 100 cells were calculated.

Figure III-3. *net1-13M* phosphosite mutant was synthetic lethal with MEN mutants

net1-PS13M is synthetic-lethal with cdc15-2. 0.3 $O.D._{600}$ unit of cells of *NET1-WT*, *cdc15-2*, *net1-PS13M*, and *cdc15-2 net1-PS13M* double mutation background were diluted with five-fold serial dilution and plotted on YP-2% dextrose from left to right. Plates were incubated at the indicated temperature for 2-3 days before the picture was taken.

Figure III-4. Cells with Dbf2-Mob1 phosphosite mutant cells were defective in exit from mitosis

Wild-type (WT) and phosphosite mutated (*PS11M* and *PS13M*) Net1 were harvested at indicated time points after released from alpha factor arrest.

(a-c) WT and phosphosite mutant cells showed similar cell cycle progression under permissive temperature. 1 $O.D._{600}$ unit of *WT*, *PS11M*, and *PS13M* cells were grown at 30 °C and collected by adding ethanol to a final 70% concentration to each time points, as indicated. Time "0" was the time right before cells released from alpha factor treatment. 0.2 ug/ml of alpha factor was added back at 90 minutes time point. Time points were labeled as

minutes after released from alpha factor. Cell types were indicated at the bottom and the chromosome copy number was labeled at the top with arrow to indicate the specific peak.

(d-f) Phosphosite muatant cells were defective in exit from mitosis under restrictive temperature. 1 $O.D._{600}$ unit of *WT*, *PS11M*, and *PS13M* cells were grown at 38 °C and collected by adding ethanol to a final 70% concentration to each time points, as indicated. Time "0" was the time right before cells released from alpha factor treatment. 0.2 ug/ml of alpha factor was added back at 80 minutes time point. Time points were labeled as minutes after released from alpha factor. Cell types were indicated at the bottom and the chromosome copy number was labeled at the top with arrow to indicate the specific peak.

(g) Phosphosite mutant cells exhibited defects of Clb2 degradation and the re-accumulation of Sic1 under restrictive temperature. 8 $O.D._{600}$ unit of *WT* and *PS13M* cells were grown at 30 °C or 38 °C after 2-3 hours of alpha factor arrest. Time "0" was the time right before cells released from alpha factor treatment on filter. Cells were collected followed the time point as indicated. The time points labeled with blue color were the time we added 0.2 ug/ml of alpha factor back to the culture to prevent cells entering 2^{nd} round of cell cycle. Temperature and the strain type were labeled at the top of each blot. Protein extract from each time point were analyzed by 10% SDS-PAGE and blotted with polyclonal anti-Net1, polyclonal anti-Clb, polyclonal anti-Sic1, and monoclonal anti-Cdc28.

Figure III-1. *net1* phosphosite mutant cells were low and high temperature-sensitive

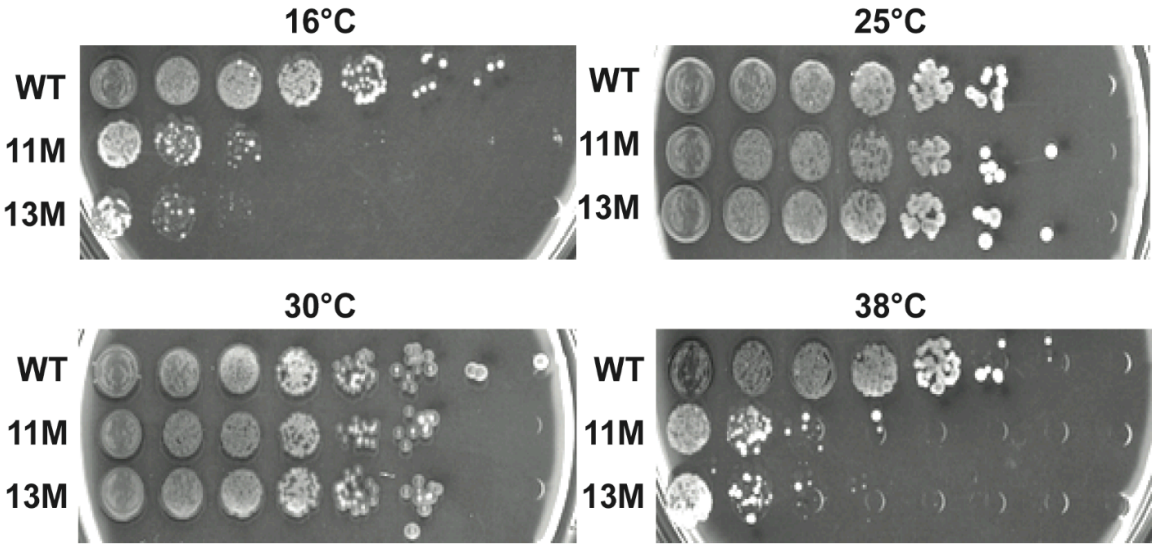
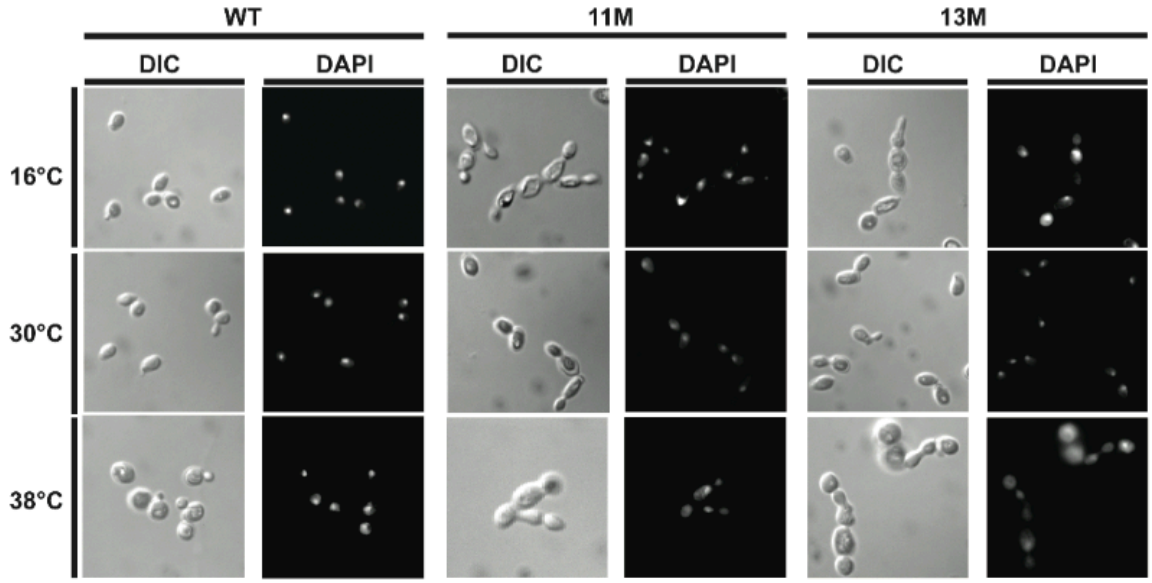


Figure III-2. Microsopic terminal morphology of *net1* phosphosite mutant cells

(a)



(b)

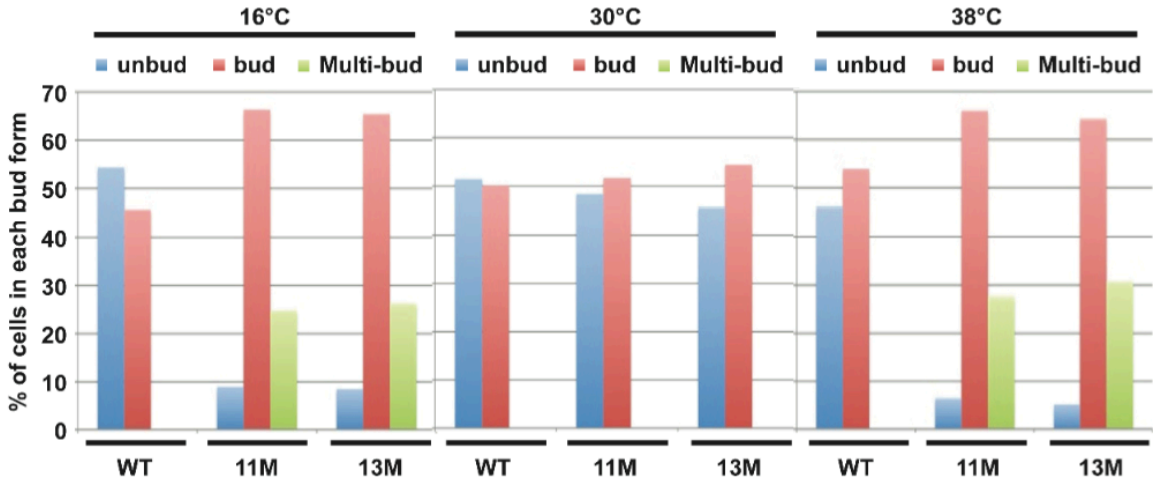


Figure III-3. *net1-13M* phosphosite mutant was synthetic lethal with *cdc15-2* mutant

