THE ROLE OF NET1 PHOSPHORYLATION IN REGULATING CDC14 RELEASE DURING MITOTIC EXIT

Thesis by

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ABSTRACT

Exit from mitosis is as an important phase in the cell cycle. The molecular event that triggers the cell cycle transition from anaphase into the G1 state involves the inactivation of the cyclin-dependent kinase complex (Cdk) through multiple mechanisms that lead to both destruction of the cyclin subunit co-activator and direct Cdk kinase inhibition. These multiple mechanisms indicate the importance of regulating the inactivation of Cdk to ensure proper cell cycle progression and cytokinesis. We set out to examine the regulation of the protein phosphatase Cdc14. Cdc14 is thought to act through reversal of phosphorylation on key Cdk substrates that promote mitotic exit by stimulating the destruction and inactivation of Cdk. In *Saccharomyces cerevisiae*, activation of Cdc14 is achieved via release from its nucleolar inhibitor Net1/Cfi1. This activation is correlated with multi-site phosphorylation of Net1 in cells where Cdc14 appears to be released from the nucleolus. We set out to identify new components of the nucleolar complex known as RENT (Regulator of Nucleolar Silencing and Teleophase) which holds Cdc14 in an inactive state. This led to the identification of Casein Kinase II (CKII) as a new component of RENT. CKII was verified to co-immunoprecipitate with Net1; and mutants in CKII arrest in anaphase with unreleased Cdc14 and unsegregated rDNA. Interestingly, phospho-peptide mapping experiments from *in vivo* Net1 samples revealed phosphorylation of a CKII consensus sequence within Net1. *In vivo* mapping also revealed another subset of sites that matched the consensus sequence established for Cdk phosphorylation. Mutational analysis of these sites unveiled their involvement in Cdc14 release during early anaphase and a role for a network of genetically interacting proteins involved in Fourteen Early Anaphase Release (FEAR) in promoting these phosphorylations.

In summary, the regulation of Cdc14 release via phosphorylation of its nucleolar inhibitor Net1 as demonstrated by this work highlights the importance of nucleolar sequesteration and regulated release as a mechanism of controlling important cell cycle factors and events. It also points to a fascinating role for Cdk in insuring its own destruction at the end of the cell cycle, thus promoting transition back into the G1 state.

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Chapter I- The End of the Cell Cycle

Overview: The motor of the cell cycle; Cyclin-dependent kinase (Cdk)

The ability of cells to coordinate important events such as spindle disassembly (Li and Cai, 1997), chromosomal condensation (Loidl, 2003), and DNA replication (Piatti, 1997) with cellular division rely on the activity of the cyclin-dependent kinase complex (Amon et al., 1994; Holloway et al., 1993; Surana et al., 1993). The Cdk complex consists of at least two components in *Saccharomyces cerevisiae*. The first is a cyclin subunit that both activates and is thought to impart substrate specificity to the second component, the kinase subunit. The cyclin subunit appears to be interchangeable during various phases of the cell cycle where G1 cyclins (Cln1,2,3) (Levine et al., 1995); S-phase cyclins (Clb5,6) (Kuntzel et al., 1996; Toone et al., 1997); and G2 cyclins (Clb1,2,3,4) (Fitch et al., 1992) activate the kinase subunit to effect phosphorylation on cell cycle-specific substrates. Direct regulation of the kinase subunit in *cis* involves the post-translational phosphorylation of key residues that lead to modification of enzymatic activity (Mendenhall and Hodge, 1998). Regulation of the Cdk complex in *trans* also plays a key role in *S. cerevisiae*. This is achieved through the action of Cdk inhibitors (Sic1) (Donovan et al., 1994) and (Cdc6) (Calzada et al., 2001) which directly bind to and inhibit Cdk complexes.

This work focuses on the regulation of Cdk activity at the end of the cell cycle during mitotic exit. Specifically, the regulation of the molecular trigger both in *cis* and in *trans* that signals the cells to initiate Cdk inactivation via the various mechanisms listed previously. We will demonstrate the ability of the cell cycle motor Cdk to act as its own

break, in effect, by regulating the activation of the molecular trigger that leads to the ultimate extinction of Cdk activity, thus paving the road for the return back to the G1 state.

Exit from mitosis in *Saccharomyces cerevisiae*

The Mitotic Exit Network (MEN)

To achieve the goal of Cdk inactivation by the various mechanisms listed previously, a signal transduction network has been identified to be involved in mitotic exit. This group of proteins consists of at least 10 genetically interacting proteins collectively known as the mitotic exit network (MEN) (Jaspersen et al., 1998; McCollum and Gould, 2001). They include 4 protein kinases (Cdc15, Cdc5, Dbf2, and Dbf20) (Johnston et al., 1990; Kitada et al., 1993; Schweitzer and Philippsen, 1991; Toyn et al., 1991), a spindle pole body (SPB) protein (Nud1) (Adams and Kilmartin, 1999), a protein phosphatase (Cdc14) (Wan et al., 1992), a GTPase (Tem1) (Shirayama et al., 1994), a GTP/GDP exchange protein (Lte1) (Shirayama et al., 1994), a negative regulator of Cdc15 (Amn1) (Wang et al., 2003), and a protein of unknown function (Mob1) (Luca and Winey, 1998). All of the MEN proteins, with the exception of Lte1, are essential in budding yeast and many are found to be conserved between yeast and higher eukaryotic organisms (Li et al., 1997; Li et al., 2000).

A model for how exit from mitosis is controlled involves the retention of Cdc14 in an inactive form within the nucleolus while tethered to the nucleolar protein (Net1/Cfi1) (Shou et al., 1999; Visintin et al., 1999). Upon activation of the MEN, Cdc14 is released from Net1, and diffuses throughout the cell. The active form of Cdc14 is thought to activate

cyclin proteolysis by removing inhibitory phosphorylation from Hct1/Cdh1, an activator of APC in late anaphase (Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998). Cdc14 is also thought to be responsible for promoting Sic1 accumulation and stability by reversal of Cdk phosphorylation events on Swi5 (a Sic1 transcriptional activator) and Sic1, respectively (Moll et al., 1991; Toyn et al., 1991; Toyn et al., 1997; Verma et al., 1997; Visintin et al., 1998).

Evidence suggests that the MEN acts as part of the Bub2-dependent spindle positioning checkpoint that monitors spindle pole body (SPB) position with respect to the bud neck (Bardin et al., 2000; Bloecher et al., 2000; Pereira et al., 2000). Upon spindle pole body duplication, the old spindle pole body is thought to contain the Tem1 GTPase which migrates to the bud. Activation of Tem1 occurs as the SPB interacts with the Bud cortex containing the GTP/GDP exchange protein Lte1 (Bardin et al., 2000; Bloecher et al., 2000; Pereira et al., 2000). Tem1 then in turn is thought to activate Cdc15 kinase (Asakawa et al., 2001; Bardin et al., 2003) which in turn leads to the activation of the Dbf2/Dbf20 kinase complex bound to the cyclin-like subunit Mob1 (Mah et al., 2001; Visintin and Amon, 2001). It remains unclear how the Dbf2/Dbf20/Mob1 complex then controls the activation of Cdc14 to promote mitotic exit. One possible role for the action of MEN involves the control of Cdc14's ability to transition between the nucleus and cytoplasm where the majority of its identified substrates reside. Indeed, genetic screens have identified a few nuclear transporters as suppressors of MEN mutants such as *SUP1, KAP104, and MTR10* (Asakawa and Toh-e, 2002; Shou and Deshaies, 2002; Shou et al., 2001; Shou et al., 1999), yet it remains unknown as to whether these transporter act directly on Cdc14 or indirectly through general perturbation of nuclear/nucleolar architecture to

allow precocious release of Cdc14 independently of MEN function. Indeed, Net1 mutations such as *net1-1* have a significant impact on general nucleolar structure as judged by localization of multiple nucleolar antigens and regulation of rDNA morphology (Shou et al., 2001), which helps to explain this allele's ability to bypass *cdc15Δ* cells (Shou et al., 1999). It is clear though that the cell cycle function of Net1 can be uncoupled from its other nucleolar functions as demonstrated by the dominant mutation in *CDC14* (*TAB6*) (Shou et al., 1999).

Fourteen Early Anaphase Release (FEAR) Network

Recently, a new network of proteins has been identified in regulating the release of Cdc14 from the nucleolus in early anaphase (Stegmeier et al., 2002). This network consists of a separase (Esp1), the polo-like kinase (Cdc5), Spo12, and Slk19 which have unknown functions.

The first component of this network is a protease known as separase (Esp1) (Uhlmann et al., 2000). Esp1 is held inactive by securin (Pds1) (Cohen-Fix and Koshland, 1999; Yamamoto et al., 1996) until the metaphase to anaphase transition, at which point it is activated to promote destruction of cohesin (Scc1) (Ciosk et al., 1998). Cohesin holds sister chromatids together after DNA replication and its enzymatic cleavage by separase allows separation of the sister chromatids to the opposite SPB ends (Uhlmann et al., 2000). Notably, the enzymatic activity of Esp1 does not appear to be required for its function in the FEAR pathway as catalytically inactive Esp1 is still able to promote Cdc14 release in metaphase-arrested cells (Sullivan and Uhlmann, 2003). Also, over-expression of Esp1

allows metaphase-arrested cells to complete mitosis and cycle to the next G1 phase but is dependent on Spo12, Slk19, and to a lesser extent Cdc5 (Sullivan and Uhlmann, 2003).

The second component is (Cdc5), the only known homolog to the polo kinase in *Saccharomyces cerevisiae*. Cdc5 is interesting because it acts as both a component of the MEN as well as the FEAR networks. Cdc5 is thought to act in the MEN pathway through inhibitory phosphorylations of the Bub2/Bfa1 complex which leads to the activation of Tem1 (Hu et al., 2001). Cdc5 is also thought to act on the RENT complex to promote Cdc14 release from Net1, although this is thought to be primarily through an indirect mechanism (Shou et al., 2002). The function of Cdc5 in the FEAR pathway is less clear, whereas over-expression of Cdc5 can promote Cdc14 release in metaphase-arrested cells, it appears to be independent of the other FEAR network genes (Sullivan and Uhlmann, 2003; Visintin et al., 2003).

The third component of the FEAR is Spo12, a protein of unknown function. Spo12 was originally identified as playing an important role in the proper progression of meiosis (Klapholz and Esposito, 1980a; Klapholz and Esposito, 1980b). Interestingly, Spo12 levels appear to be cell-cycle regulated (Shah et al., 2001) and functions as a multi-copy suppressor of *cdc15-2* temperature sensitive strains (Jaspersen et al., 1998). Also, the role of Spo12 in promoting Cdc14 release appears to be in parallel to the other components of the FEAR network, as over-expression of Spo12 still drives Cdc14 out of the nucleolus in FEAR mutants (Visintin et al., 2003). Recently, Spo12 has been suggested to antagonize the function of the nucleolar protein (Fob1), thus promoting the premature release of Cdc14 from the RENT complex in metaphase-arrested cells (Stegmeier et al., 2004).

The fourth component of this network is Slk19, another protein of unknown function. Slk19 was originally identified and later confirmed to be required for the proper maintenance of mitotic anaphase spindles (Sullivan et al., 2001; Zeng et al., 1999). Slk19 localizes to kinetochores and the spindle mid-zone during anaphase and is a substrate of Esp1, yet interestingly cleavage of Slk19 by Esp1 does not seem to be necessary for Cdc14 release from the nucleolus (Stegmeier et al., 2002). The function of Slk19 in the FEAR pathway could also be complicated by the fact that Slk19 appears to be required upstream to promote Cdc14 release through the FEAR pathway yet also required for Cdc14 function downstream to promote anaphase spindle integrity through Cdc14 mediated localization of the INCENP-Aurora B complex to the spindle midzone (Pereira and Schiebel, 2003).

Roles of FEAR

In a relatively short amount of time, the FEAR network has been implicated to play a key role in organizing multiple events during early anaphase. FEAR was initially identified as a group of proteins required for the timely progression through mitosis by promoting the release of Cdc14 in early anaphase (Stegmeier et al., 2002). Cdc14 is then able to positively feed back and activate the kinase Cdc15 as part of the MEN pathway as has been proposed previously (Jaspersen and Morgan, 2000). FEAR mutants, in the context of mitosis, displayed a delay in proper progression through mitosis, and synthetic phenotypes when combined with MEN mutants (Stegmeier et al., 2002).

Recent studies have illuminated another role for FEAR in the coordination of MI and MII phases during meiosis (Buonomo et al., 2003; Marston et al., 2003) and proper

segregation of rDNA (Buonomo et al., 2003). Anaphase I spindle disassembly is delayed in *cdc14-1*, *spo12Δ*, and *slk19Δ* mutants to the point where anaphase II equational division occurs on meiosis I spindles (Marston et al., 2003). Remarkably, meiosis II events still take place and deletion of *SPO11*, preventing recombination, rescues the nuclear division defect of these mutants (Marston et al., 2003). Both Spo12 and Slk19 appear to be critical for executing meiosis than mitosis as judged by the sporulation and meiotic defects of *spo12Δ* and *slk19Δ* mutant cells (Buonomo et al., 2003; Grether and Herskowitz, 1999; Klapholz and Esposito, 1980b; Zeng et al., 1999). Mutant *spo12Δ* asci contain only two spores and half of *slk19Δ* contain two and half three or four spores (unpublished observations) (Marston et al., 2003). More so, Spo12 and Slk19 are required for the division of the nucleolus (specifically, the segregation of newly replicated rDNA) and release of Cdc14 during Anaphase I of meiosis implying that Cdc14 release and activation is important for nucleolar segregation as has been suggested previously for cells exiting mitosis (Granot and Snyder, 1991). The polo kinase Cdc5 has also been linked to controlling chromosomal segregation during meiosis I (Lee and Amon, 2003). Removal of meiotic cohesin from chromosomes and sister-kinetochore co-orientation during meiosis I are coupled through their dependence on Cdc5 (Lee and Amon, 2003).

More recently, a new function of FEAR has been attributed to the controlling the proper transfer of the INCENP-Aurora B complex from the kinetochore to the spindle midzone (Pereira and Schiebel, 2003). Release of Cdc14 in early anaphase leads to the dephosphorylation of Sli15, a component of the INCEP-Aurora complex composed of Sli15-Ipl1. This dephosphorylation occurs on Cdk sites of Sli15 through Cdc14's

phosphatase activity *in vitro* (Pereira and Schiebel, 2003) which corresponds with Cdc14 being a proline-directed phosphatase (Gray et al., 2003). Sli15 dephosphorylation is sufficient to trigger microtubule binding of the INCEP-Aurora complex as demonstrated by the Sli15-6A mutant which remarkably is able to rescue the spindle defect of *cdc14-2* mutant cells. Mutant *Sli15-6A* cells also displayed a 1000-fold increase in chromosome loss rate due to constitutive localization of Ipl1 and consequent stabilization of microtubules (Pereira and Schiebel, 2003) again arguing that proper activation of Cdc14 in early anaphase is important for timely progression through the cell cycle towards mitotic exit.