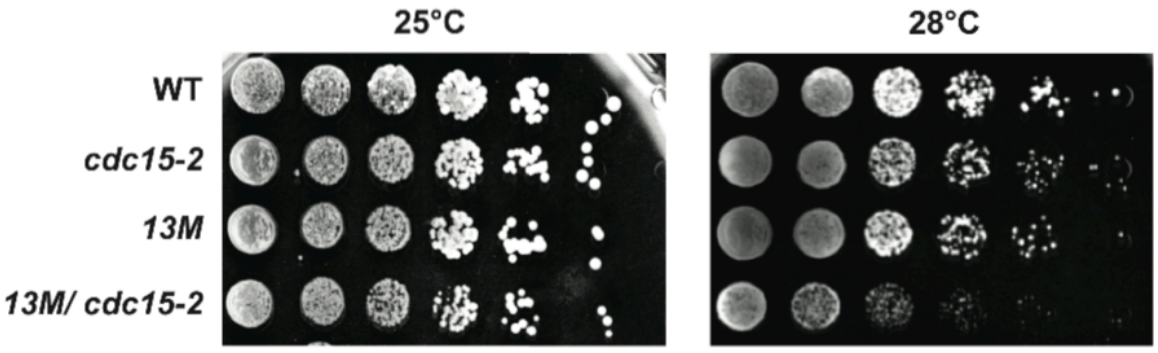
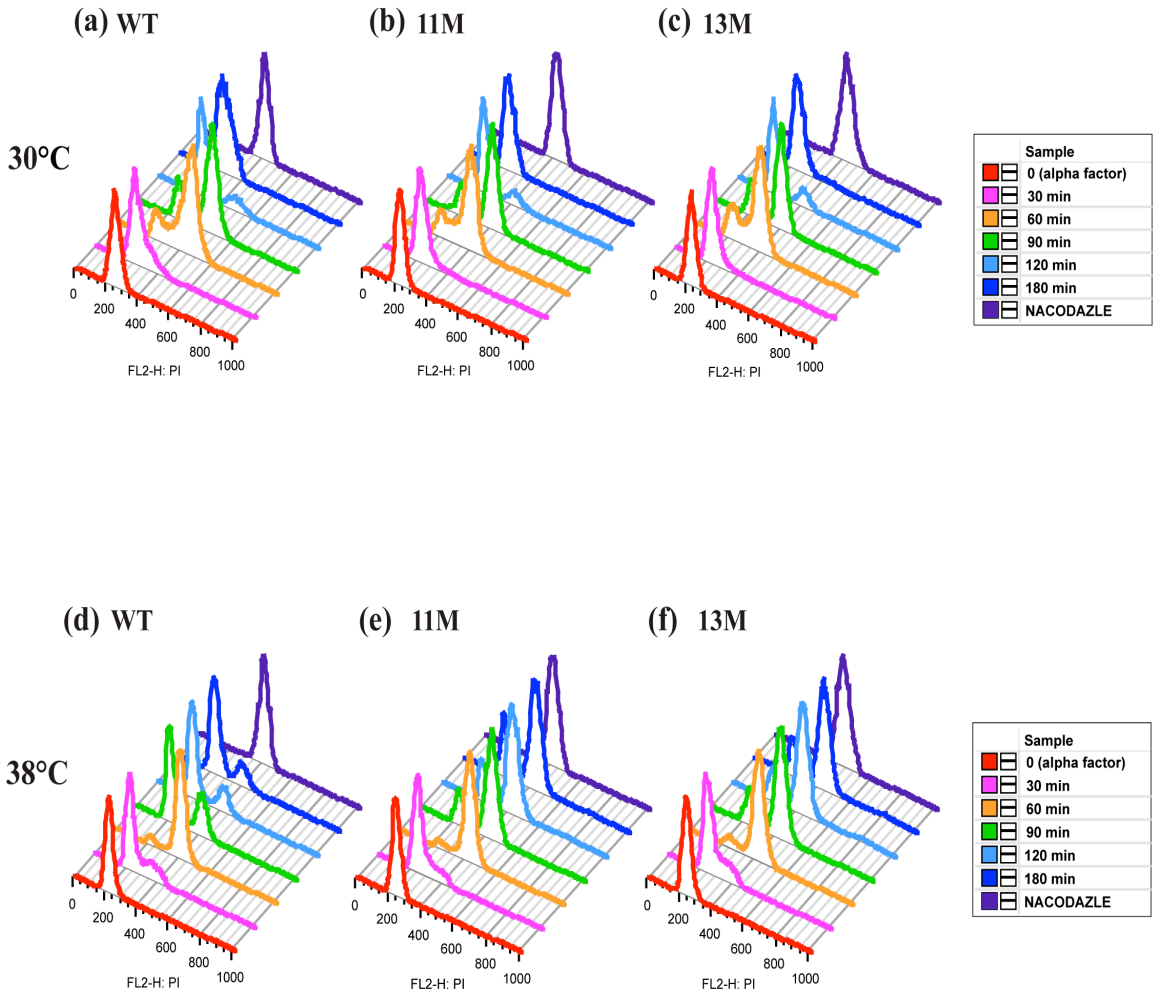
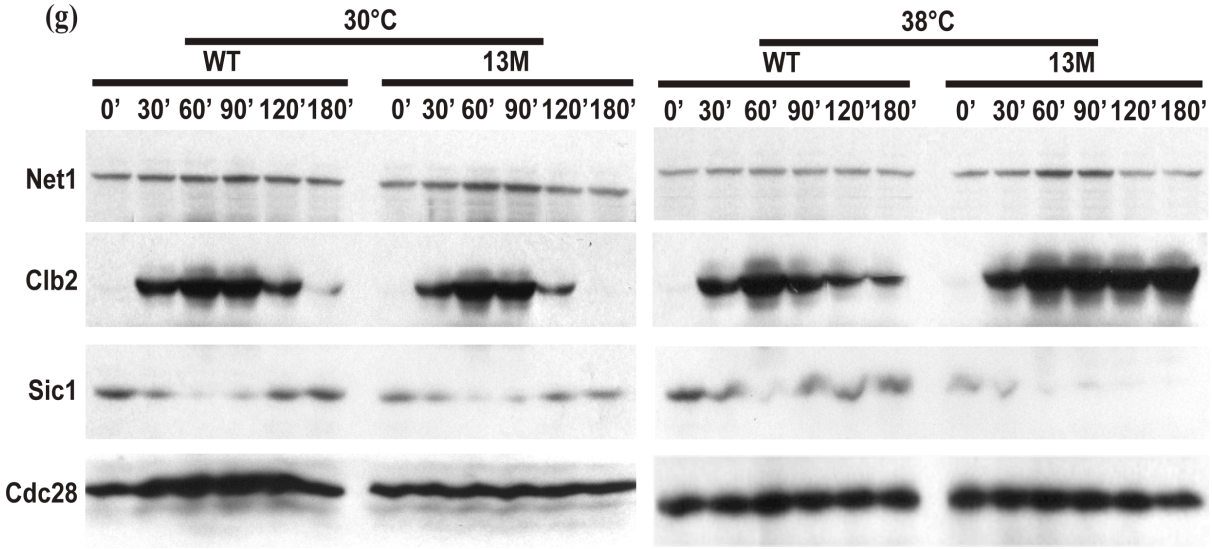


Figure III-4. Cells with Dbf2-Mob1 phosphosite mutant Net1 was defective in exit from mitosis




(g)
30°C
38°C
WT
13M
WT
13M
0' 30' 60' 90' 120' 180'
0' 30' 60' 90' 120' 180'
0' 30' 60' 90' 120' 180'
0' 30' 60' 90' 120' 180'
Net1
Clb2
Sic1
Cdc28

Chapter IV

net1 Phosphosite Mutant Cells Were Defective in Releasing Cdc14

Introduction

In *Saccharomyces cerevisiae*, the localization of Cdc14 is tightly regulated throughout cell cycle, and Net1 is responsible for the regulation of the activity and the location of Cdc14. Net1 binds to Cdc14 and sequesters it in the nucleolus during the G1 phase, S phase, and metaphase. It has been shown that overexpression of Net1 causes the distribution of Net1 protein throughout the cell. In such a cell, Cdc14 is no longer sequestered in the nucleolus but present throughout the cell. This indicates that Net1 controls the cellular localization of Cdc14. Mislocalized Net1 leads to mislocalization of Cdc14 (Visintin, et al., 1999, Shou et al., 1999).

In addition, Net1 inhibits the activity of Cdc14 when they form a complex *in vitro* (Traverso et al., 2001, Shou et al., 1999). It has been shown that Net1 binds to the active site of Cdc14 and inhibits Cdc14 phosphotase activity *in vitro* (Traverso et al., 2001). This mechanism also been reported in *in vivo* system. Deletion of Net1 leads to a delay of bud formation, elongation of bud morphology, and delay of mitotic spindle formation. Cells in the Net1 deletion background escaped from nocodazle arrest and prematurely exited from mitosis. These phenotypes are similar to those phenotypes caused by overexpression of Cdc14, although Net1 deletion is not lethal but overexpression of Cdc14 is. These results suggest that Net1 is a negative regulator of Cdc14 activity *in vivo* (Visintin, et al., 1999). This idea was tested by overexpression of Net1, which is lethal and leads to cellular arrest at telophase with high Clb2 and low Sic1 levels, similar to the phenotype with Cdc14 deleted. Furthermore, deletion of Net1 rescues the temperature-sensitive lethality of tem1-

1, cdc15-2, and dbf2-2. This implies that promoting the dissociation of Cdc14 from Net1 is likely to be a key for mitotic exit pathway.

Cdc14 is initially released from the nucleolus in early anaphase when Net1 is phosphorylated by FEAR network. However, the FEAR network cannot maintain the release state of Cdc14 because Cdc14 moves back to nucleolus when the MEN is defective. The way to regulate the release of Cdc14 and sustain the cytoplasmic Cdc14 in the late anaphase is not clear.

Recently, a mechanism for sustaining cytoplasmic Cdc14 was discovered. Dbf2-Mob1 inhibits the function of the nuclear localization signal (NLS) of Cdc14 through phosphorylation. This leads to the blocking of the reuptake of the cytoplasmic Cdc14 back to the nucleus. When the Dbf2-Mob1 phosphorylation sites in the NLS are mutated (Cdc14-PS1,2 A), cells cannot sustain the cytoplamic release of Cdc14 in the late anaphase (Mohl et al., 2009). This mechanism explains why Cdc14 is phosphorylated, and it identifies which kinase is in charge of Cdc14 phosphorylation at the end of mitosis. However, it is still unclear what phosphorylates Net1 and how Net1 phosphorylation maintains the release of Cdc14 from nucleolus at the end of mitosis. In our previous chapters, we demonstrated that Dbf2-Mob1 is the kinase that regulates the mitotic exit and we proved that Dbf2-Mob1 is responsible for Net1 phoshorylation *in vitro* and *in vivo* by genetic interaction assays. It would be interesting to test whether this process is the answer for late anaphase Cdc14 release.

Methods

Growth assay

This method is described in the Chap III.

Cell growth and synchronization procedure

Cells were grown overnight in YP-2% Dextrose and diluted into 0.25 $O.D._{600}$ YP-2% Dextrose. Diluted cells were grown at permissive temperature for about 1 doubling cycle then synchronized in G1 phase with alpha-factor added at 10 ug/ml for *BAR1* Cells and 0.3 ug/ml for *bar1*Δ cells for at least 2.5 hours at 30 °C. The arrested condition was judged when greater than 90% of cells displayed the elongated "shmoo" phenotype under microscope. For cells that were going to test the phenotype under restrictive temperature, cells were moved to 38 °C for another 2 hours added with 0.2 ug/ml of alpha factor after the initial 2 hours treatment with alpha factor. Cells were released from alpha factor arrest condition by filtered through a 0.2 μm filter jar by washed and resuspend with 50 ml of YP-2% dextrose twice, then resuspended in pre-warmed YP-2% dextrose. "Time 0" was collected before the release procedure. A 0.2 ug/ ml of alpha factor was added into cultures at either 90 minutes or 75 minutes after the first alpha factor release for cultures at the permissive or restrictive temperature, respectively. For western blot boiling extract experiment, 8 $O.D._{600}$ unit of cells was collected at each time point as indicated. For Fluorescence Activated cell sorting experiment, 1 $O.D._{600}$ unit of cells was collected at each time point.

Immunofluorescent staining

The growth and synchronize condition was performed as previously described in method for FACS. 1 $O.D._{600}$ units of cells was collected at indicated time points. Cells are fixed with formaldehyde (HCHO) at a final concentration of 4% for 15 minutes at the original growth condition with constant shaking. Cells were collected by centrifugation and wash with 500 µl of Wash Buffer (1% β-mercaptoethanol in 1x PBS). After the wash, cells were digested with 100 µl of lyticase solution to make spheroblast at 30°C. Cells condition were checked under microscope before proceed further staining. Spheroplastes were diluted with 10X volume PBS and spot 30 µl of cell suspension to polyethyleneimine (Sigma P-3143) coated slides and leave in a moisture chamber for 30 min at RT. Slides were then washed with 1xPBS and fixed with MeOH and Acetone. After fixation, slides were washed with 0.1% Triton X-100 and blocked with blocking buffer [2% BSA, 0.1% Tween20 in DPBS] and labeled with primary antibodies. Goat polyclonal anti-Cdc14 antibody was used to detect cellular localization of Cdc14. Mouse anti-Nop1 monoclonal antibody and rat anti-tubulin monoclonal antibody YOL1/34 were used to detect nucleolus and cell cycle stages, correspondingly. Secondary antibodies conjugated with Alexa fluor® 488, Alexa fluor® 633, and Alexa fluor® 546 were used to detect Cdc14, Nop1, and Tubulin, respectively. Images of cells were taken on a Zeiss LSM510 META inverted confocal microscope with 2-photon laser. Cell images were processed using ImageJ software. Tubulin, Nop1 and DAPI are presented in green, red, and blue, respectively. Spindle length measurements were also performed using ImageJ software. More than 100 cells are counted for Cdc14 localization profile.

Immunoprecipitation and Dbf2-Mob1 release/ kinase assay

100 $O.D._{600}$ units of a log phase cell culture were harvested to prepare extracts for immunoprecipitation. Cells were washed once with 2 ml of ice-cold 1x PBS plus 5 mM NEM and frozen in liquid N_2. Cell pellets were resuspended in 600 µl of lysis buffer [25 mM HEPES/KOH (pH7.5), 300 mM NaCl, 2 mM DTT, 0.5% Triton X-100, 1 mM EDTA, 2 mM EGTA, freshly add 1x Protease Inhibitor Cocktail (Roche), 5 mM NEM, and 1 mM AEBSF] and supplemented with 400 µl volume of acidwashed glass beads (500 µm). Samples were lysed using Bio 101 multi-beads vortexer at setting 6.5 (speed) and 60 sec (time) for three times with 3 minutes on ice in between. Lysates was collected and transfered to another eppendorf tube. The glass beads was washed once with 600 µl of lysis buffer without sodium chloride and Triton X-100 and combined with previous lysates into the same tube. Combined lysates were then centrifuged for 5 minutes at top speed and the supernatant was collected. At the same time, wash acid-washed anti-Flag M2 affinity gel with wash buffer [25 mM HEPES/KOH (pH7.5), 150 mM NaCl, 2 mM DTT, 0.25% Triton X-100, 1 mM EDTA, 2 mM EGTA, freshly add 1x Protease Inhibitor Cocktail (Roche), 5 mM NEM, and 1 mM AEBSF] for three times before mixed with cell extracts. Clarified extract was incubated with 250 µl of 50% slurry of equilibrated affinity gel on rotator for 1.5 hours at 4 °C. Affinity gel were collected and washed five times with wash buffer and divided to 25 µl beads for each reaction. When affinity gel was incubated with cell lysates, we started to assemble the preactivated Dbf2-Mob1 in vitro. We incubate purified Dbf2-Mob1 with purified Cdc15 in Cdc15 kinase buffer [50 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM beta-glycerophosphate, 1 mM DTT, and 20 µM ATP] for 20