FEAR and Mitotic Exit Networks in Meiosis and Mitosis

Exit from anaphase is similar in meiosis and mitosis in that it requires the down-regulation of Cdk1 activity. However, in contrast to the exit from mitosis which is brought about by a complete loss of M-phase Cdk activity, the exit from meiosis I is accompanied by only a partial reduction perhaps because modest levels of Cdk1 activity appear to be required in the interval between meiosis I and II to prevent DNA replication and origin resetting (Iwabuchi et al., 2000; Noton and Diffley, 2000). Interestingly, the FEAR network appears to perform similar functions in both M-phases: it creates a brief window of Cdc14 activity that antagonizes Clb-Cdk to initiate a collapse of the M-phase state. In meiosis, the transient reduction in the M-phase state appears to be brief and only necessary for partial reversal of the M-phase state to allow for the remodeling of the microtubule spindle to support equational segregation of sister chromosomes during meiosis II. For this, the FEAR appears to be both necessary and sufficient, and the MEN does not appear to play a critical role (Buonomo et al., 2003; Marston et al., 2003). However, in mitotic cycles, Clb-

Cdk activity must be completely extinguished to enable an irreversible return to an interphase state that can support DNA replication. This requirement in turn may necessitate a more extended release of Cdc14 from the nucleolus, which requires the MEN. Positive feedback loops built into the MEN (Jaspersen and Morgan, 2000) may act to ensure that once the MEN is switched on, Clb-Cdk activity will inevitably be extinguished completely to allow the cell to divide and enter a subsequent S phase. Owing to these positive feedback loops, even a very modest initial stimulus could snowball into a complete mobilization of Cdc14, which could explain why the FEAR is not essential in mitotic cycles.

Conversely, the budding lifestyle of *Saccharomyces cerevisiae* offers a simple explanation for why the FEAR may not be sufficient to drive exit from mitosis (in contrast to the situation at the completion of meiosis I). Accurate partitioning of sister chromosomes to mother and daughter yeast cells is supported by a post-anaphase checkpoint that monitors the position of the spindle relative to the bud neck (Bardin et al., 2000; Pereira et al., 2000). If the FEAR by itself could drive exit from mitosis, then the cell would be irreversibly committed to exit mitosis upon activation of separase at the metaphase-anaphase transition, regardless of whether the two sets of sister chromatids were properly distributed to the two daughter cells.

Thesis Overview

Although the action of the FEAR network is not essential for mitotic cell cycles, its activity helps to determine the timing of exit from mitosis (Stegmeier et al., 2002). Chapter

II describes how phosphorylation of Net1 by Clb-Cdk underlies disruption of the RENT complex during anaphase and illustrates a fascinating aspect of the switch that governs the return of mitotic cells to an interphase state. Although Clb-Cdk initiates feedback loops that help sustain a mitotic state with high Clb-Cdk activity (Deshaies, 1997), there must be mechanisms for subverting the reign of Clb-Cdk to allow growing cells to continue to cycle. Fittingly, at least two of these mechanisms – activation of Cdc20 binding to APC (Rudner et al., 2000; Rudner and Murray, 2000) and disruption of the Cdc14/Net1 complex – are initiated directly by the very enzyme whose activity helps to specify the mitotic state in the first place. Chapter III describes the identification of new interactors with the RENT complex such as Casein kinase II (CKII) and RNA polymerase I in mitotic cells. Given that a CKII site was identified and mapped from *in vivo* samples of Net1, and CKII was found co-associated with RENT complexes by mass spectrometry and this association was confirmed by co-immunoprecipitation; CKII could be playing an important role in regulating Cdc14 release and possibly rDNA segregation. Also, given the location of the RENT complex within the nucleolus, interactions of Net1 with RNA Pol I help explain the multi-functionality displayed by *net1-1* mutants. Chapter IV will outline a potential line of experiments that address how regulation of the FEAR and MEN pathways could be investigated through the use of Clb2–Cdk phosphorylation assays on Net1 and how these phosphorylations lead to the activation of Cdc14.

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Chapter II- Net1 Phosphorylation by Clb1,2–Cdk Regulates Cdc14 Release from the Nucleolus during Exit from Mitosis

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Summary

The Cdc14 Early Anaphase Release (FEAR) network promotes a transition in the M-phase state that demarcates meiosis I and II, and together with the mitotic exit network enables timely exit from mitosis. These functions derive from FEAR's ability to promote release of active Cdc14 from the nucleolar anchor Net1/Cfi1. We report here a molecular basis for the release of Cdc14 during early anaphase. Mitotic cyclin Clb2–Cdk complexes phosphorylate Net1 *in vitro* and *in vivo*. Underscoring the relevance of these phosphorylations, *net1* phosphosite and *clb1Δclb2Δ* mutants are deficient in FEAR, and *net1* phosphosite mutants fail to execute proper meiosis. Over-expression of non-degradable Clb2 induces ectopic release of Cdc14 from the nucleolus, and purified Clb2-Cdk dissociates Cdc14 from Net1 *in vitro*. Finally, FEAR network components Spo12, Slk19, and Cdc5 promote Net1 phosphorylation by Clb–Cdk. These results suggest that the FEAR network mobilizes Clb1,2-Cdk to phosphorylate Net1 and dislodge Cdc14, which initiates a transient (meiosis I) or sustained (mitosis) departure from M-phase via down-regulation of Clb-Cdk activity. Thus, Clb-Cdk sows the seeds of its own demise.

Introduction

Exit from mitosis is an essential step in the progression of cells through the cell cycle. In late mitosis, inactivation of mitotic cyclin–Cdk complexes causes mitotic spindle disassembly (Li and Cai, 1997), chromosome decondensation, and return of cells to G1 phase (Amon et al., 1994; Holloway et al., 1993; Surana et al., 1993). In budding yeast, this inactivation is achieved by accumulation of the Cdk inhibitor Sic1 (Donovan et al., 1994; Schwab et al., 1997; Visintin et al., 1998), and destruction of the mitotic cyclins by the Anaphase Promoting Complex (APC), a multi-subunit ubiquitin protein ligase that catalyzes the ubiquitination of substrates containing a destruction-box sequence (Glotzer et al., 1991; Irniger et al., 1995; King et al., 1995; Morgan, 1999). Both of these processes require a network of genetically interacting proteins collectively known as the mitotic exit network (MEN) (Jaspersen et al., 1998; McCollum and Gould, 2001). The most upstream component is the spindle pole body (SPB) protein Nud1 (Adams and Kilmartin, 1999), which tethers MEN components to the SPB. Among those components is the GTPase Tem1 (Shirayama et al., 1994b), which is activated by the guanine nucleotide exchange factor Lte1 (Shirayama et al., 1994a). The location of Lte1 in the bud cortex is thought to render Tem1 activation dependent upon successful orientation of the anaphase spindle along the mother-bud axis (Bardin et al., 2000; Pereira et al., 2000). Activated Tem1 then promotes activation of the protein kinase Cdc15 (Schweitzer and Philippsen, 1991), which in turn activates the homologous protein kinases Dbf2 and Dbf20 (Johnston et al., 1990; Toyn et al., 1991). Proper activation of Dbf2 requires its association with its binding partner Mob1 (Luca and Winey, 1998; Mah et al., 2001). MEN components Cdc15, Dbf2, Dbf20, and Mob1, like Tem1, localize to the SPB (Luca

et al., 2001; Pereira et al., 2002; Visintin and Amon, 2001; Yoshida and Toh-e, 2001). Activation of Cdc14, a protein phosphatase (Wan et al., 1992) that serves as a key mediator of mitotic exit, appears to be the eventual target of the MEN, yet the mechanism by which this activation occurs remains unknown.

During interphase, Cdc14 is retained in an inactive form within the nucleolus as a component of the RENT complex, which comprises Cdc14 and Sir2 tethered to the nucleolar protein Net1/Cfi1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). During anaphase, active Cdc14 is released from Net1, and diffuses throughout the cell. Cdc14 is thought to activate cyclin proteolysis by removing inhibitory phosphate groups from Hct1/Cdh1, an activator of APC in late anaphase (Jaspersen et al., 1999; Visintin et al., 1998; Zachariae et al., 1998). Cdc14 also promotes Sic1 accumulation and stability by removal of Cdk phosphorylations on Swi5 (a Sic1 transcriptional activator) and Sic1, respectively (Moll et al., 1991; Toyn et al., 1997; Verma et al., 1997; Visintin et al., 1998).

Recently, Cdc5, Esp1, Spo12, and Slk19 have been proposed to comprise a signaling network (dubbed the FEAR network for Fourteen Early Anaphase Release) that instigates transient release of Cdc14 from Net1 at the onset of anaphase (Stegmeier et al., 2002). Cdc5 and Esp1 are, respectively, the polo kinase and separase orthologs of budding yeast. The biochemical functions of Spo12 and Slk19 are unknown. The FEAR network is required for proper coordination of chromosome segregation during meiosis I and II (Buonomo et al., 2003; Marston et al., 2003) but is not essential for exit from mitosis (Stegmeier et al., 2002). Nevertheless, FEAR network mutants exhibit a modest delay in exiting mitosis, and strong synergy with MEN mutants.

Although the FEAR network plays a critical coordinating role in the M-phases of mitosis and meiosis, the molecular mechanisms underpinning FEAR's ability to promote Cdc14 activation are not fully understood. Investigations on the role of separase in this process demonstrated that ectopic disassembly of RENT induced by over-expression of separase is not affected by a point mutation that disrupts separase's endoproteolytic activity, but is blocked completely by deletion of *SPO12* or *SLK19*, and partially in *cdc5-1* temperature sensitive (*ts*) mutants (Sullivan and Uhlmann, 2003). These observations suggest that a novel activity of Esp1 stimulates Spo12, Slk19, and Cdc5 to promote release of Cdc14 from Net1. In contrast, over-expressed Cdc5 can promote ectopic release of Cdc14 from the nucleolus in *spo12Δ*, *slk19Δ*, and *esp1-1* mutants, and over-expressed Spo12 accelerates the same event in *slk19Δ*, *esp1-1*, and Cdc5-depleted cells. These observations suggest that Spo12 and Cdc5 collaborate on parallel pathways to dislodge Cdc14 from Net1 (Visintin et al., 2003). Interestingly, separase- and Cdc5-induced disassembly of RENT correlates with hyper-phosphorylation of Net1 (Shou et al., 2002a; Sullivan and Uhlmann, 2003; Visintin et al., 2003). It has long been thought that phosphorylation of Net1, Cdc14, or both underlies disassembly of the RENT complex during early and late anaphase (Shou et al., 2002a; Shou et al., 1999; Sullivan and Uhlmann, 2003; Visintin et al., 2003). Among the protein kinases in the MEN, Cdc5 has been implicated in controlling Net1 phosphorylation state and RENT disassembly (Shou et al., 2002a; Visintin et al., 2003; Yoshida and Toh-e, 2002), and has been postulated to be the direct effector of FEAR-induced Net1 phosphorylation (Ross and Cohen-Fix, 2003). However, the identity of the relevant protein kinases that phosphorylate RENT and the sites of *in vivo* phosphorylation remain uncertain. Thus, it has not been possible to test directly

the appealing hypothesis that phosphorylation underlies the release of Cdc14 from Net1 during anaphase. We propose that mitotic Clb-Cdk phosphorylates Net1 to bring about Cdc14 release and activation and that this phsophorylation is regulated by components of the FEAR network.

Results

Net1 N-terminus mediates regulated localization of Cdc14 and is highly phosphorylated in *cdc14-1* cells.

To map determinants that sustain regulated disassembly of the RENT complex, we examined a Net1 (1-621) truncation mutant along with a set of strains that contain transposon insertions in *NET1* (Burns et al., 1994; Ross-Macdonald et al., 1999). Immuno-fluorescence analysis of asynchronous cell cultures using anti-Cdc14 antibodies revealed that the N-terminal 621 amino acid fragment of Net1 was both sufficient and necessary for proper nucleolar sequestration of Cdc14 in G1 phase and its release in anaphase cells (Figure 1). Net1 is heavily phosphorylated in *cdc14-1 ts* cells arrested in anaphase (Shou et al., 1999), but less so in other MEN mutants (Shou et al., 2002b). This observation suggested that the MEN controls phosphorylation of Net1, which in turn might underlie the release of Cdc14 during anaphase. To test this hypothesis we purified Net1 from *cdc14-1* arrested cells, and mapped sites of phosphorylation as described (Shou et al., 2002b). Nineteen sites of phosphorylation were identified (Table 1). Of these, we focused our attention on the N-terminal phosphorylations because the corresponding region was found to be necessary and sufficient to mediate proper cell cycle-regulated localization of Cdc14 (Figure 1).

Net1 phosphorylation is required for the transient release of Cdc14 in early anaphase and proper meiosis.

Endogenous *NET1* was substituted by a mutant (*net1-13m*) allele in which thirteen, N-terminal, *in vivo* phosphorylation sites were converted to Alanine (Table1). The mutant protein, designated Net1-13m, localized to the nucleolus (data not shown) and directed the nucleolar localization of Cdc14 (Figure 7A). However, *net1-13m* cells were deficient in the transient release of Cdc14 from the nucleolus that occurs during early anaphase (i.e., FEAR; compare Figure 7B to Figure 2B). To determine which phosphorylation sites were responsible for the phenotype of *net1-13m*, we constructed mutants lacking subsets of sites. This effort yielded a mutant lacking three CDK consensus sites (hereafter referred to as *net1-3Cdk*) that almost completely recapitulated the phenotypes of *net1-13m* (Figures 2A, 2B), whereas another mutant (*net1-3Ax*) lacking the 3 non-Cdk consensus sites in the first 341 aa of Net1 displayed a very minor reduction in FEAR (Figure 7C). Upon further analysis of the phosphosite mapping data (see Experimental Procedures), we constructed an additional mutant lacking all six Cdk consensus sites in the first 341 aa of Net1 (hereafter referred to as *net1-6Cdk*).

Although FEAR is typically assayed in the context of a MEN mutant (e.g., *cdc15ts*), deletion of *SPO12* in a wild-type background results in a delay in both release of Cdc14 from the nucleolus and exit from mitosis (Stegmeier et al., 2002). To evaluate the role of Cdk phosphorylation on Net1 in an unperturbed cell cycle, we monitored Cdc14 localization and spindle elongation in wild-type, *spo12Δ*, and *net1-6Cdk* cells released synchronously from α factor block in G1 phase. There was a modest (10') delay

in Cdc14 release from the nucleolus in *net1-6Cdk* mutants similar to that seen for *spo12Δ* cells (Figure 8 and (Visintin et al., 2003). A third phenotype exhibited by FEAR mutants is that they exhibit synthetic genetic phenotypes when combined with MEN mutants (Stegmeier et al., 2002). Likewise, the *net1-3Cdk* and *net1-6Cdk* mutants showed synthetic-lethal interactions with the MEN mutants *dbf2-2* (Figure 2C) and *cdc15-2* (Figure 2D), respectively. For example, whereas *cdc15-2* and *net1-6Cdk* mutants were viable at 30.7°C, *cdc15-2 net1-6Cdk* double mutants were not. Taken together, these observations implicate *net1-6Cdk* as a FEAR mutant.

Recent work has implicated the FEAR network in proper segregation of rDNA and cell cycle progression during meiosis (Buonomo et al., 2003; Lee and Amon, 2003; Marston et al., 2003). Given that *net1-6Cdk* mutants exhibited phenotypes reminiscent of FEAR network mutants, we evaluated their ability to segregate rDNA and form meiotic spores. In parallel with the 10' delay in Cdc14 release from the nucleolus, *spo12Δ* and *net1-6Cdk* mutants exhibited a 10' delay in rDNA segregation during mitosis (Figure 8). Likewise, *net1-6Cdk* mutants exhibited a meiotic defect: whereas greater than 90% of nitrogen-starved wild-type cells produced tri- or tetranucleate asci, *net1-6Cdk* mutants produced 43% binucleate and 57% tri- or tetranucleate asci with the majority of those being tri-nucleate (data not shown). The meiotic defect of *net1-6Cdk* mutants is less severe than seen for *spo12Δ*, but closely resembles that previously reported for *slk19Δ* mutants undergoing meiosis (Buonomo et al., 2003).

Mitotic cyclin-Cdk phosphorylates sites on Net1 required for FEAR.

Given the striking phenotypes of *net1-3Cdk* and *net1-6Cdk* cells, we prepared phospho-specific antibodies using phosphorylated peptides corresponding to the three *in vivo* phosphorylation sites that were mutated in *net1-3Cdk* (designated PP-A, PP-B, and PP-C). All three antibodies reacted with Net1 isolated from arrested *cdc14-1* cells (when Net1 is maximally phosphorylated) but not with Net1-3Cdk (Figure 3A). Moreover, the antibodies failed to detect Net1 from α factor-arrested cells (when Net1 is not detectably phospho-shifted) (Figure 3B). In many of the subsequent experiments we chose to focus on the PP-B epitope (which is formed by phosphorylation on Threonine 212) because it yielded a better signal in western blot analyses.

In vitro protein kinase assays provided the first concrete evidence that Net1 is a specific Cdk substrate. Recombinant Clb2–Cdk both generated the PP-B and PP-C epitopes on (Figure 3C), and incorporated radiolabel from γ-^{32}P-ATP into recombinant Net1 (Figure 3D). Remarkably, phosphorylation of Net1 by Clb2–Cdk was exquisitely specific, in that an equivalent amount of Clb5–Cdk histone H1 kinase activity did not phosphorylate Net1 (Figure 3D).

If Clb2-Cdk is indeed a physiological Net1 kinase, then Net1 phosphorylation should be diminished and/or delayed in a *clb2Δ* mutant. Indeed, in *clb2Δ* cells released from a G1 cell cycle block, the timing of appearance of the PP-B antigen was delayed by approximately 30-minutes as compared to wild-type cells, but the accumulation of Clb3 was not significantly affected (Figure 4A). We speculated that in the absence of Clb2, Clb1 compensated to phosphorylate Threonine 212 and promote FEAR, but both processes were delayed. To test this prediction, Threonine 212 phosphorylation was evaluated in a *clb1Δ* mutant and in a *clb1Δclb2Δ* mutant kept alive by a glucose-

repressible *GAL1p-CLB2* construct. Whereas the timing and level of PP-B epitope formation were normal in *clb1Δ* cells (data not shown), *clb1Δclb2Δ GAL1p-CLB2* cells depleted of the majority of Clb2 by growth on glucose after centrifugal elutriation, proceeded from G1 phase through anaphase but arrested in late anaphase/telophase without undergoing either detectable phosphorylation on Threonine 212 (Figure 4B) or release of Cdc14 from the nucleolus (Figures 4B, 4C, 4D). This observation was further corroborated by using an analog-sensitive version of the yeast Cdk (*cdc28-as1*) (Bishop et al., 2000; Ubersax et al., 2003) to demonstrate that phosphorylation of Net1 on Threonine 212 was dependent on Cdk activity as cells exited mitosis (Figure 9). Moreover, *CLB2* fulfilled the criteria established for FEAR network genes in that *clb2Δ cdc15-2* cells failed to exhibit FEAR (Figures 4C and 4D), and *clb2Δ* enhanced the *ts* phenotype of *cdc15-2* (Figure 4F) and *dbf2-2* mutants (data not shown). Interestingly, Cdc14 localization in both *clb1Δclb2Δ* and *clb2Δ cdc15-2* cells appeared to remain nucleolar despite arresting in late anaphase (Figure 4C, 2nd panel). Moreover, upon meiotic segregation we could not obtain viable colonies containing *clb2Δ* in combination with *cdc14-1*, *cdc5-1*, or *msd2-1* (data not shown) (Yuste-Rojas and Cross, 2000). Importantly, the synthetic phenotype of *clb2Δ* MEN *ts* double mutants arose from an exacerbation of the MEN defect because the terminal phenotype of *cdc15-2 clb2Δ* remained a late anaphase arrest (Figure 4C, data not shown), even though the double mutant cells exhibited a 30-minute delay at metaphase, as expected for a *clb2*Δ mutant (Figures 4C, 4E).

Over-expression of non-degradable Clb2 is sufficient to drive Cdc14 out of the nucleolus in metaphase-arrested cells.

Our results with *cis* mutations in sites of Net1 *in vivo* phosphorylation coupled with *trans* mutations in *CLB1* and *CLB2* provide strong evidence that phosphorylation of Net1 on a set of N-terminal CDK sites by Clb1/Clb2-Cdk is necessary for FEAR. Over-expression of a stable form of Clb2 (*GAL1p-CLB2Δdb*) has been reported to arrest cells in late anaphase with Cdc14 released from the nucleolus at the onset of anaphase (Stegmeier et al., 2002; Surana et al., 1993). To determine if regulation of Clb2-Cdk activity might normally govern the timing of FEAR, we tested whether elevated levels of Clb2 are sufficient to bring about ectopic release of Cdc14 from the nucleolus in cells arrested in metaphase. Whereas endogenous levels of Clb2 did not sustain Threonine 212 phosphorylation in metaphase-arrested cells (Figure 5A, Lane 1), over-expression of a hyper-stable form of Clb2 ($CLB2C_2DK_{100}$) (Hendrickson et al., 2001) was sufficient to drive Net1 phosphorylation and release of Cdc14 into the nucleus (Figures 5A, 5B). By contrast, induction of stable Clb2 in cells arrested in G1 phase with alpha factor did not bring about formation of the PP-B epitope on Net1 or disrupt association of Cdc14 with Net1 (data not shown).

The ability of over-expressed Clb2 to induce premature Net1 phosphorylation and release of Cdc14 from the nucleolus prompted us to test whether Clb2–Cdk activity is sufficient to bring about disassembly of RENT *in vitro*. Pre-phosphorylation of a 600 amino acid N-terminal fragment of Net1 blocked its binding to Cdc14 (Figure 5C). The 6 Cdk consensus sites contained within the N-terminal region of Net1 were required for efficient disruption of the assembled RENT complex *in vitro* in that complexes isolated from

strains expressing wild-type Net1 were dismantled by Clb2–Cdk in a dose-dependent manner, whereas complexes isolated from *net1-6Cdk* cells were not (Figure 5D). Taken together, the *in vivo* and *in vitro* data indicate that Cdk phosphorylation sites on Net1 can drive RENT disassembly.

Slk19, Spo12, and Cdc5 modulate phosphorylation of Net1 on Threonine 212.

Analysis of Threonine 212 phosphorylation in synchronized cells revealed an unexpected result: appearance of Clb2 antigen preceded appearance of PP-B epitope by 10-20 min (Figure 3B). Because Clb2-associated protein kinase activity rises in parallel with Clb2 antigen (Stegmeier et al., 2002), we concluded that the ability of Clb2–Cdk to promote accumulation of phosphorylated Net1 must somehow be regulated. Moreover, because FEAR requires both the FEAR network genes and phosphorylation of Net1 by Clb1 or Clb2–Cdk complexes, and because ectopic induction of FEAR with *GAL1p-ESP1* induces Net1 hyperphosphorylation (Sullivan and Uhlmann, 2003), we hypothesized that the FEAR network acts, at least in part, through induction of Net1 phosphorylation by Clb2–Cdk. To test this hypothesis, we examined the Threonine 212 phosphoepitope in synchronized wild-type, *slk19Δ* and *spo12Δ* strains. The timing of phosphorylation was delayed in both mutants for at least 10-minutes, and there was a noticeable increase in duration of the phosphoepitope in the *slk19Δ* strain (Figure 6A). To distinguish the effects of the FEAR network and MEN on Cdc14, FEAR is typically monitored in a *cdc15-2* background (Stegmeier et al., 2002). Thus, we examined the kinetics of phosphorylation of Threonine 212 in *spo12Δ cdc15-2* and *slk19Δ cdc15-2* cells released from an α factor block. Both phosphorylation of Net1 on Threonine 212

(Figure 6B) and FEAR (Figure 6C) were greatly diminished in the double mutant cells compared to wild-type and *cdc15-2* cells.

We have previously reported that Cdc5 influences the phosphorylation state of Net1 and promotes the release of Cdc14 *in vivo* (Shou et al., 2002a). This regulation appears to operate at least in part through Clb-Cdk, because phosphorylation of Threonine 212 was absent in *cdc14-1 cdc5-1* double mutant cells (Figure 6D) and over-expression of stable Cdc5 promoted phosphorylation of Threonine 212 in nocodazole-arrested cells (Figure 6E). Thus, Cdc5, like Spo12 and Slk19, promotes the phosphorylation of Net1, at least in part, through Clb-Cdk.

Discussion

Phosphorylation of Net1 by Clb-Cdk underlies FEAR

Protein phosphatase Cdc14 plays a critical role in promoting exit from the M-phase state during both mitotic and meiotic cell cycles (Buonomo et al., 2003; Jaspersen et al., 1998; Marston et al., 2003). Cdc14 is kept under negative control during most of the cell cycle by binding to its nucleolar partner Net1, but is released from Net1 during anaphase through the actions of the FEAR and mitotic exit (MEN) networks (Shou et al., 1999; Stegmeier et al., 2002; Visintin et al., 1999). Because the transient release of Cdc14 from Net1 during anaphase is a signature event that drives exit from M-phase, we sought to investigate how this association is regulated in the context of the more well defined pathway for mitotic exit.

Net1 is a phosphoprotein whose phosphorylation correlates inversely with the activity of Cdc14 (Shou et al., 1999; Visintin et al., 2003). Mapping and mutation of *in*

vivo phosphorylation sites on Net1 (isolated from *cdc14ts* cells) revealed that a set of Cdk sites in the N-terminal portion of Net1 were required for the proper release of Cdc14 in early anaphase (FEAR). Phosphorylation of these Cdk sites in Net1 was catalyzed by Cdk complexes formed by the late mitotic cyclins Clb1 and Clb2, in that they were completely absent in *clb1Δ clb2Δ* and *cdc28-as1* mutants and that Clb2–Cdk was sufficient to bring about phosphorylation of Net1 and disassembly of the RENT complex both *in vivo* and *in vitro*. Interestingly, a proteomic screen by Morgan and colleagues revealed that Net1 is one of the best substrates for Clb2-Cdk *in vitro* (Ubersax et al., 2003). In accordance with the observation that the FEAR network is important for proper progression through meiosis, the FEAR-deficient *net1-6Cdk* mutant also displayed a sporulation defect reminiscent of the *slk19Δ* FEAR mutant. Net1 phosphorylation by Clb-Cdk is likely to be a recurrent theme of M-phase progression in budding yeasts, because the majority of the N-terminal Cdk phosphorylation sites in Net1 are highly conserved among orthologs found in other fungal species (Figure 10). A similar phosphorylation-based mechanism may likewise control the transient association of human Cdc14A with the nucleolus (Mailand et al., 2002).

FEAR network promotes phosphorylation of Net1 by Clb1,2-Cdk

Release of Cdc14 from Net1 during early anaphase requires the FEAR network components Spo12, Slk19, Cdc5, and Esp1 in addition to phosphorylation of Net1 by Clb1,2-Cdk. Since the phosphorylation of Net1 by Clb-Cdk lagged behind the accumulation of Clb2, we sought to test whether the FEAR network acts by switching on the phosphorylation of Net1 by pre-formed Clb-Cdk. Whereas phosphorylation of Net1

was only moderately delayed in both *spo12Δ* and *slk19Δ* mutants, it was severely impaired in *cdc5-1* mutants. We propose that either the FEAR network or the MEN can dissociate Cdc14 from Net1, and they act by independent mechanisms. FEAR network brings about RENT complex disassembly by promoting the phosphorylation of Net1 by Clb-Cdk. In contrast, the mild phenotype of the *net1-6Cdk* mutant indicates that the MEN can bring about release of Cdc14 from Net1 even in the absence of these same phosphorylations. Nevertheless, the MEN promotes phosphorylation of Net1 by Clb-Cdk, but possibly as an indirect consequence of exposing phosphorylation sites on Net1 that are normally shielded by bound Cdc14. Thus, whereas Net1 phosphorylation by Clb-Cdk is required for FEAR, we propose the same phosphorylations can occur as a passive consequence of Cdc14 release that is triggered by the MEN via a distinct mechanism. Consistent with this interpretation, in the absence of both the FEAR and mitotic exit networks (*spo12Δ cdc15-2*, *slk19Δ cdc15-2*, and *cdc5-1* mutants), Net1 phosphorylation on Cdk sites was drastically reduced. It remains unknown how the FEAR network promotes phosphorylation of Net1 by Clb-Cdk. The putative 'effector' components of the FEAR network, Cdc5 and Spo12 (Sullivan and Uhlmann, 2003; Visintin et al., 2003), may modulate Net1, Clb-Cdk, or a Net1 phosphatase to promote accumulation of phosphate groups on Net1.

Is Net1 phosphorylation by Clb–Cdk sufficient for FEAR?