minutes at room temperature. Then, mixed with equal total volume of Dbf2 Kinase buffer [50 mM Tris, pH 7.4, 60 mM potassium acetate, 10 mM magnesium chloride, 1mM DTT, and 10 μM ATP]. We used 0 μg, 0.025 μg, 0.05 μg, 0.1 μg, and 0.2 μg of preactivated Dbf2-Mob1 to treat affinity gel with NET1/Cdc14 complex on it in total 30 μl volume for each reaction. After 30 minutes incubation on rotator, we add 70 μl of HEBD buffer [20 mM HEPES (pH 7.2), 150 mM NaCl + 2 mM EDTA + 0.1 mg/ml BSA + 1 mM DTT] and spin the affinity gel down to collect the supernatant. We washed affinity gel with another 100 μl of HEBD and spin down to collect supernatant. Proteins in the combined supernatant was precipitated with 15% TCA on ice for 30 min and pellets was collected by spin down at top speed for 10 minutes at 4 °C. Pellets was washed twice with ice-cold acetone and resuspend with 40 μl of 1X SDS buffer. This is the sample we called "Sup". The affinity beads was mixed with 50 μl of 1X SDS buffer and labeled with "Beads". Samples were boiled for 5 minutes on dry base and fractionated on a 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane in wet chamber for 2 hours. Western blot analysis was performed with the primary antibodies at the indicated dilutions: polyclonal anti-Cdc14 (Santa cruz sc-12044, 1:500), monoclonal anti-Flag (Sigma F1804, 1:1000).

Results

The temperature-sensitive phenotype of *net1-PS13M* cells was rescued by *CDC14^{P116L}*

Given that (i) Dbf2 function is required for release of Cdc14 from the nucleolus in anaphase, which in turn is required for exit from mitosis, (ii) Dbf2–Mob1 directly

phosphorylates Net1, and (iii) a mutant of Net1 lacking Dbf2 phosphorylation sites was defective in exit from mitosis, an attractive hypothesis that emerges is that phosphorylation of Net1 by Dbf2–Mob1 promotes release of Cdc14. We therefore sought to determine whether the Net1 phophosite mutant has an altered ability to sequester Cdc14 in the nucleolus.

To address this hypothesis, we first looked for genetic interaction between *net1-PS13M* and $CDC14^{TAB6}$. The dominant *TAB6* allele produces a Cdc14 protein with a $Pro^{116} \rightarrow Leu^{116}$ point mutation the reduces the binding affinity for Net1 (Shou et al., 1999). Because of this, $CDC14^{TAB6}$ serves as a dominant bypass suppressor of the late anaphase arrest caused by inactivation of the MEN. If *net1-PS13M* fails to exit mitosis efficiently due to a defect in releasing Cdc14, we predicted that it too would be suppressed by $CDC14^{TAB6}$.

To perform genetic interaction assays, we spotted five-fold serial dilutions of single and double mutant cells on on YP-2% dextrose, and grew for 2-3 days under different temperatures. We found that at the permissive temperature, all the cells grew normally. At the restrictive temperature, the *net1-PS13M* cells exhibited poor growth whereas the *net1-PS13M* $CDC14^{TAB6}$ double mutant cells grew far better (Figure III-4a). The double mutants essentially grew as well as the $CDC14^{TAB6}$ cells. Thus, $CDC14^{TAB6}$ potently suppressed the growth defect of *net1-PS13M*.

Defective Cdc14 release in the *net1* phosphosite mutant cells

To further test our hypothesis that phosphorylation of Net1 by Dbf2–Mob1

mediates release of Cdc14 from the nucleolus, we performed immunofluorescence assays. We arrested WT and *net1-PS13M* cells at G1 stage with alpha-factor and collected time points after removing alpha-factor. Cells were fixed with formaldehyde and then permeabilized on pre-coated microscope slides with methanol/ acetone. The cellular localization of Cdc14 was detected by staining with polyclonal anti-Cdc14. Cell cycle stages were evaluated by tubulin staining and the nucleolar location was shown by Nop1 staining.

The length of the microtubule spindle is indicative of different cell cycle stages (Winey et al., 2001; Yeh et al., 1995). Tubulin forms a spindle, which then elongates to direct the segregation of chromosomal DNA. In budding yeast, the length of metaphase spindles is 1.5-2 μm. The metaphase spindles elongate during anaphase and reach a length of 10 μm during telophase. Ten to twelve micron spindles are observed at the terminal arrest of MEN mutants.

In WT cells, Cdc14 was sequestered in the nucleolus in the G1 and S phases up through metaphase of mitosis (0-2 μm), and released from the nucleolus in cells with spindles ranging from 4-10 μm in length. This indicates that Cdc14 was released from the nucleolus in early anaphase and remained in the released state throughout anaphase and telophase. On the other hand, Cdc14 in *net1-PS13M* cells had a different releasing pattern. Cdc14 was released from nucleolus to nucleus with spindles 2-4 μm in length in a small percentage of the cells, but it was sequestered back to nucleolus with mitotic spindles 6-10 μm in length with focal staining of Cdc14. This indicated that Cdc14 is partially released in early anaphase but cannot sustain the release stage throughout anaphase and telophase (Figure. IV-2a).

We quantified the localization of Cdc14 in about 100 cells in each spindle length category. We found that when spindle length is 2-6 μm there is partial released Cdc14 in *net1-PS13M* cells and in wild-type cells. However, the percentage of cells with spindles 6-8 μm in length and Cdc14 confined to the nucleolus was greatly increased in the *net1-PS13M* strain compared to wild type. This was further exacerbated in cells with 8-10 μm spindles (Figure. IV-2b). These data are consistent with the hypothesis that phosphorylation of Net1 by Dbf2–Mob1 triggers the release of Cdc14 from the nucleolus during late anaphase.

Phosphorylation of Net1 by Dbf2-Mob1 regulated the release of Cdc14

Our previous data suggested that mitotic exit is regulated through phosphorylation of Net1 by Dbf2-Mob1. The immunofluorescence experiments further proved that this phosphorylation reaction is involved in the regulation of Cdc14 release from the nucleolus in late anaphase. Therefore we sought to test whether the phosphorylation of Net1 by Dbf2-Mob1 can directly facilitate the dissociation of Cdc14 from the immunoprecipitated RENT complex.

To address this question, we performed a release assay, in which Net1-3xFlag and the associated RENT subunits were immunopurified from extracts of wild-type or *net1* phosphosite mutant cells by capturing Net1-3xFlag on anti-Flag M2 affinity beads. Then, we treated these beads with Dbf2-Mob1 plus ATP. If phosphorylation of Net1 by Dbf2-Mob1 can loosen the binding between Net1 and Cdc14, Cdc14 should be released from the beads into the supernatant. With wild type RENT complex, Cdc14 began to appear in the

supernatant when the immobilized complex was treated with 0.025 ug of Dbf2-Mob1 and the amount of released Cdc14 increased progressively as more kinase was added. On the other hand, Dbf2–Mob1 was unable to induce release of Cdc14 from *net1-PS11M*, even when 0.2 ug of Dbf2-Mob1 was used (Figure. IV-3). Taken together, these data suggest that Dbf2-Mob1 kinase is sufficient to release Cdc14 from wild type Net1, but not from the Net1 phosphosite mutant.