Our data indicate that FEAR components Spo12, Slk19, and Cdc5 act, at least in part, through phosphorylation of Net1 by Clb–Cdk to promote disassembly of the RENT complex. This raises two important, related questions: is activation of Net1

phosphorylation the only function of the FEAR, and is phosphorylation of Net1 on Cdk sites sufficient to dislodge Cdc14? Mutant *spo12Δ* cells have a sporulation defect that is more severe than that of *net1-6Cdk* mutants, suggestive of additional roles for Spo12 that go beyond phosphorylation of Net1. Moreover, the early anaphase release of Cdc14 promoted by the FEAR network in a *cdc15-2* mutant is transient (Stegmeier et al., 2002), whereas the phosphorylation of Net1 on Threonine 212 is not (Figure 6). Thus, although the FEAR network promotes phosphorylation of Net1 and dissociation of Cdc14 from Net1, the former does not appear to be sufficient for the latter. On the other hand, Clb2–Cdk was sufficient to dislodge Cdc14 from Net1 *in vitro*, and over-expression of Clb2 brought about release of Cdc14 from Net1 in nocodazole-arrested cells (in which the FEAR network is inactive due to inhibition of Esp1 by securin). One possible explanation is that the FEAR network mobilizes Clb–Cdk to promote stable phosphorylation of Net1 and labile phosphorylation of a second target (possibly Cdc14?), and that both of these phosphorylations are needed to sustain release of Cdc14 from Net1 *in vivo*. Attempts to resolve this issue through expression of *NET1* mutants bearing phosphomimetic substitutions have been thwarted by the apparent hypomorphic nature of these alleles (data not shown).

Regardless of whether phosphorylation of Net1 by Clb2–Cdk is sufficient to dismantle RENT *in vivo*, there clearly is yet another mysterious dimension to this regulatory mechanism, in that Clb2 over-expressed in G1 phase promoted neither release of Cdc14 from Net1 nor phosphorylation of Net1. Thus, there appears to be at least one cell cycle-regulated factor in addition to the FEAR network that controls the ability of Clb2–Cdk to promote accumulation of phosphate on Net1. Despite these unsolved

issues, it is clear that phosphorylation of Net1 by Clb–Cdk is a key mechanism by which the FEAR network instigates disassembly of the RENT complex during mitosis.

On the roles of Cdc5 and Clb-Cdk protein kinase activities in FEAR

In previous work, we reported that Cdc5 influences the phosphorylation of Net1 and disassembly of the RENT complex (Shou et al., 2002a). Our current observations that Cdc5 is necessary and sufficient for phosphorylation of Net1 by Clb–Cdk lead us to propose that Cdc5 controls Net1 phosphorylation primarily through Clb–Cdk. However, to evaluate the relative role of Cdc5 in more detail requires careful consideration of our prior findings. To address the role of Cdc5 in RENT disassembly *in vivo*, we originally constructed a mutant Net1 (*net1-7m*) lacking the 7 sites of *in vitro* phosphorylation that contributed most prominently to the Cdc5-induced dissociation of recombinant Cdc14–Net1 complexes (Shou et al, 2002). Mutant *net1-7m* cells exhibited both the FEAR and MEN components of Cdc14 release from the nucleus during mitosis, but the former was diminished by approximately 25%. Although this result could arise directly from failure of Cdc5 to phosphorylate Net1, it could also be an indirect consequence of mutating multiple sites in Net1, given that none of the sites mutated in *net1-7m* were found in this study to be phosphorylated *in vivo*. Interestingly, of the 17 sites on Net1 that were definitively identified as being phosphorylated by Cdc5 *in vitro* (Loughrey Chen et al., 2002), only two (S231 and S259) were confirmed here to be modified *in vivo*. A mutant lacking S169, S231 and S259 (*net1-3Ax*) (the non-Cdk sites mutated in *net1-6m*) displayed an extremely modest (~20%) defect in FEAR (Figure 7C). Thus, Cdc5 may promote FEAR both by enabling the action of Clb-Cdk, and by direct

phosphorylation of Net1. However, our *cis* mutant analysis of Net1 clearly indicates that the former role is critical, and the latter role is, at best, minor. We wish to note that the putative Cdc5 phosphorylation sites (S231 and S259) do not lie in the recently reported consensus for Cdc5/Polo kinases (Nakajima et al, 2003), and thus it remains possible that these sites may normally be phosphorylated by an unknown protein kinase *in vivo*, and Cdc5 may act solely through Clb–Cdk.

Although the action of the FEAR network is not essential for mitotic cell cycles, its activity helps to determine the timing of exit from mitosis (Stegmeier et al., 2002). Our finding that phosphorylation of Net1 by Clb-Cdk underlies disruption of the RENT complex during anaphase illustrates a fascinating aspect of the switch that governs the return of mitotic cells to an interphase state. Although Clb-Cdk initiates feedback loops that help sustain a mitotic state with high Clb-Cdk activity (Deshaies, 1997), there must be mechanisms for subverting the reign of Clb-Cdk to allow growing cells to continue to cycle. Fittingly, at least two of these mechanisms – activation of Cdc20 binding to APC (Rudner et al., 2000; Rudner and Murray, 2000) and disruption of the Cdc14/Net1 complex – are initiated directly by the very enzyme whose activity helps to specify the mitotic state in the first place. Given that *clb1Δ clb2Δ* mutants display an anaphase defect far more severe than *net1-6Cdk* indicates that other aspects of this crucial switch await discovery.

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Experimental Procedures

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*) except where noted in the strain table (Supplementary Table 1). A strain expressing a stable form of Clb2 lacking both the KEN and destruction boxes ($Clb2C_2DK_{100}$)HA3 was used in over-expression experiments with Clb2 (Hendrickson et al., 2001).

Net1 mutant constructs were created as previously described (Shou et al., 2002a). Briefly, a wild-type *NET1-myc9* epitope tagged construct was cloned into a modified pRS304 vector containing 300bp upstream of the ATG translation start site using NcoI and EagI. Site-directed mutagenesis of Serine/Threonine to Alanine was carried out

using QuikChange Site-Directed mutagenesis kit from Stratagene (La Jolla, CA). The indicated Serine/Threonine were mutated to Alanine in Net1-13m (166,169,212,231,252,259,356,362,384,385,497,611,676), Net1-6m (166,169,212,231,252,259), Net1-3-Cdk (166,212,252), and Net1-6Cdk (62*, 166,212,252,297,304) where * indicates that residue 62 was mutated to ensure complete elimination of all Cdk consensus sites even though it was not determined to be phosphorylated *in vivo*. Mutagenesis was confirmed using restriction digests followed by DNA sequencing. All constructs were targeted by linearization with BstXI to the *trp1* locus in a *NET1*/*net1::his5*$^{+}$ heterozygous diploid. The strains were sporulated and tetrads were dissected to obtain a haploid isolate of the integrant over *net1::his5*$^{+}$. Copy number of integrants was estimated by normalizing extract protein from transformed and wild-type cells and blotting for Net1 levels. Proper localization of all *net1* phosphosite mutants was confirmed by indirect immuno-fluorescence against the myc epitope (data not shown).

Production and purification of antibodies made against phosphorylated peptides were performed by Abgent (San Diego, CA). A pair of peptides – phosphorylated or not at the indicated, underlined residue – was synthesized to generate and purify each of the following antibodies against Net1: anti-phosphopeptide A corresponding to aa 159-173 (RSKLNNG<u>S</u>PQSVQPQC); anti-phosphopeptide B corresponding to aa 205-219 (NGSMRVW<u>T</u>PLARQIYC); and anti-phosphopeptide C corresponding to aa 245-259 (PPPTQPQ<u>S</u>PPIRISSC). All peptides contained a Cysteine at the C-terminus, and anti-phosphopeptide B was modified by replacing Serine with Tryptophan at the –1 position relative to the underlined Threonine to optimize the phosphoepitope presentation.

Antibodies specifically reactive against the phosphopeptides were positively selected on a resin derivatized with the phosphopeptide immunogen and negatively selected by passage through a resin derivatized with the unphosphorylated version of the peptide. Anti-phosphopeptide B (α-PP-B) was used in all experiments described since it generated the strongest signal against Phospho-Net1 (Figure 3A).

Cell Growth and Synchronization Procedures

Cells were grown in yeast extract-peptone (YP) or in yeast minimal (YM) media containing 2% glucose (YPD,YMD), 2% raffinose (YPR,YMR) or 2% galactose (YPG,YMG) as carbon source. Where appropriate, minimal media were supplemented with leucine, histidine, tryptophan, uracil, and adenine to complement auxotrophies. Synchronization of cells in G1 phase was achieved with α-factor added at 10 μg/ml for *BAR1* cells and 0.1 μg/ml for *bar1Δ* cells for at least 3 hrs at 25°C. Cells were judged to be arrested when greater than 90% of cells displayed the elongated "shmoo" phenotype. Cells were released from α factor by filtration through a 0.2 μm filter followed by a wash with 150 ml of YP, then resuspended in the desired volume at a density of 1 $O.D._{600}$/ml. For elutriation, cells were grown overnight in YP containing 2% raffinose and 2% galactose and harvested at log phase. Elutriation was performed as described (Amon, 2002; Johnston and Johnson, 1997; Walker, 1999) for the collection of small, unbudded G1 cells; contamination with budded cells was measured to be no more than 2%. For galactose induction experiments, cells were grown overnight in either YMR or YPR until an $O.D._{600}$ of 1.0 was reached, then induced with 2% galactose followed by time point collection.

Cell Extract Preparation and Western Blotting

Cells were grown to an $O.D._{600}$ of 1.0, and for every time point 2 ml of culture was collected and TCA added to a final concentration of 20%. Cells were collected by centrifugation and washed with 2 ml of Tris-HCl (pH 7.5). SDS loading buffer [70 µl of 100mM Tris-HCl (pH 7.5), 20% glycerol, 4% SDS, 2M Urea, 200mM DTT] was added, tubes were boiled for 3-minutes, and 100 µl of acid-washed glass beads (500 µm) were added to each tube followed by boiling for an additional 2-minutes. Tubes were vortexed for 45 sec using a Bio 101 multi-bead vortexer at setting 5.5. Tubes were boiled again for 2-minutes and 5 µl of sample was fractionated on a 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane. Western blot analysis was performed with the following primary antibodies at the indicated dilutions: All anti-phospho Net1 antibodies (α-PP-A, α-PP-B, α-PP-C) at 2 µg/ml; anti-Clb2 (1:3000), anti-Cdc28 (1:5000), anti-Clb3 (1:2000), anti-myc (9E10) (1:5000), anti-His (1:250), anti-HA (1:5000), and anti-Cdc14 (1:1000).

Immunoprecipitation and Clb2–Cdk release/kinase assay.

To prepare extracts for immunoprecipitation, 10 $O.D._{600}$ units of a log phase cell culture was harvested and washed with 2 ml of Tris-HCl (pH 7.5). Cells were re-suspended in 500 µl lysis buffer [25 mM HEPES/KOH (pH7.5), 150 mM NaCl, 1 mM DTT, 0.2% Triton, 1 mM EDTA, 1 mM PMSF, 1 mM Benzamidine, 1x Protease Inhibitor Cocktail (Aprotinin, Chymostatin, Leupeptin, and PepstatinA all at 5 µg/ml in 90% DMSO)], transferred to a flat-bottom 2 ml tube and supplemented with 100µl of

acid-washed glass beads (500 μm). Samples were vortexed using a Bio 101 multi-beads vortexer at setting 5.5 (speed) and 45sec (time). Tubes were then centrifuged for 5-minutes at 14,000 rpm and the supernatant was collected. Clarified extract (400 μl) was incubated with 60 μl of 9E10-coupled protein A beads for 1 hour on a rotator at 4°C. Beads were collected and washed ten times in wash buffer [25 mM HEPES/KOH (pH7.5), 150 mM NaCl, 1 mM DTT, 0.2% Triton], and divided to approximately 15 μl beads per reaction condition. For protein kinase assays, 3 μl of either Clb2–Cdk or Clb5–Cdk was used with either 1 μg of myc9-Net1 (purified from insect cells infected with a recombinant baculovirus) (Shou et al., 2001), or 5 μg of Histone H1. For assays that monitored release of Cdc14 from bead-bound Net1-myc9, varying concentrations of *in vitro* assembled Clb2–Cdk in 30μl kinase buffer [25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 0.1 mg/ml BSA, 50 mM NaCl] were mixed with 15 μl 9E10 beads coated with RENT complex. Reactions were allowed to proceed for 30-minutes on a rotator at 25°C. Supernatant and beads were processed for Western blot analysis as previously described (Shou et al., 2002a). For *in vitro* assays with bacterially expressed constructs, approximately 116 ng of Net1 per 20 μl of Ni^{+2}-NTA beads, 10 ng Cdc14, and 5 μl of roughly 30 ng/μl stock of Clb2–Cdk was used per reaction.

Immuno-fluorescence and Cdc14 release quantification

Immuno-fluorescence was performed as previously described (Shou et al., 2002a; Shou et al., 1999). The analysis of Cdc14 localization for (Figure 1) was performed in haploid cells carrying the transposon-mutagenized *net1* allele. Rabbit anti-Cdc14 (1/3000) and rat anti-tubulin monoclonal antibody YL1/34 (1/1000) were used at the

indicated dilutions. Images of synchronized cells at 70 to 110 minutes following release from α factor were collected on a Zeiss Axioskop or Axiovert 200M microscope using a Hamamatsu CCD digital camera. Spindle length measurements were performed using Zeiss Axiovision software.

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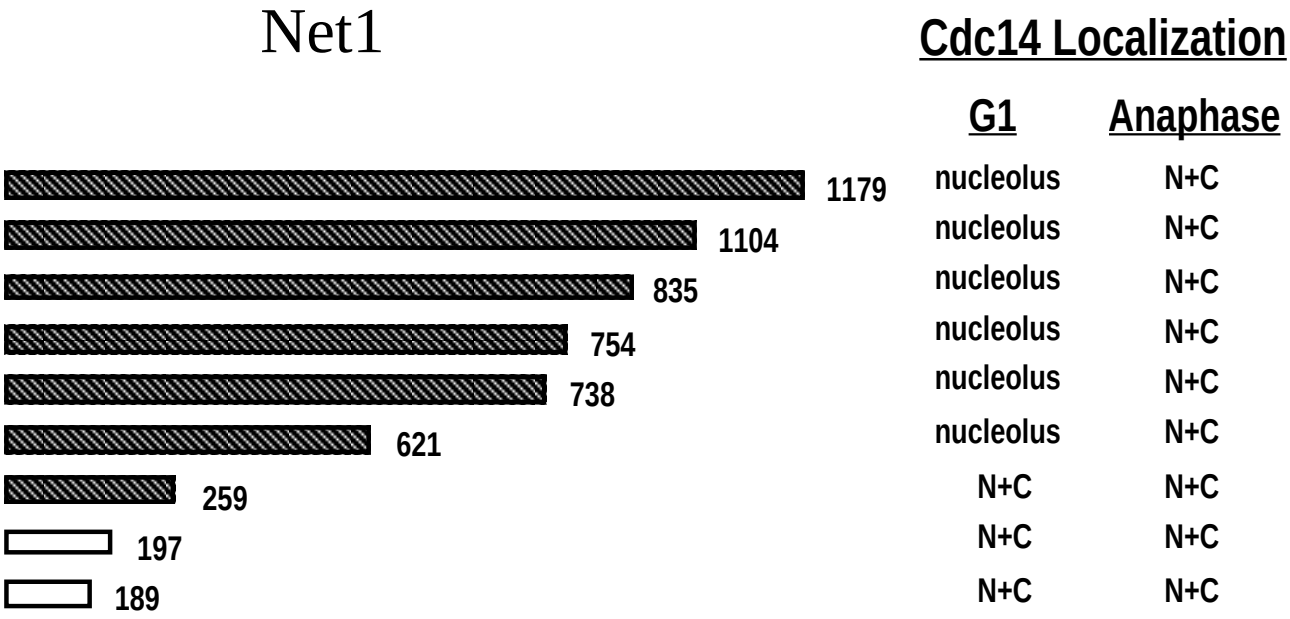

Net1
Cdc14 Localization
G1
Anaphase
1179
nucleolus
N+C
1104
nucleolus
N+C
835
nucleolus
N+C
754
nucleolus
N+C
738
nucleolus
N+C
621
nucleolus
N+C
259
N+C
N+C
197
N+C
N+C
189
N+C
N+C

Figure II-1

Figure II-1: Cell cycle-regulated binding site for Cdc14 resides in the N-terminal half of Net1

Transposon insertion and truncation mutants of Net1 define a cell cycle-regulated Cdc14–binding domain. The numbers adjacent to each construct indicate where the transposon insertion occurred in the *NET1* locus with the exception of the aa 1-621 HA-fragment of Net1 in which endogenous *NET1* was replaced by the truncated allele (RJD1783). The localization of Cdc14 and the length of microtubule spindles in asynchronous cell populations were determined by indirect immuno-fluorescence using anti-Cdc14 and anti-tubulin antibodies, respectively. Cell cycle position was estimated from the length of the microtubule spindle. The hatched bars indicate fragments of Net1 with proper nucleolar localization as reported in the TRIPLES database (Ross-Macdonald et al., 1999). 'N+C' refers to both nuclear and cytoplasmic staining.

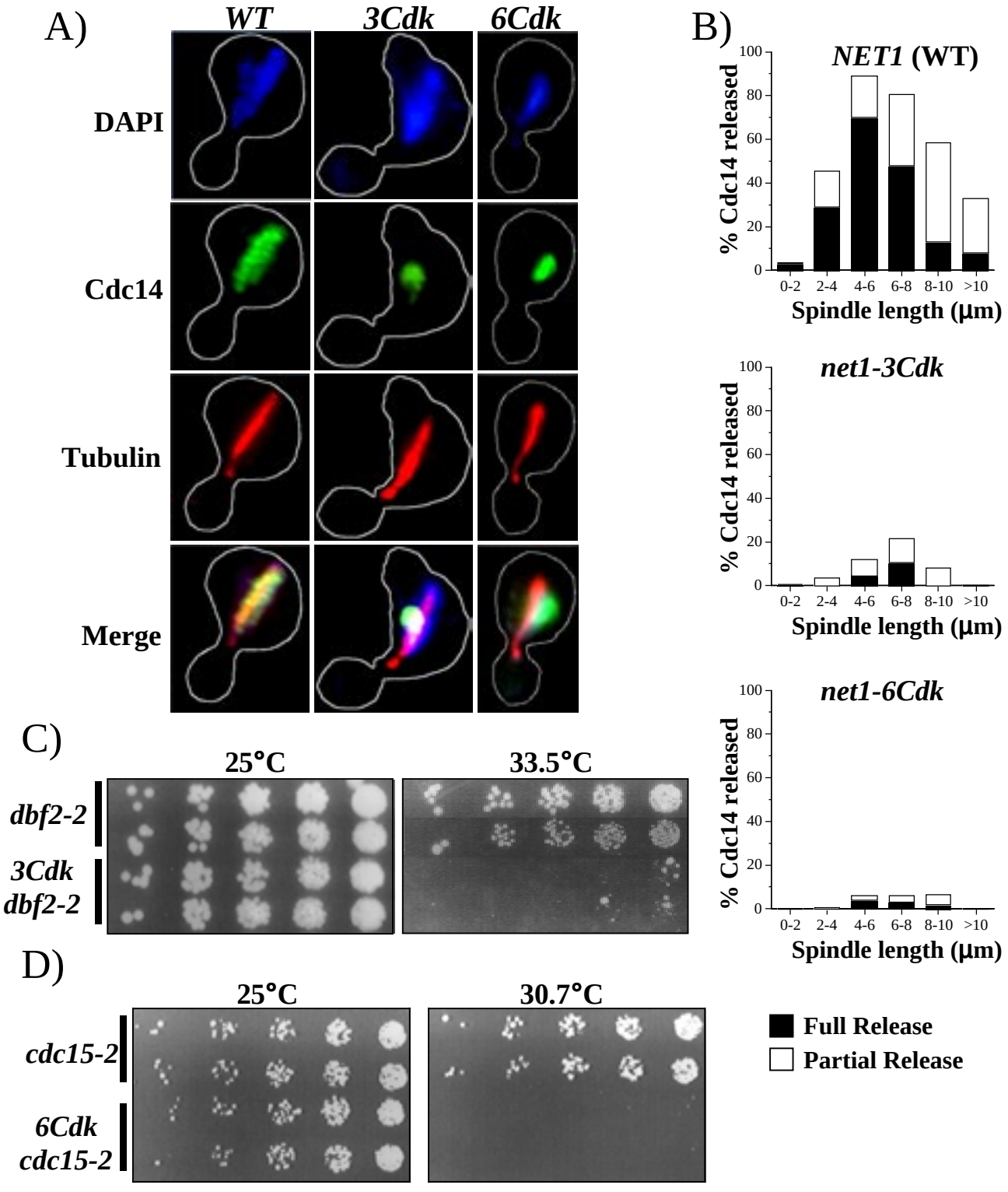

A)
WT
3Cdk
6Cdk
DAPI
Cdc14
Tubulin
Merge
B)
NET1 (WT)
net1-3Cdk
net1-6Cdk
% Cdc14 released
Spindle length (µm)
0-2
2-4
4-6
6-8
8-10
>10
Full Release
Partial Release
C)
25°C
33.5°C
dbf2-2
3Cdk dbf2-2
D)
25°C
30.7°C
cdc15-2
6Cdk cdc15-2

Figure II-2

Figure II-2: Net1 phosphorylation site mutants display Cdc14 release defects and synthetic interactions with MEN mutants.

(A) Net1 mutants are defective in Cdc14 release in early anaphase. Mutant *cdc15-2* cells carrying either *NET1* (WT) (RJD2617), *net1-3Cdk* (*3Cdk*) (RJD2613), or *net1-6Cdk* (*6Cdk*) (RJD2614) alleles were synchronized with α factor at 25°C and released in YP 2% glucose at 37°C. Cells were collected at 10 to15 minute time intervals for analysis by indirect immuno-fluorescence. Staining was performed with DAPI, anti-Cdc14 and anti-tubulin antibodies for determination of nuclear position, Cdc14 localization, and microtubule spindle length, respectively. Cell outlines are indicated for comparison.

(B) Quantitation of Fourteen Early Anaphase Release (FEAR) defect of Net1 phosphorylation site mutants. Synchronized cells collected at 70 to110 minutes after release from α factor were double-labeled with anti-Cdc14 and anti-tubulin antibodies. Spindle length was measured and localization of Cdc14 was determined to be either: 1, full release (black boxes; complete release of Cdc14 from the nucleolus into the nucleus); or 2, partial release (white boxes; Cdc14 was nuclear in one of the DAPI masses and nucleolar in the other DAPI mass in the same cell, or Cdc14 was not completely restricted to the nucleolus in either DAPI mass). Over 350 cells were counted for each panel.

(C) Mutant *net1-3Cdk* enhances the temperature-sensitive growth phenotype of *dbf2-2*. Starting with 3000 cells, 3-fold serial dilutions of *dbf2-2* (RJD2625) and *dbf2-2* carrying a *net1-3Cdk* allele (RJD2626) were spotted on YPD plates from right to left, and

incubated at the indicated temperature for 2-3 days before the picture was taken. Two independent isolates of each strain were used. The first two isolates in the 33.5°C panel were compiled from different sections of the same plate.

(D) Mutant *net1-6Cdk* exacerbates the temperature-sensitive growth phenotype of *cdc15-2*. Starting with 3000 cells, 3-fold serial dilutions of *cdc15-2* (RJD2610) and *cdc15-2* carrying a *net1-6Cdk* allele (RJD2614) were spotted on YPD plates as described for panel (C).

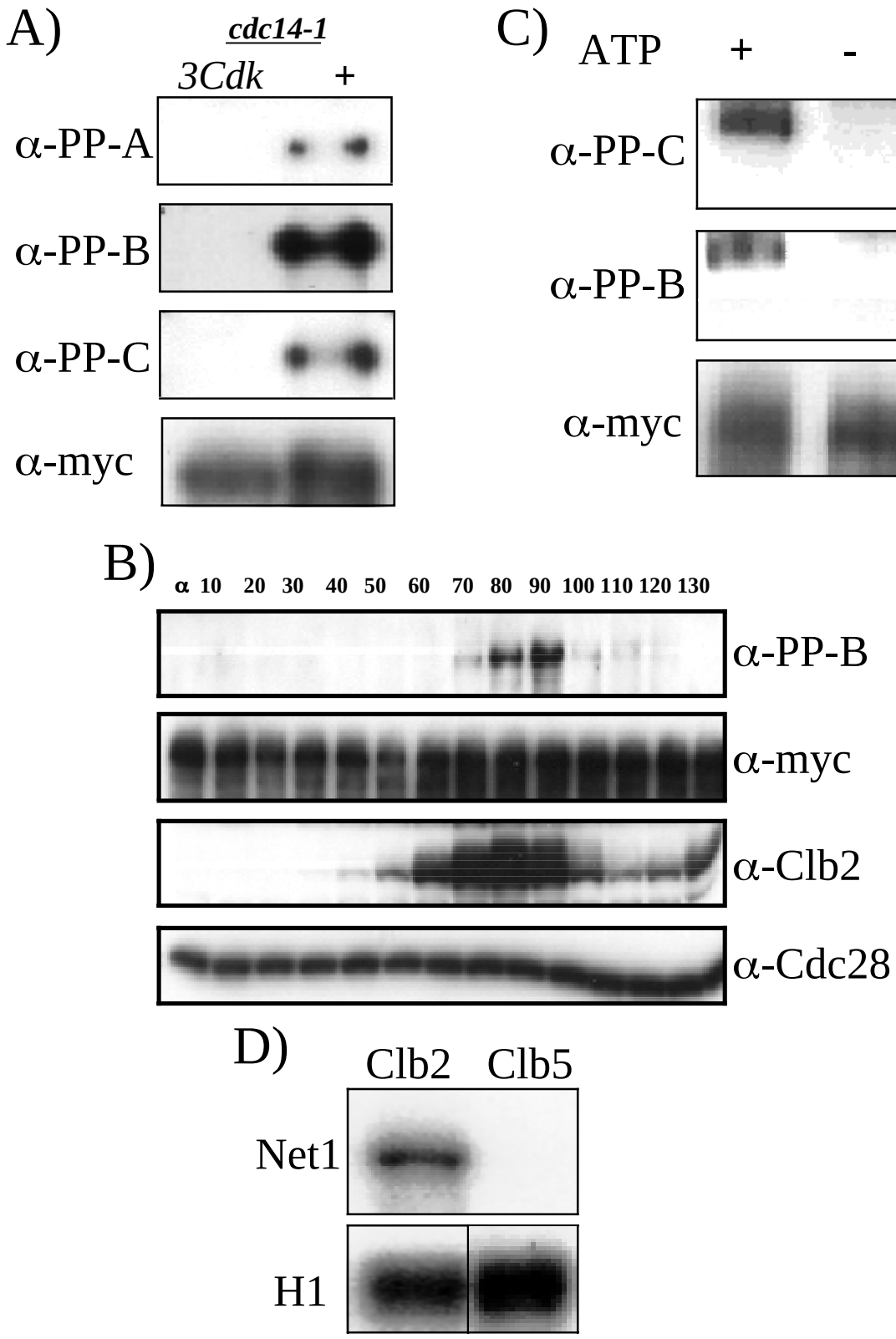

A)
cdc14-1
3Cdk
+
α-PP-A
α-PP-B
α-PP-C
α-myc
C)
ATP
+
-
α-PP-C
α-PP-B
α-myc
B)
α 10 20 30 40 50 60 70 80 90 100 110 120 130
α-PP-B
α-myc
α-Clb2
α-Cdc28
D)
Clb2
Clb5
Net1
H1

Figure II-3

Figure II-3: Net1 phosphorylation by Clb2–Cdk is cell cycle regulated and sufficient for disruption of Cdc14 binding *in vitro*.

(A) Phospho-specific antibodies to Net1. Three phospho-specific antibodies were raised against peptides containing either phosphorylated Serine166 (α-PP-A), Threonine 212 (α-PP-B), or Serine 252 (α-PP-C). Crude extracts from *cdc14-1* strains carrying either a wild-type (+) (RJD2615) or a mutant (3Cdk) *NET1-myc9* allele (RJD2616) in which the three sites were converted to Alanine were fractionated by SDS-PAGE and immunoblotted with the different antibodies to evaluate specificity. Anti-myc (9E10) detection of Net1-myc9 was used as a loading control.

(B) Net1 phosphorylation on Threonine 212 is cell cycle regulated. Cells arrested in α factor were released from G1 block and samples were collected at the indicated time points (min). Phosphoepitope formation, Net1-myc9, and Clb2 levels were monitored by fractionating crude cell extracts by SDS-PAGE and immunoblotting with α-PP-B, α-myc (9E10), and α-Clb2 antibodies, respectively. Cdc28 levels (α-Cdc28) were used as a loading control.

(C) Clb2–Cdk generates the *in vivo* phosphoepitopes *in vitro*. Net1-myc9 was immunoprecipitated from α factor-arrested cells and the bead-bound protein was treated with protein kinase Clb2–Cdk in the presence (+) or absence (-) of ATP. The bead-bound material was fractionated by SDS-PAGE and immunoblotted with α-PP-B and α-PP-C

antibodies to monitor formation of the phosphoepitopes on Net1. Anti-myc (9E10) detection of Net1-myc9 was used as a loading control.

(D) Net1 is a substrate for Clb2–Cdk *in vitro*. Clb2–Cdk and Clb5–Cdk protein kinase complexes were incubated *in vitro* with Net1 and radiolabeled ^{32}P (top panel). The amounts of each kinase complex used for this experiment were matched using histone H1 as a substrate (bottom panel).