Temperature-sensitive MEN mutants are unable to grow at 37°C, and MEN gene knockouts are inviable at normal growth temperatures (e.g. 25°C or 30°C). If phosphorylation of Net1 by Dbf2–Mob1 is the principal means by which the MEN induces exit from mitosis, we would expect that a phosphosite mutant of Net1 would be inviable at all growth temperatures. However, *net1-PS13M* grows normally at 30°C. This suggested that there must be at least one other target for Dbf2–Mob1 that accounts for the essential nature of this protein kinase. One candidate for a 'second' target is Cdc14 itself. In prior work, this lab showed that Dbf2–Mob1 phosphorylates Cdc14 adjacent to a nuclear localization sequence (NLS) in the C-terminal region, and this inactivates the function of the NLS. By this mechanism, Dbf2–Mob1 promotes the redistribution of Cdc14 from the nucleoplasm to the cytoplasm during late anaphase. We speculated that the MEN thereby triggers exit from mitosis by a two-stage mechanism. Dbf2–Mob1 first phosphorylates Net1 to induce release of Cdc14 from the nucleolus to the nucleoplasm. Normally, this Cdc14 could enter the cytoplasm but it immediately returns to the nucleus due to the action of the C-terminal NLS. However, upon phosphorylation of Cdc14 by Dbf2–Mob1, the C-terminal NLS is inactivated, allowing Cdc14 to accumulate in the cytoplasm where it can dephosphorylate and activate its targets such as Cdh1. If this idea is correct and Dbf2–

Mob1 has two substrates that account for its essential nature, mutation of the phosphorylation sites in both Net1 and Cdc14 would be expected to result in synthetic lethality. To test this possibility, we constructed a mutant that combined net1-PS13M and cdc14-PS1,2A (the latter mutant is lacking the phosphorylation sites adjacent to the NLS). Double mutant spores readily germinated upon dissection of tetrads, indicating that the double mutant is not inviable. This was further substantiated by spotting five-fold dilutions of single and double mutants cells on YPD and incubating the plates at 30°C and 38°C (Fig. IV-3).

Discussion

In order to exit from mitosis, it is necessary to phosphorylate both Cdc14 and Net1 (Vinsitin et al., 2003). The mechanism responsible for Cdc14 phosphorylation in late mitosis has been illustrated (Mohl et al., 2009). However, it is unclear how Net1 is phosphorylated at the end of mitosis. In this work, we showed that Dbf2-Mob1 is responsible for phosphorylation of Net1 in late mitosis. This is also the key to promote late anaphase Cdc14 release, leading to mitotic exit. Besides regulating mitotic exit, this mechanism may also be involved in promoting cytokinetic exit. Net1 phosphosite mutant strains show chain-like budding morphology, when cells were grown at both cold and restrictive temperatures (Figure III-2a). This morphology indicates that cells suffer from defects in premature of mitotic exit before closing of the bud neck, and thus cells continue the next round of budding cycle. The chain-like morphology has been reported in tem1-1, cdc15-2, dbf2-2, and cdc14-1 cells (Grandin et al., 1998; Jimenez et al., 1998; Jimenez et

al., 2005). We also found a small portion of cells containing higher amount of chromosome DNA (4N) with FACS technology (data not shown).

One possible reason of why phosphosite mutant cells showed morphology of mitotic exit defect and growth defect is that it failed to release Cdc14. We test this idea with genetic interaction assays using a *Cdc14* mutant (*cdc14*^{P116L}), which has a lower binding affinity to Net1. The *net1-PS13M cdc14*^{P116L} double mutant cells rescued the temperature sensitive phenotype of *net1-PS13M* cells alone, which indicated that net1-PS13M might have defects in Cdc14 release. On the other hand, double mutation of *net1-PS13M* and *cdc14-PS (1,2A)* cells cannot rescue the temperature sensitive phenotype of *net1-PS13M* cells. However, unlike the *cdc15-2*, *net1-13M* double mutant, *net1-PS13M*, *Cdc14-PS (1,2A)* double mutant showed similar growth defect as shown in *net1-PS13M* cells alone. This indicated that the defect caused by *net1-PS13M* is down stream of the *cdc14-PS (1,2A)*. It is also possible that there is another target of Db2-Mob1 that must be mutated in order to cause lethality phenotype.

In our previous data, cells with phosphosite-mutated net1 grew normally, similar to that of WT cells at permissive temperatures. Surprisingly, we found focal localization of Cdc14 in the mutant cells in our immunostaining assay. Similar observations have been reported. Shou et al. showed that Cdc14 localized in nucleolus in *cdc5* mutant cells but the cells showed no obvious growth defects (Shou et al., 2002). Since Cdc5 is responsible for activating the MEN, the reason why cells show focal Cdc14 in the cdc5 mutated cells may be due to the failure of Dbf2-Mob1 kinase activation. It is possible that there is still some baseline release of Cdc14 that escaped our detection by our indirect immunostaining method, and thus this baseline release is sufficient for cell to exit from mitosis under

permissive temperatures. It is also possible that there is another minor pathway to support the basal growth even when majority of Cdc14 is sequestered.

We found the focal nucleolus localization of Cdc14 in the cells with Dbf2-Mob1 phosphosite mutated Net1. We further tested the ability of release Cdc14 from the RENT complex by adding activated Dbf2-Mob1 to the immunoprecipitated RENT complex on the beads. Significantly, we found that about an eight-fold difference in the ability to release Cdc14 between WT and mutant Net1. This result is positively related to the data from the *in vitro* kinase reaction where the phosphorylation level of the phosphosite mutant Net1s are about 80% lower than that of WT. Taken together, it strengthened our hypothesis that Dbf2-Mob1 is in charge of Net1 phosphorylation and promoting the release of Cdc14.

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Figure legends

Figure IV-1. *net1* phosphosite mutant displayed genetic interactions with *cdc14* mutants

(a) *cdc14*^{P116L} mutant rescued temperature-sensitive growth of cells with *net1-PS13M*. 0.3 O.D.$_{600}$ unit of cells of *NET1-WT, cdc14*^{P116L}*, net1-PS13M*, and *cdc14*^{P116L} *net1-PS13M* double mutation background were diluted with five-fold serial dilution and plotted on YP-2% dextrose from left to right. Plates were incubated at the indicated temperature for 2-3 days before the picture was taken.

(b) Double mutation of *net1-PS13M* and *cdc14 (PS1,2A)* cells showed temperature sensitive phenotype as *net-PS13M* cells alone. 0.3 O.D.$_{600}$ unit of cells of *NET1-WT, cdc14 (PS1,2A), net1-PS13M*, and double mutation of *cdc14 (PS1,2A) net1-PS13M* background were diluted with five-fold serial dilution and plotted on YP-2% dextrose plates as described for panel (a).

Figure IV-2. Dbf2-Mob1 phosphorylation site mutant cells were defective in releasing Cdc14 in late anaphase

Wild-type (WT) and phosphosite mutated (PS13M) *net1* cells were harvest at stationary growth and immuntained as described in the method section.

(a) *net1-PS13M* cells shows nucleolus localization phenotype of Cdc14. Strain types were labeled at the right side of each panel. Images were grouped by the spindle lengths as indicated at the left side. Cells were collected and fixed with 4-5% of

formaldehyde and fixed on the slides with Methanol/ Acetone after lysozyme treatment. The cellular localization of Cdc14, Tubulin, and Nop1 were monitored by staining with polyclonal anti-Cdc14, monoclonal anti-Tubulin, and monoclonal anti-Nop1 and shown with green, red, and blue color, respectively.

(b) Quantification of the immunostaining results in each spindle length group. We divided spindle length into 0-2, 2-4, 4-6, 6-8, and 8-10 μm group as labeled at the bottom. " No release " means the Cdc14 signal is completely overlap with the Nop1 signal. "Partial release" means the Cdc14 signal is overlap but also out side of the Nop1 signal. "Full release" means the Cdc14 signal decorate the whole cell and not specifically localized with the Nop1 signal. Data is collected from about 100 cells in each spindle length category.