A)

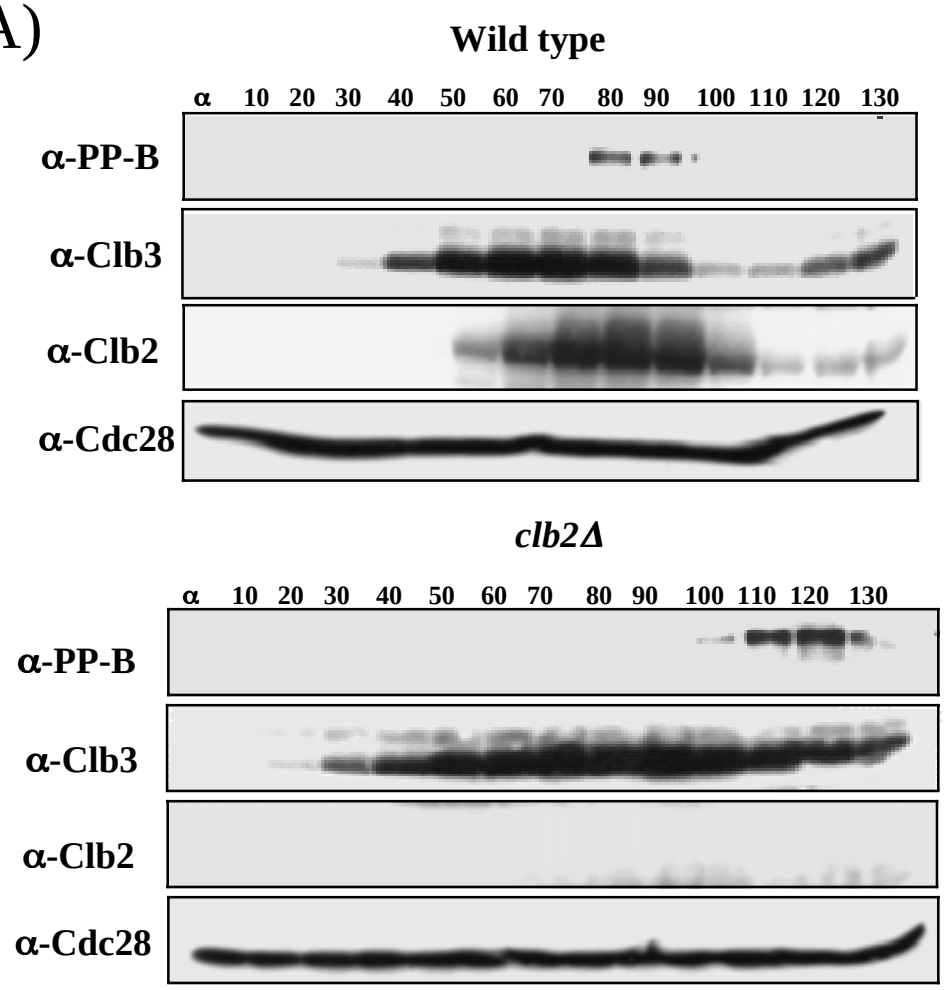


Figure II-4A

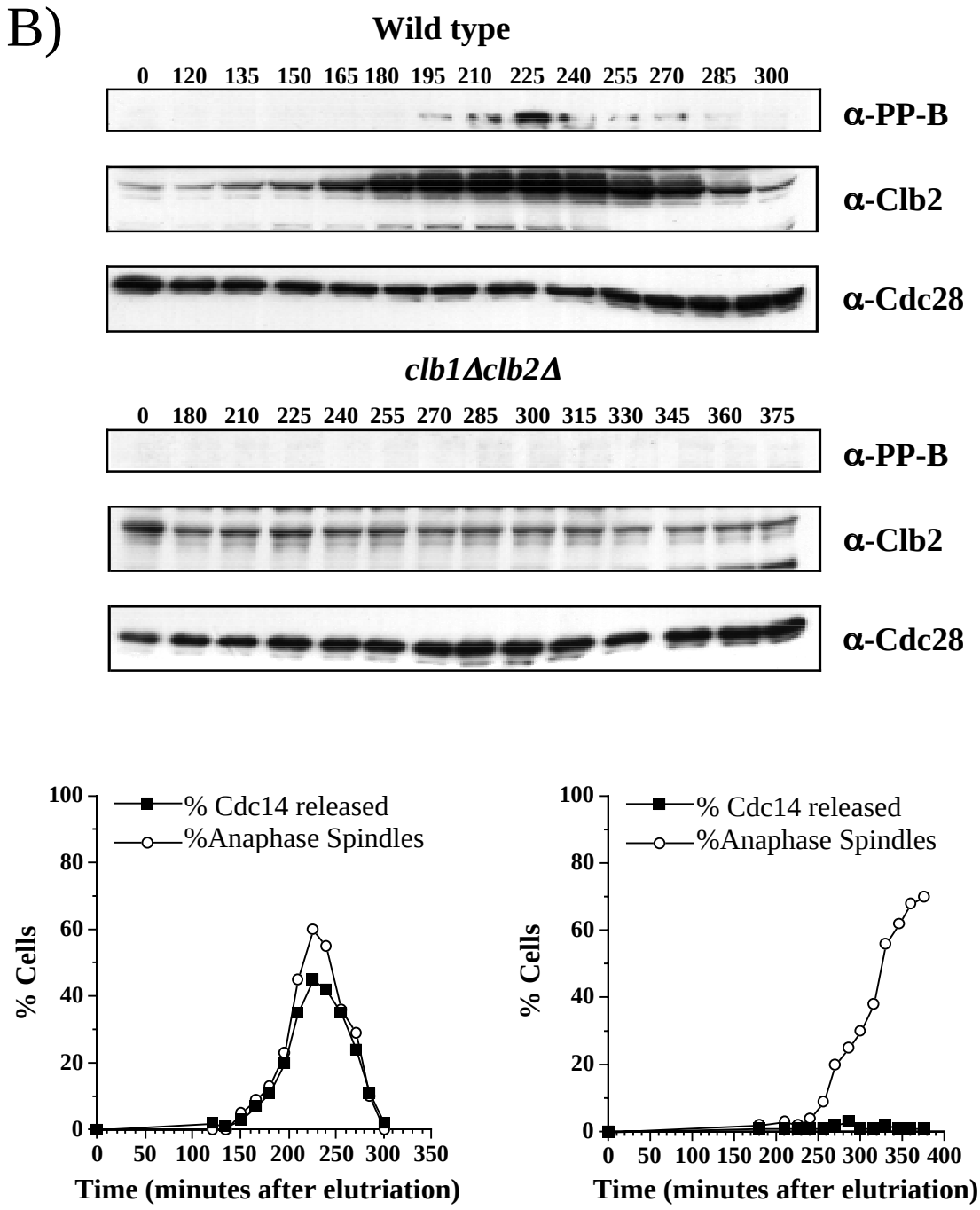

B)
Wild type
0 120 135 150 165 180 195 210 225 240 255 270 285 300
α-PP-B
α-Clb2
α-Cdc28
clb1Δclb2Δ
0 180 210 225 240 255 270 285 300 315 330 345 360 375
α-PP-B
α-Clb2
α-Cdc28
% Cdc14 released
%Anaphase Spindles
% Cells
Time (minutes after elutriation)
% Cdc14 released
%Anaphase Spindles
% Cells
Time (minutes after elutriation)

Figure II-4B

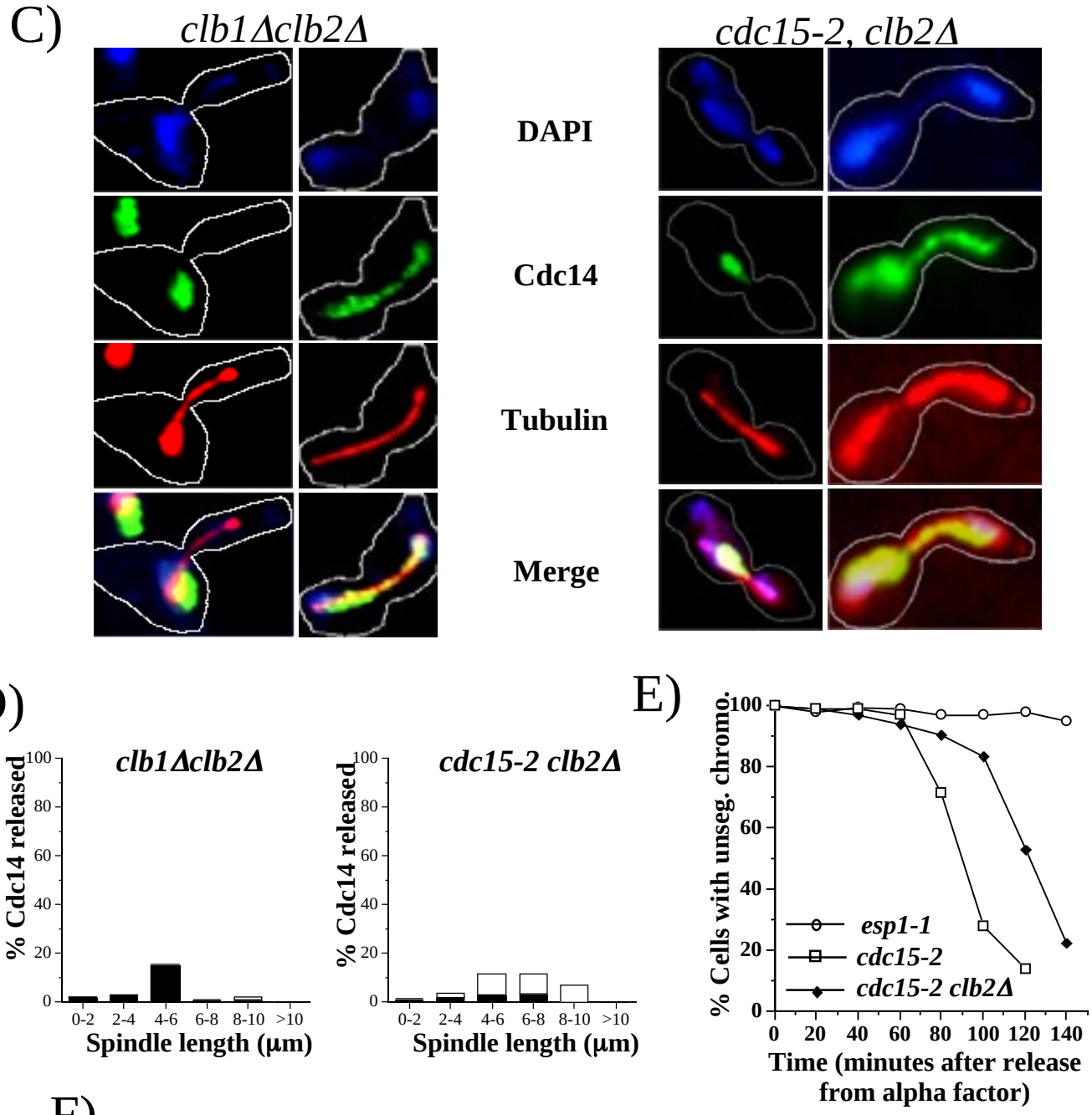


Figure II-4C, D, E, F

Figure II-4: Clb1 and Clb2 control the timing and appearance of the phosphoepitope on Net1.

(A) Mutant *clb2Δ* cells have a prolonged delay in phosphoepitope formation. Isogenic wild-type (RJD2617) and *clb2Δ* (RJD2623) strains were synchronized in α factor and released. Samples were collected at 10-minute intervals, analyzed by SDS-PAGE, and immunoblotted with the indicated antibodies. Alpha factor was added back after 70-minutes to prevent cells from entering into a second cell cycle. Phosphoepitope formation was monitored by α-PP-B. Appearance of Clb3 antigen (α-Clb3) was used as a marker for cell cycle progression. Cdc28 levels (α-Cdc28) were used as a loading control.

(B) Phosphoepitope formation on Net1 is abolished in *clb1Δ clb2Δ* cells. Wild-type or *clb1Δ clb2Δ GAL1p-CLB2* (*clb1Δ, clb2Δ*) (RJD2624) cells were grown overnight in YP 2% raffinose plus 2% galactose and harvested for elutriation. Elutriation was performed as described (Johnston and Johnson, 1997; Walker, 1999). Elutriated G1 phase cells were inoculated into YP 2% glucose. Alpha factor was added to wild-type cells after 180-minutes in YP 2% glucose. Alpha factor was also added to *clb1Δ, clb2Δ* cells after 300-minutes in YP 2% glucose to prevent them from initiating a second cell cycle. At each indicated time point, cells were collected for Western blot analysis.

Phosphoepitope formation and levels of Clb2 were monitored by α-PP-B and α-Clb2, respectively. Cdc28 levels (α-Cdc28) were used as a loading control. Cells were also subjected to indirect immuno-fluorescence with anti-Cdc14 and anti-tubulin antibodies as previously described.

(C) Impaired release of Cdc14 in early anaphase in *clb1Δ clb2Δ* and *cdc15-2 clb2Δ* mutants. Samples of *clb1Δ clb2Δ GAL1p-CLB2* and *cdc15-2 clb2Δ* cells (RJD 2622) were collected after elutriation/*GAL*-shutoff or α factor block/release, respectively, for analysis by indirect immuno-fluorescence with DAPI, anti-Cdc14, and anti-tubulin antibodies. Two panels are shown for each mutant cell line, representing early anaphase (1st panel) and late anaphase (2nd panel). Cell outlines are indicated for comparison.

(D) Fourteen Early Anaphase Release (FEAR) defect of *clb1Δ clb2Δ* and *cdc15-2 clb2Δ* cells. Cells were collected after elutriation and *GAL* shutoff (*clb1Δ clb2Δ GAL1p-CLB2*) or α factor release (*clb2Δ cdc15-2*) and double-labeled with anti-Cdc14 and anti-tubulin antibodies. Spindle length was measured and release of Cdc14 from the nucleolus was determined to be either complete (black boxes) or partial (white boxes; see legend to Fig. 2B). Over 350 cells were counted for each panel.

(E) Mutant *cdc15-2 clb2Δ* cells arrest in late anaphase. Mutant *cdc15-2* cells (open squares) (RJD2630), *cdc15-2 clb2Δ* cells (closed diamonds) (RJD2631), and *esp1-1* cells (open circles) (RJD2629) containing a *tetO112* array at the *URA3* locus and expressing 3tetR-GFP from the *HIS3* locus were synchronized in G1 phase with α factor and released into YP 2% glucose at 37°C. Samples were taken at the indicated time points to determine the percentage of cells with unsegregated chromosomes (one GFP dot) compared to cells that have segregated their chromosomes (two GFP dots).

(F) Mutant *clb2Δ* exacerbates the temperature-sensitive growth phenotype of Mitotic Exit Network (MEN) mutants. Starting with 3000 cells, 3-fold serial dilutions of *cdc15-2* (RJD602) and *cdc15-2 clb2Δ* cells (RJD2622) were spotted on YPD plates as described in the legend of Fig. 2C. Three independent isolates of each strain were used.