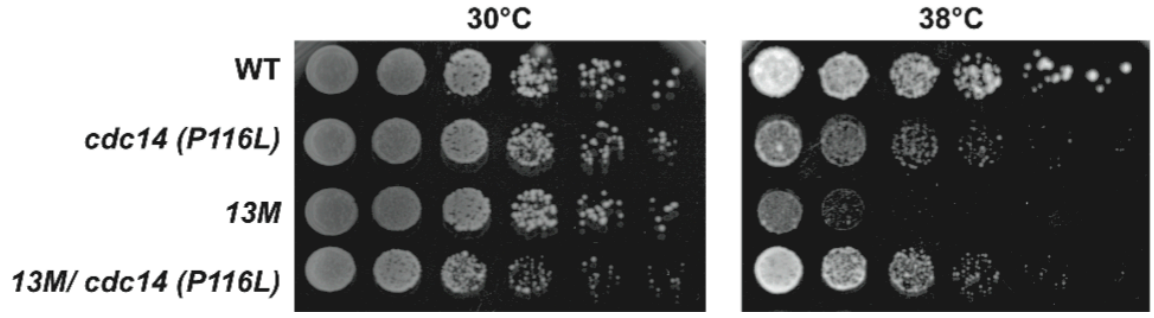
Figure IV-3. Dbf2-Mob1 phosphorylation site mutant cells were defective in releasing Cdc14 in *in vitro*.

WT, *net1-PS11M*, and *net1-PS13M* cells were collected at their stationary growth. Net1 in both strains are tagged with 3xFlag tag so that we can capture the Net1/ Cdc14 complex by anti-Flag beads. Immunoprecipitated protein extracts were incubated with various amount of purified and pre-activated Dbf2-Mob1 as indicated on the top. Strain type is labeled at the left side. Proteins collected from the supernatant were labeled as "Sup", while proteins extracted from the beads by boiling were labeled as "Beads". Extracts from either Sup or Beads were analyzed on the 10% SDS-PAGE and blot for monoclonal anti-Flag for Net1 and polyclonal anti-Cdc14 as indicated at the right side. The rough quantification data comparing the amount of Cdc14 released at the highest Dbf2-

Mob1 used was marked at the bottom of each panel.

Figure IV-1. *net1* phosphosite mutant displayed genetic interactions with cdc14 mutants

(a)



(b)

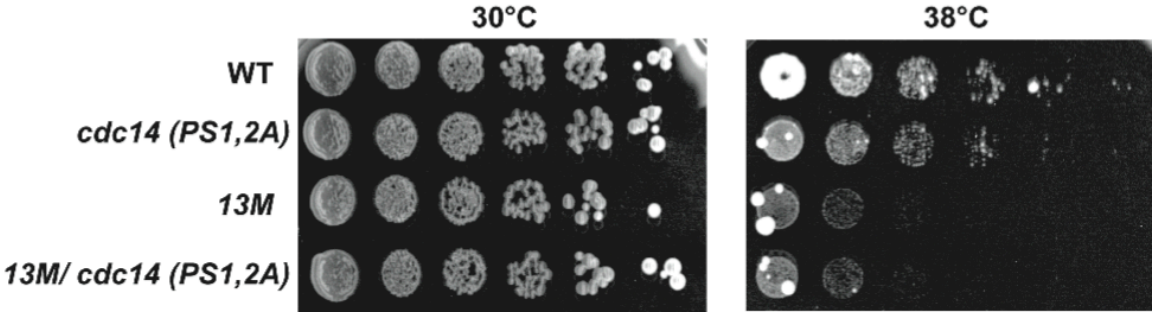
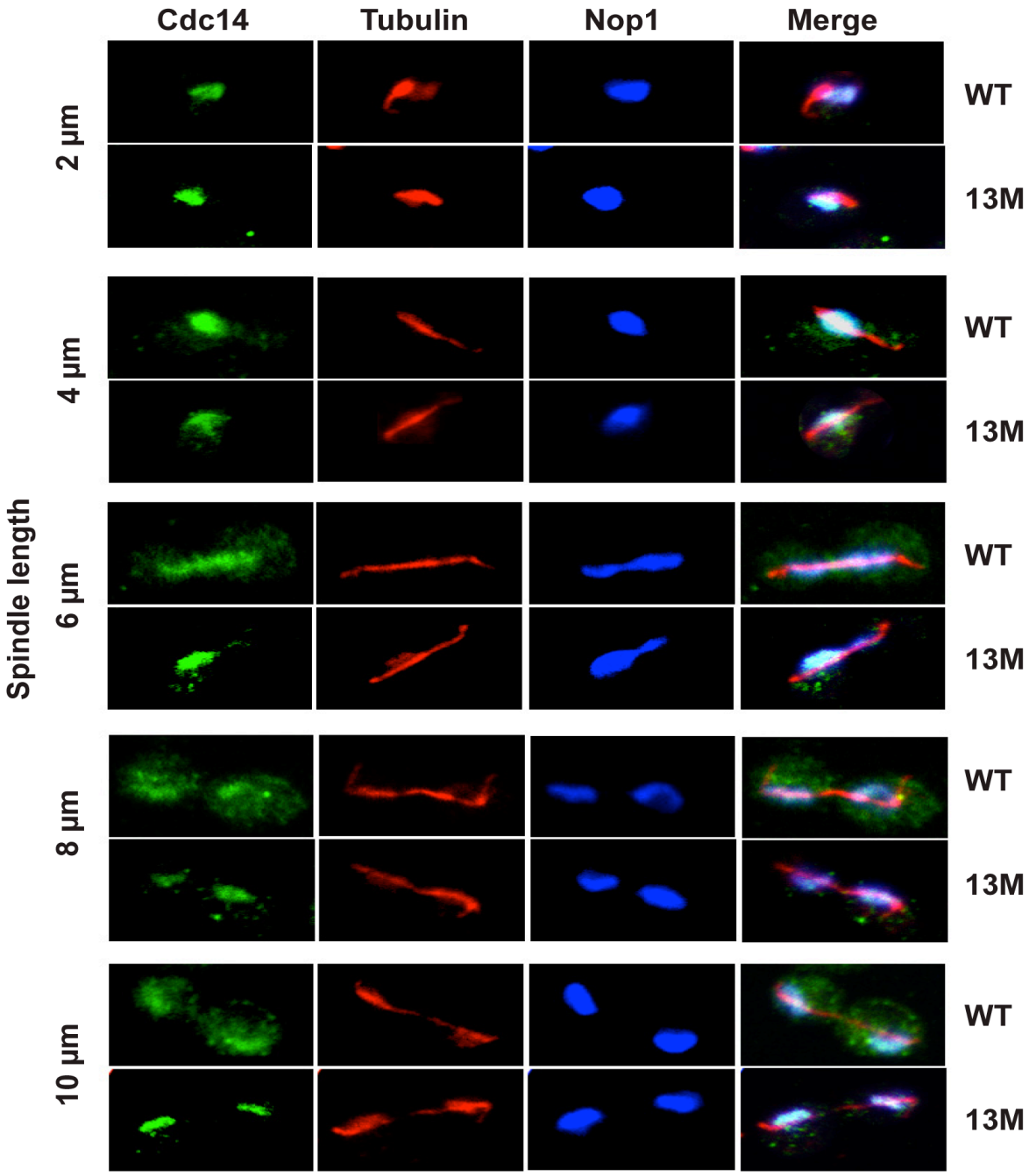


Figure IV-2. Dbf2-Mob1 phosphorylation site mutant cells were defective in releasing Cdc14 in late anaphase

(a)



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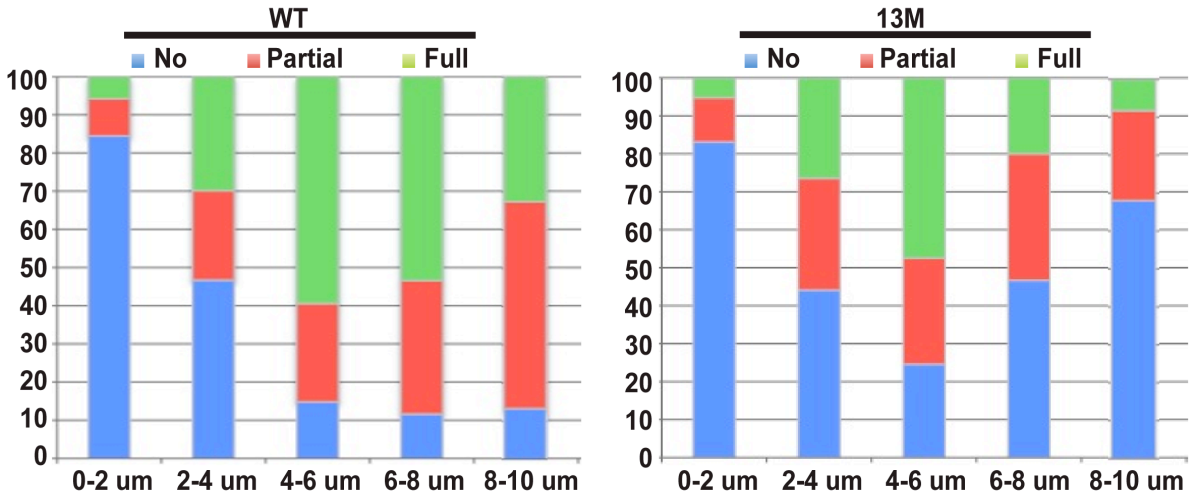
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6-8 um
8-10 um
13M
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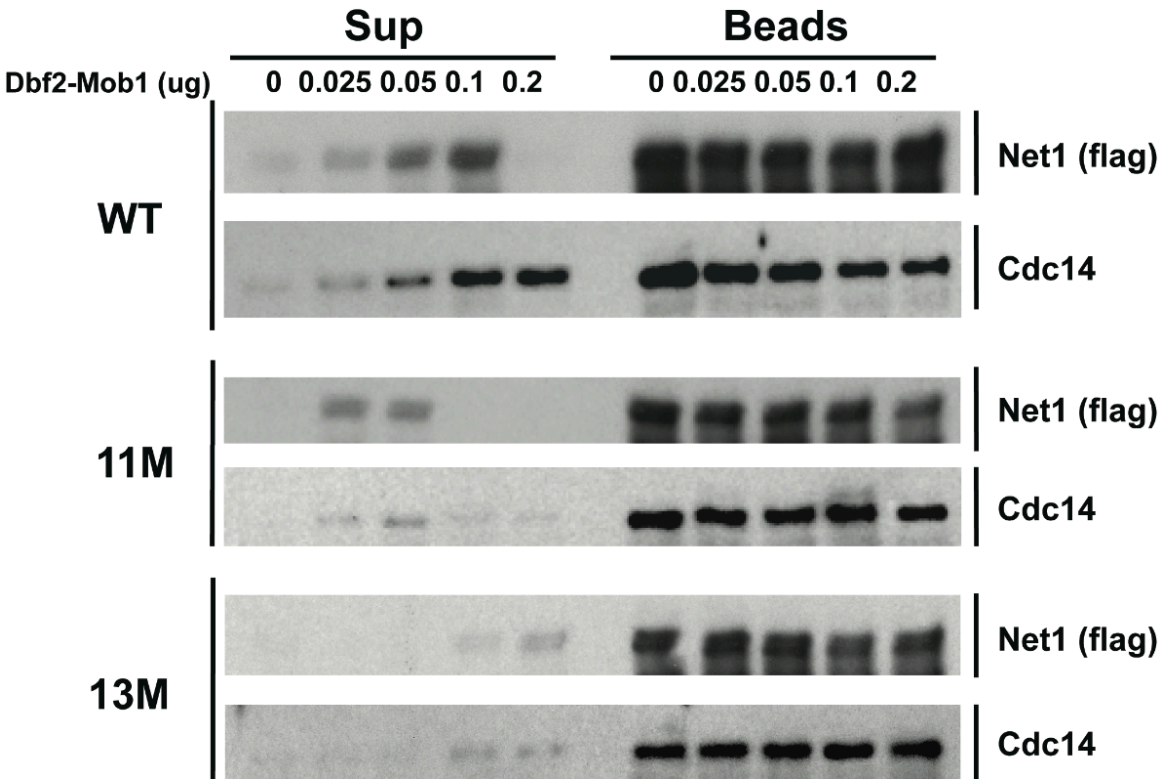
Figure IV-3. Dbf2-Mob1 phosphorylation site mutant cells were defective in releasing Cdc14 in *in vitro*.



Chapter V

Future Directions

Summary

In this thesis, we showed that Dbf2-Mob1 phosphorylates Net1 in the *in vitro* assay, and we found the effective phosphorylation sites in Net1. We also showed that phosphosite mutant cells exhibited defects in mitotic exit by growth assays, morphology assays, Fluorescence-activated cell sorting (FACS), and western blots. The phoshosite mutant cells also exhibited defects in Cdc14 release in genetic assays, immunofluorescent assays, and the bead releasing assays. We conclude that Dbf2-Mob1 is the key kinase phosphorylating Net1 and promoting Cdc14 release in the late anaphase.

Future questions

The mechanism we proposed may be applied to other unsolved mechanisms.

(1) Exit from cytokinesis. In the morphology assay, a small portion of the cells show undivided, chain-like multibudded shape with DNA distributed into each bud. This is the signature of cells that go on to the second round of the mitosis but exhibit defects in completing cell division process. This morphology has been reported in all the MEN component mutant cells and the *cdc14-1* cells. However, the detailed mechanism is still unclear. It would be interesting to test whether Net1 phosphorylation is also the key mechanism for cytokinesis.

(2) **Regulation of meiosis.** It has been reported that Cdc14 cellular localization is tightly regulated in meiosis and that *cdc14* mutant cells (*cdc14-1*) exhibit dyads morphology and uneven distribution of the DNA into each spore (Marston et al., 2003). In addition, the MEN component, Cdc15, plays an important role in spore formation (Pablo-

Hernando et al., 2007; Diamond et al., 2009). Therefore we sought out to test whether Dbf2-Mob1 regulates meiosis through regulating Cdc14 release by phosphorylating Net1.

In our preliminary work, we test the sporulation rate and the morphology of the spores in the *WT*, *PS10M*, and *PS13M* cells in w303 background. We found that the WT cells started to sporulate within 24 hours, while more than 95% of the *PS10M* and *PS13M* cells were still unsporulated. Less than 50% of the cells in *PS10M* and *PS13M* were sporulated in day 4 (Figure V-1). In addition, we found that the phosphosite mutant cells exhibit abnormal spore morphology and uneven DNA distribution (Figure V-2a-b). Furthermore, we found that $cdc14^{P116L}$ rescued both the abnormal morphology and the sporulation rate in 10M and 13M cells (Figure V-3). However, $Cdc14^{P116L}$ WT double mutants show defects in meiosis. Taken together, we propose that Dbf2-Mob1 could regulate meiosis through the control of Cdc14 cellular localization by phosphorylation of Net1.

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Figure Legends

Figure V-1. Phosphosite mutant cells exhibited defects in sporulation

net1 phosphosite mutants showed delayed in sporulation. *Net1-WT*, *PS10M*, and *PS13M* cells grew on YP-2% dextrose plates at 25 °C for 24 hours and transferred into sporulation media. Cells were formaldehyde fixed and stained with DAPI to indicate DNA localization. DAPI staining is presented in bright white dot. The days in the sporulation media is labeled at the left side of the images. Cell type is marked on the top of each image.

Figure V-2. Phosphosite mutant cells exhibited abnormal spore morphology

(a) The sporulation morphology and nucleus distribution in WT cells. *Net1-WT* cells grew on YP-2% dextrose plates at 25 °C for 24 hours and transferred into sporulation media. Cells were formaldehyde fixed and stained with DAPI to indicate DNA localization. DIC represents the light field imaging.

(b) Phosphosite mutant cells exhibited abnormal spore morphology and uneven distribution of the nucleus. *net1-PS10M* cells grew on YP-2% dextrose plates at 25 °C for 24 hours and

transferred into sporulation media. Cells were formaldehyde fixed and stained with DAPI to indicate DNA localization. DIC represents the light field imaging.

Figure V-3. *Cdc14^{P116L}* rescued the sporulation defects in *net1* phosphosite mutant cells

Diploid cells with either *NET1 WT/ net1*Δ or *net1-PS* mutant/ *net1*Δ were combined with *Cdc14 WT/ Cdc14^{P116L}*. Cells grew on YP-2% dextrose plates at 25 °C for 24 hours and transferred into sporulation media. Cells were formaldehyde fixed and stained with DAPI to indicate DNA localization. DAPI staining is presented in bright white dot. The days in the sporulation media is labeled at the left side of the images. Cell type is marked on the top of each image.