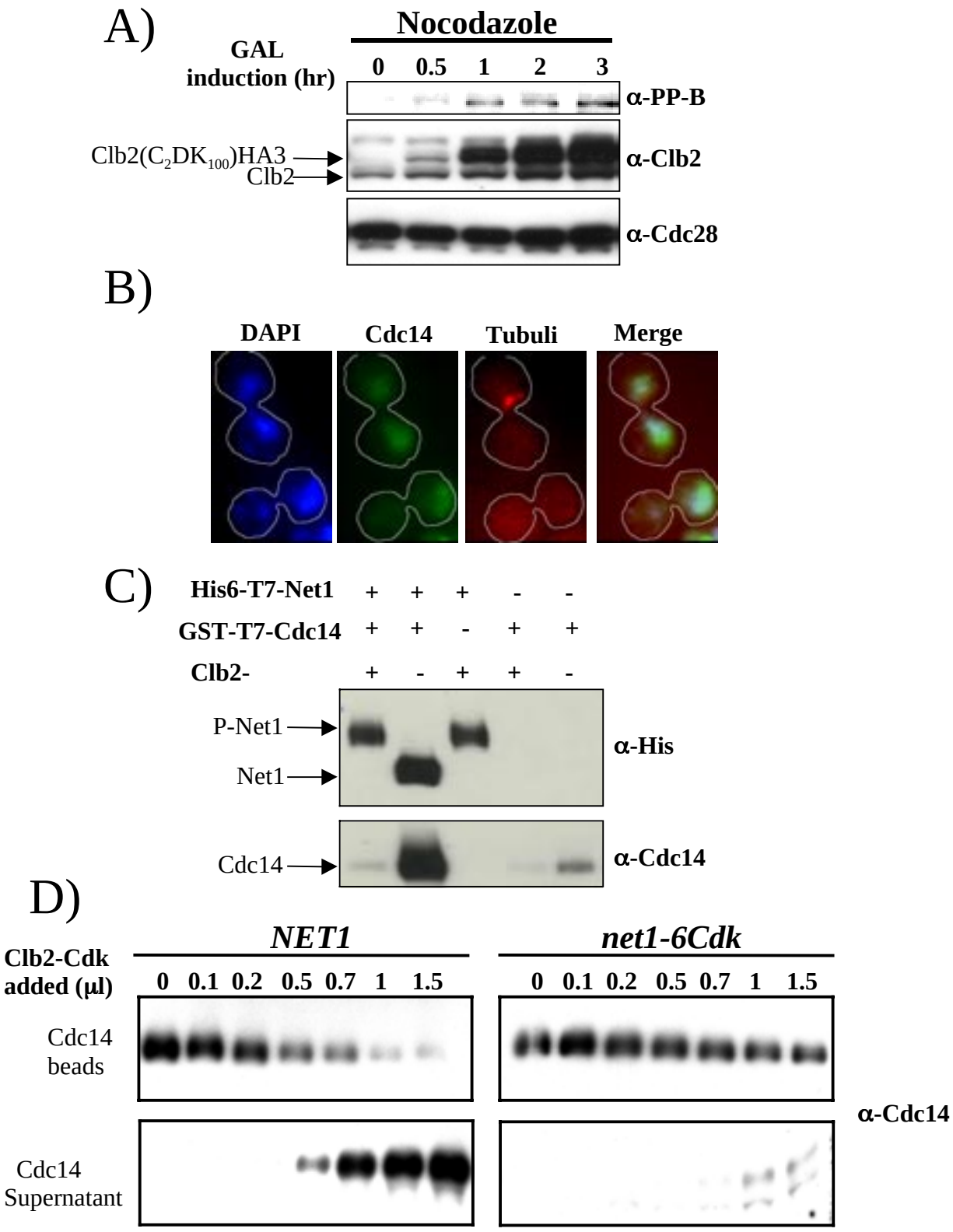

A)
Nocodazole
GAL induction (hr)
0 0.5 1 2 3
α-PP-B
Clb2(C2DK100)HA3
Clb2
α-Clb2
α-Cdc28
B)
DAPI
Cdc14
Tubuli
Merge
C)
His6-T7-Net1 + + + - -
GST-T7-Cdc14 + + - + +
Clb2- + - + + -
P-Net1
Net1
α-His
Cdc14
α-Cdc14
D)
NET1
net1-6Cdk
Clb2-Cdk added (μl)
0 0.1 0.2 0.5 0.7 1 1.5
Cdc14 beads
Cdc14 Supernatant
α-Cdc14

Figure II-5

Figure II-5: Over-expression of Clb2 is sufficient to drive Cdc14 out of the nucleolus in cells arrested in mitosis with microtubule poisons.

(A) Over-expression of a stable form of Clb2 promotes Net1 phosphorylation on Threonine 212. *NET1-myc9* cells carrying a galactose-inducible cassette that encodes a stabilized form of Clb2 that lacks both the destruction and KEN boxes (*GAL1p-CLB2C$_2$DK$_{100}$*) (RJD2627) were arrested with 30μg/ml benomyl plus 15μg/ml nocodazole at 23°C for 3 hours in YP 2% raffinose. At various times after galactose was added to induce expression of Clb2C$_2$DK$_{100}$, samples were withdrawn, fractionated by SDS-PAGE, and immunoblotted with α-PP-B, α-Clb2, and α-Cdc28 as previously described.

(B) Over-expression of a stable form of Clb2 promotes release of Cdc14 from the nucleolus in cells arrested in mitosis. Cells were arrested in mitosis with benomyl and nocodazole as described for panel (A), and samples were collected at the indicated time points for indirect immuno-fluorescence. Staining was performed using DAPI together with anti-Cdc14 and anti-tubulin antibodies as previously described. Cell outlines are indicated for comparison.

(C) Phosphorylation of Net1 (aa 1-601) by Clb2–Cdk disrupts Cdc14 binding *in vitro*. Proteins were expressed and purified from bacteria as described (Shou et al., 2002a). His6-T7-Net1 was captured on Ni^{+2}-NTA beads and either treated with Clb2–Cdk or buffer prior to addition of GST-T7-Cdc14. After a 30-minute incubation at 25°C, the NTA beads were sedimented, washed 5 times with wash buffer, and processed for SDS-

PAGE analysis and subsequent Western blotting. Amounts of Net1 and Cdc14 bound to NTA beads were determined by blotting with α-His and α-Cdc14, respectively.

(D) RENT complex containing phosphosite mutant Net1-6Cdk is refractory to disassembly by Clb2–Cdk *in vitro*. RENT complexes from isogenic *NET1-myc9* and *net1-6Cdk-myc9* cells were immunoprecipitated on 9E10-coupled protein-A beads. Beads were divided into equal portions, and treated with the indicated amounts of Clb2–Cdk protein kinase (see Experimental Procedures). Western blot analysis was performed with anti-Cdc14 antibodies to as indicated.

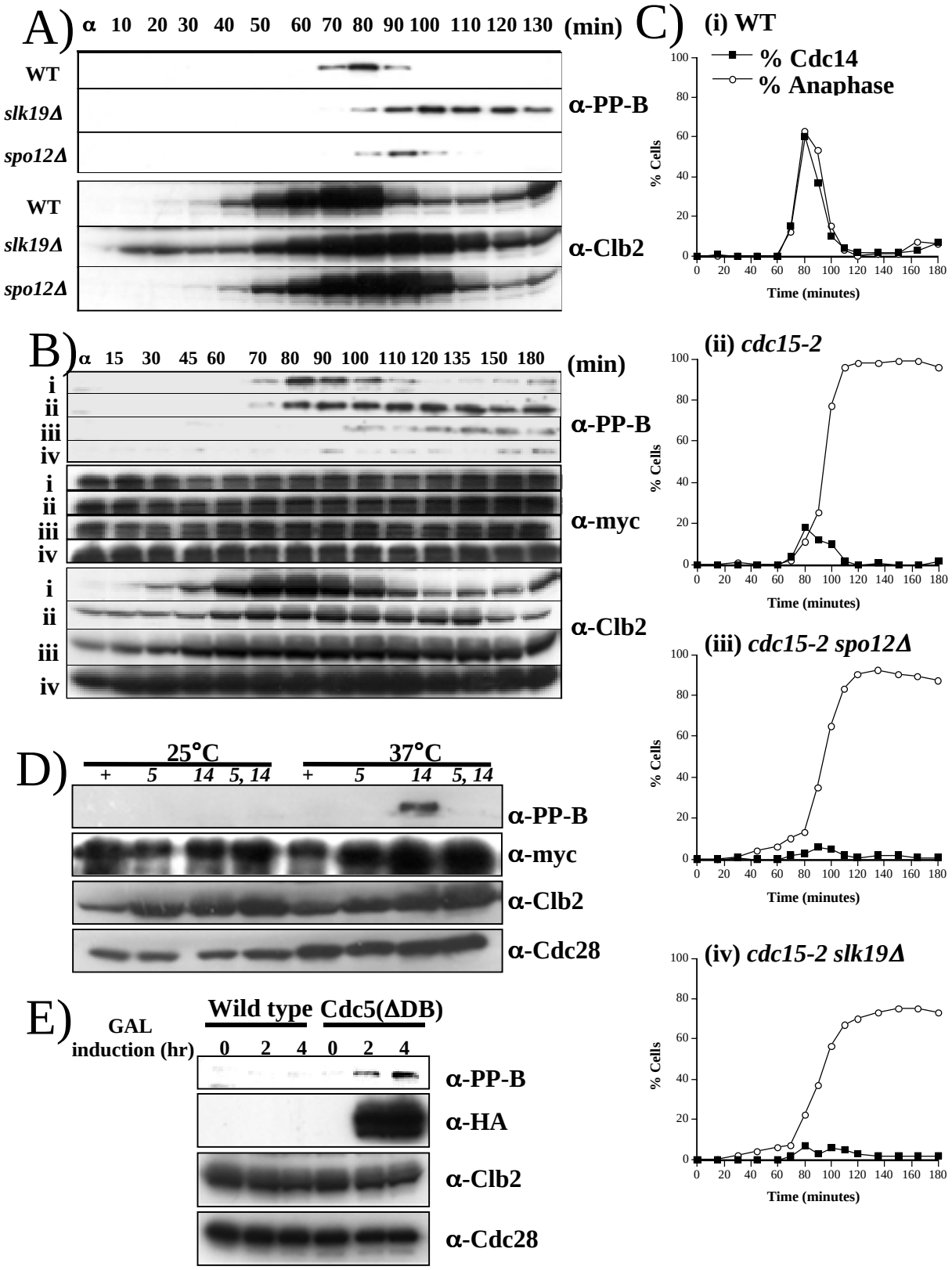

A)
α 10 20 30 40 50 60 70 80 90 100 110 120 130 (min)
WT
slk19Δ
spo12Δ
α-PP-B
WT
slk19Δ
spo12Δ
α-Clb2
B)
α 15 30 45 60 70 80 90 100 110 120 135 150 180 (min)
i
ii
iii
iv
α-PP-B
α-myc
α-Clb2
C)
(i) WT
% Cdc14
% Anaphase
% Cells
Time (minutes)
(ii) cdc15-2
(iii) cdc15-2 spo12Δ
(iv) cdc15-2 slk19Δ
D)
25°C
37°C
+ 5 14 5, 14
α-PP-B
α-myc
α-Clb2
α-Cdc28
E)
GAL induction (hr)
Wild type
Cdc5(ΔDB)
0 2 4 0 2 4
α-PP-B
α-HA
α-Clb2
α-Cdc28

Figure II-6

Figure II-6: Net1 phosphorylation on Threonine 212 is modulated by Slk19, Spo12, and Cdc5.

(A) The dynamics of Threonine 212 phosphorylation are altered in *slk19Δ* and *spo12Δ* mutants. Isogenic strains of wild-type (RJD2617), *slk19Δ* (RJD2618), and *spo12Δ* (RJD2619) carrying a *NET1-myc9* allele were synchronized by addition of α factor. Cells were released from α factor block and samples were collected at the indicated time points (min). Phosphoepitope formation, Net1-myc9, and Clb2 levels were monitored as previously described. Please refer to (Figure 11) for loading controls.

(B) Blockade of Cdc14 early anaphase release (FEAR) is accompanied by loss of Net1 phosphorylation on Threonine 212. Isogenic strains of (i) wild-type (RJD2617), (ii) *cdc15-2* (RJD2610), (iii) *cdc15-2 spo12Δ* (RJD2620), and (iv) *cdc15-2 slk19Δ* (RJD2621) were synchronized in α factor then released into pre-warmed YP 2% glucose at 36°C. Aliquots of each culture were collected at the indicated time points for analysis by Western blotting. Phosphoepitope formation, Net1-myc9, and Clb2 levels were monitored as previously described. Please refer to (Figure 11) for loading controls.

(C) Cdc15 along with either Spo12 or Slk19 is required for proper release of Cdc14 from the nucleolus during early anaphase. The same aliquots collected for panel (B) were also analyzed by indirect immuno-fluorescence with anti-Cdc14 and anti-tubulin antibodies as previously described.

(D) Net1 phosphorylation on Threonine 212 is dependent on Cdc5. Wild-type (+) (RJD1349), *cdc5-1* (5) (RJD1417), *cdc14-1* (14) (RJD1408), and *cdc5-1 cdc14-1* (5,14) (RJD2628) cells carrying a *NET1-myc9* epitope tagged allele were grown logarithmically at 25°C and samples for each culture collected. Cultures were shifted to 37°C for 3 hours and samples were collected again for analysis by Western blotting. Phosphoepitope formation, Net1-myc9 levels, Clb2, and Cdc28 levels were monitored as previously described.

(E) Over-expressed Cdc5 lacking the destruction box (DB) motif promotes Net1 phosphorylation on Threonine 212 in metaphase-arrested cells. Wild-type (RJD2634), and *GAL1p-CDC5(ΔDB)* (RJD2635) cells carrying a *NET1myc9* epitope tagged allele were grown logarithmically at 26°C and arrested with 30μg/ml benomyl plus 15μg/ml nocodazole in YP 2% raffinose. At various times after galactose was added to induce expression, samples were withdrawn, fractionated by SDS-PAGE, and immunoblotted with α-PP-B, α-Clb2, and α-Cdc28 as previously described. Levels of Cdc5Δdb induction were determined by blotting for α-HA.