Figure V-1. Phosphosite mutant cells exhibits defects in sporulation

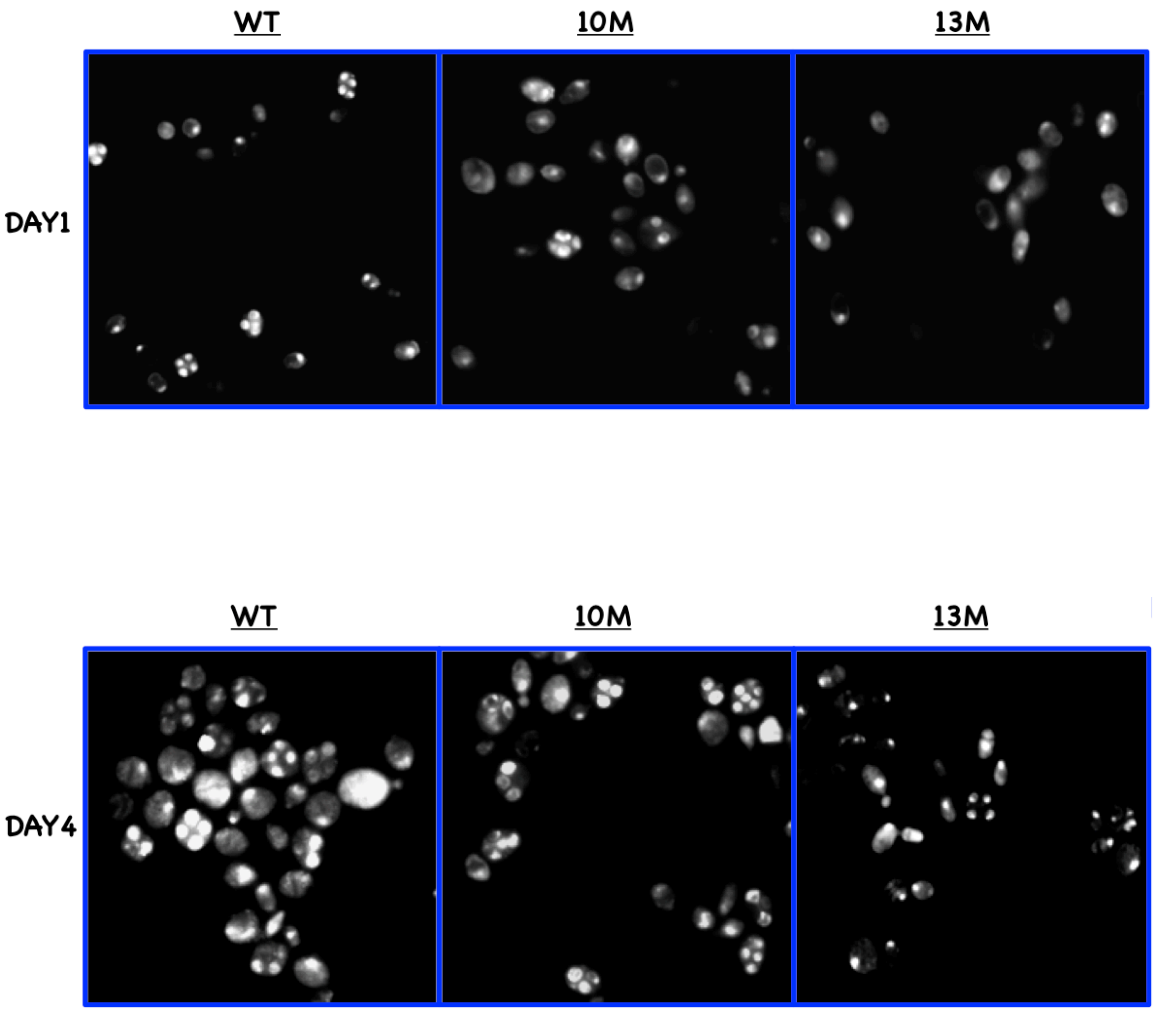
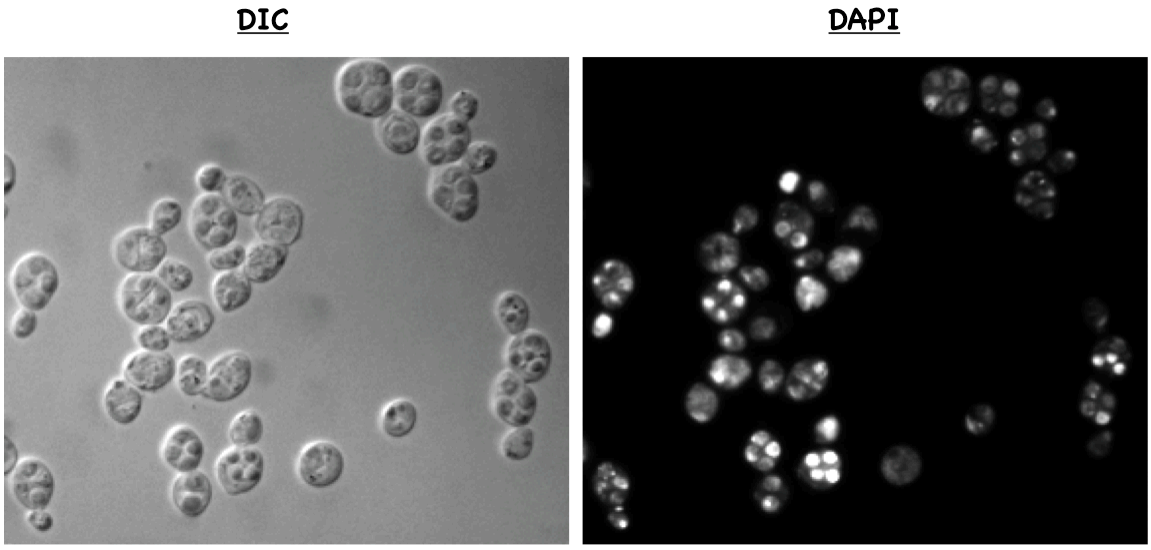


Figure V-2. Phosphosite mutant cells exhibited abnormal spore morphology

(a) WT cells



(b) net1-PS10M cells

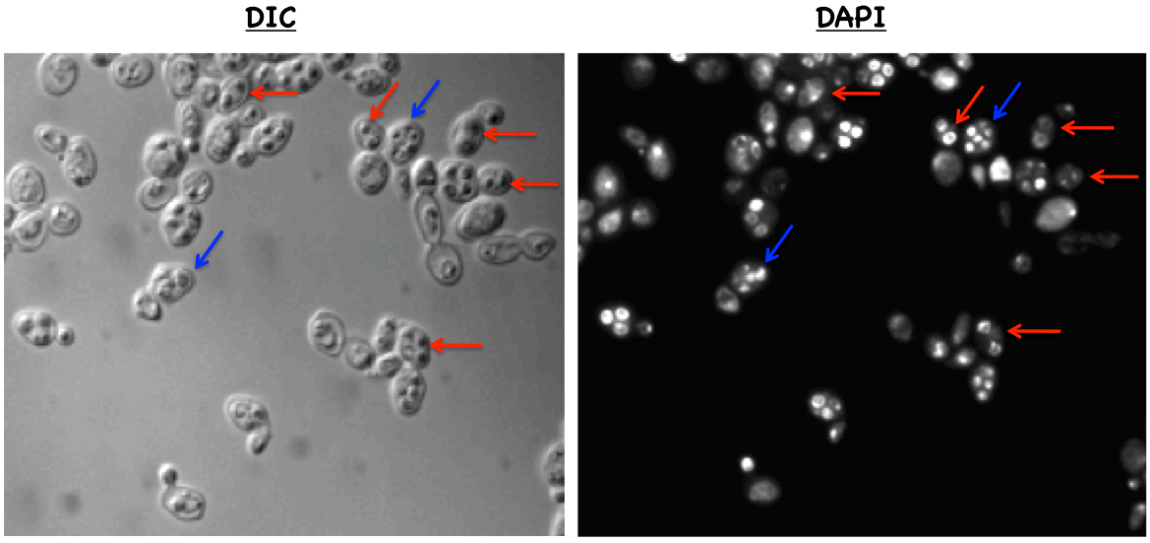


Figure V-3. Cdc14^{P116L} rescued the sporulation defects in phosphosite mutant cells