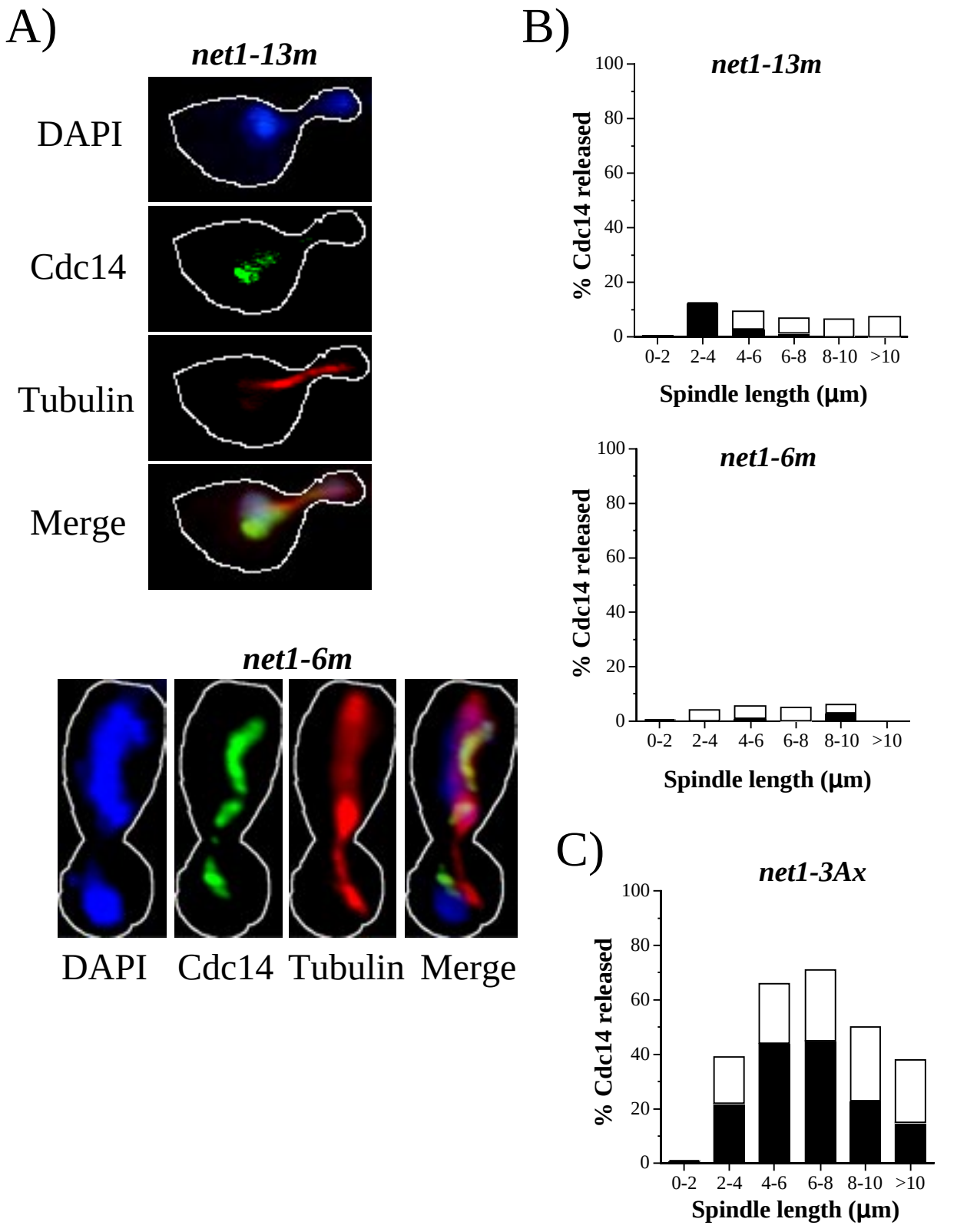

A)
net1-13m
DAPI
Cdc14
Tubulin
Merge
net1-6m
DAPI
Cdc14
Tubulin
Merge
B)
net1-13m
% Cdc14 released
Spindle length (µm)
0-2
2-4
4-6
6-8
8-10
>10
net1-6m
C)
net1-3Ax

Figure II-7

Figure II-7: Net1 mutants are defective in Cdc14 release in early anaphase.

(A) Mutant *cdc15-2* cells carrying either *net1-13m* (RJD2611) or *net1-6m* (RJD2612) alleles were synchronized with α factor at 25°C and released in YP 2% glucose at 37°C. Cells were collected for analysis by indirect immuno-fluorescence at 10 to15 minute time intervals. Staining was performed with DAPI, anti-Cdc14, and anti-tubulin antibodies for determination of nuclear position, Cdc14 localization, and spindle length, respectively. Cell outlines are indicated for comparison.

(B) Mutant *cdc15-2* cells carrying either *net1-13m*, o*r net1-6m* alleles were synchronized with α factor 25°C and released in YP 2% glucose at 37°C. Cells collected at 70 to110 minutes after α factor release were double-labeled with anti-Cdc14 and anti-tubulin antibodies. Release of Cdc14 from the nucleolus was determined to be either complete (black boxes) or partial (white boxes; see legend to Fig. 2B) and was plotted against spindle length. Over 350 cells were counted for each panel. The wild-type *NET1* control is shown in Figure 2B.

(C) Mutant *cdc15-2* cells carrying *net1-3Ax* (an allele where the 3 non-Cdk sites S169, S231, and S259 from the *net1-6m* were mutated to Alanine) were synchronized with α factor 25°C and released in YP 2% glucose at 37°C. Cells collected at 70 to 110 minutes after α factor release were double-labeled with anti-Cdc14 and anti-tubulin antibodies. Release of Cdc14 from the nucleolus was determined to be either complete (black boxes)

or partial (white boxes; see legend to Fig. 2B) and was plotted against spindle length. Over 350 cells were counted for each panel. The wild-type *NET1* control is shown in Figure 2B.

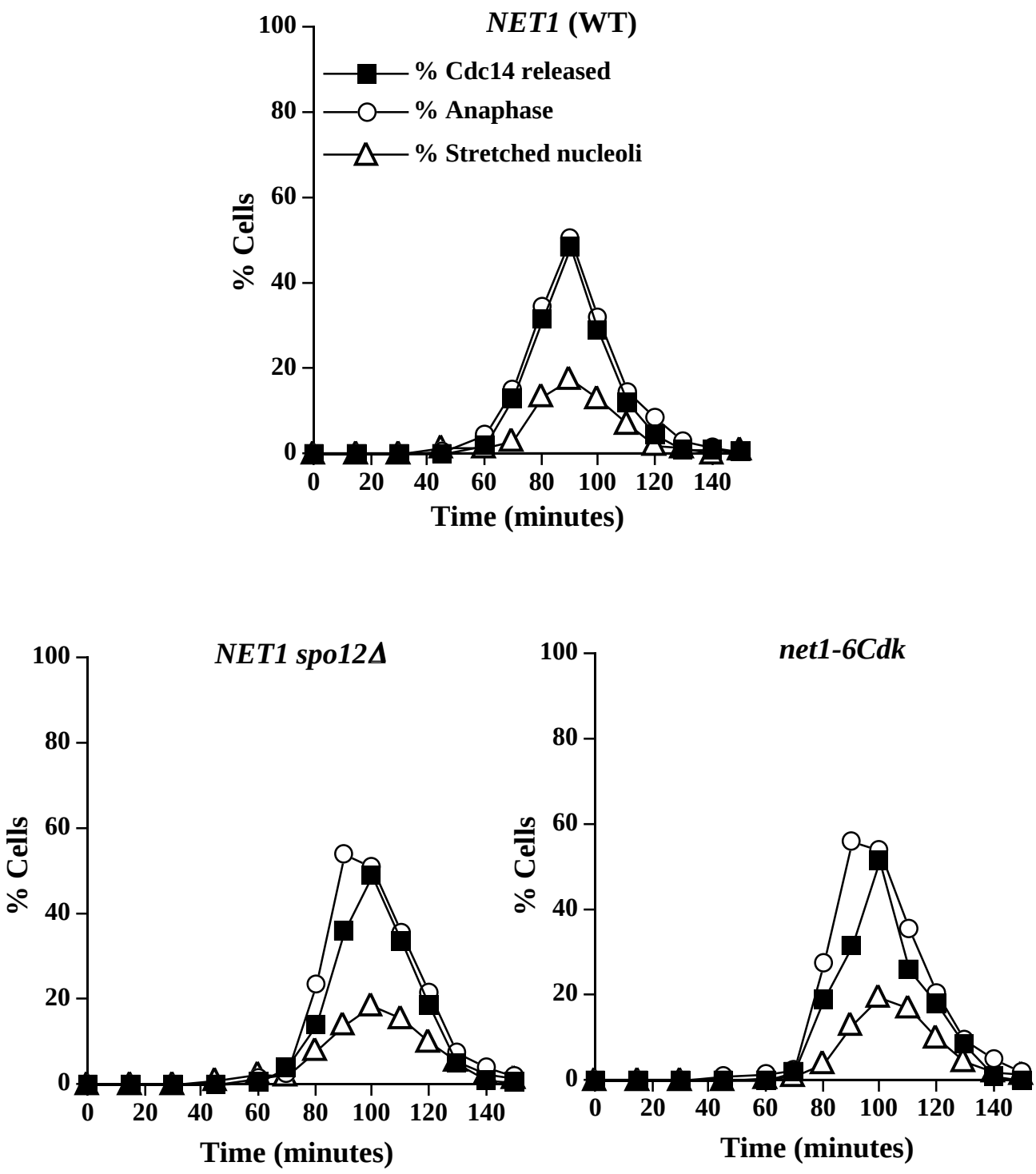

NET1 (WT)
% Cdc14 released
% Anaphase
% Stretched nucleoli
% Cells
Time (minutes)
NET1 spo12Δ
net1-6Cdk
0
20
40
60
80
100
120
140

Figure II-8

Figure II-8: Mutants *net1-6Cdk* and *spo12Δ* exhibit a delay in Cdc14 release from the nucleolus and rDNA segregation.

Wild-type (RJD2617), *net1-6Cdk* (RJD2633), and *spo12Δ* (RJD 2619) cells were synchronized in α factor then released into YP 2% glucose at 27°C. Alpha factor was added back after 70-minutes to prevent cells from entering into a second cell cycle. Aliquots of each culture were collected at the indicated time points for analysis by indirect immuno-fluorescence with anti-Cdc14, anti-RPA190 (nucleolar marker), and anti-tubulin antibodies to monitor Cdc14 release from the nucleolus, nucleolar segregation and spindle length, respectively. Percent of stretched nucleoli was determined by counting cells in which the nucleolus had stretched between mother and daughter cells but had not segregated into 2 distinct masses divided by the total number of cells counted. Over 200 cells were counted for each time point.

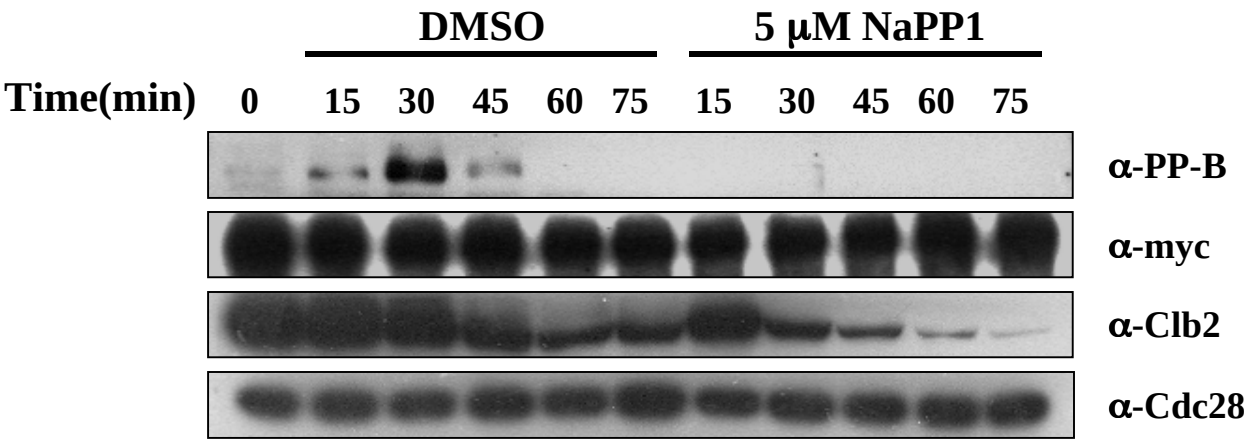

DMSO
5 μM NaPP1
Time(min)
0
15
30
45
60
75
15
30
45
60
75
α-PP-B
α-myc
α-Clb2
α-Cdc28

Figure II-9

Figure II-9: Net1 Phosphorylation on Threonine 212 is dependent on Cdk activity.

Cells carrying a wild-type *NET1-myc9* allele in combination with the *cdc28-as1* analog sensitive allele (RJD2632) were arrested at metaphase with 30μg/ml benomyl plus 15μg/ml nocodazole at 30°C for 3 hours in YP 2% glucose. Cells were then washed with and resuspended in pre-warmed YP 2% glucose and split into two flasks at 35°C either containing 5μM NaPP1 (analog inhibitor) or DMSO (vehicle). Aliquots of each culture were collected at the indicated time points and prepared for Western blot analysis. Phosphoepitope formation (α-PP-B), levels of Net1 antigen (α-myc), and levels of Clb2 (α-Clb2) were monitored by immunoblotting whole cell extracts with the indicated antibodies. Cdc28 levels (α-Cdc28) were used as a loading control.

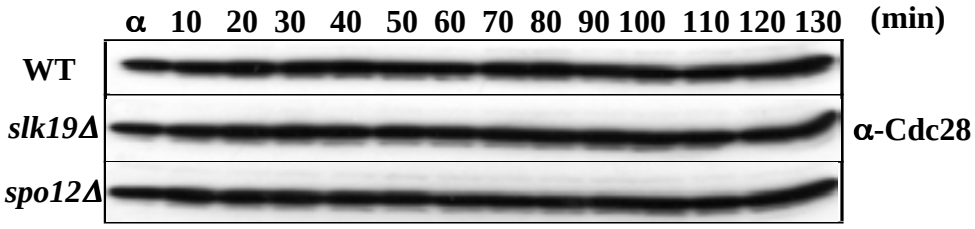
S. cerevisiae	T62P	S166P	T212P	S252P	S297P	T304P	%identities	%positives	%Gaps
S. bayanus	+	+	+	+	+	+	56	63	4
S. mikatae	+	+	+	+	+	+	60	66	2
S. paradoxus	+	+	+	+	+	+	66	69	1
S. castellii	-	-	+	+	+	+	29	42	20
S. kudriavzevii	+	+	+	+	+	+	60	65	2
Candida glabrata	+	-	+	+	+	+	36	50	12
Kluyveromyces lactis	-	-	+	+	+	+	31	44	19

Figure II-10

Figure II-10: Cdk sites are conserved in Net1 orthologs from different yeast species.

Sequence alignment of *S. cerevisiae* Net1 and its orthologs from various sequenced species of yeast show a high degree of conservation of Cdk sites mapped *in vivo*. "Percent identities" refers to exact matches in amino acid alignment for both sequences being compared divided by the total sequence length of *S. cerevisiae* Net1. "Percent positives" refers to matches where an amino acid difference exists between the two aligned sequences but both amino acids belong to the same family (acidic, basic, uncharged polar, nonpolar) divided by the total sequence length of *S. cerevisiae* Net1. "Percent gaps" refers to the number of spaces introduced into an alignment to compensate for insertions and deletions in one sequence relative to another divided by the total sequence length of *S. cerevisiae* Net1. Each parameter is shown for each species compared to Net1 from *Saccharomyces cerevisiae*.

A)



B)

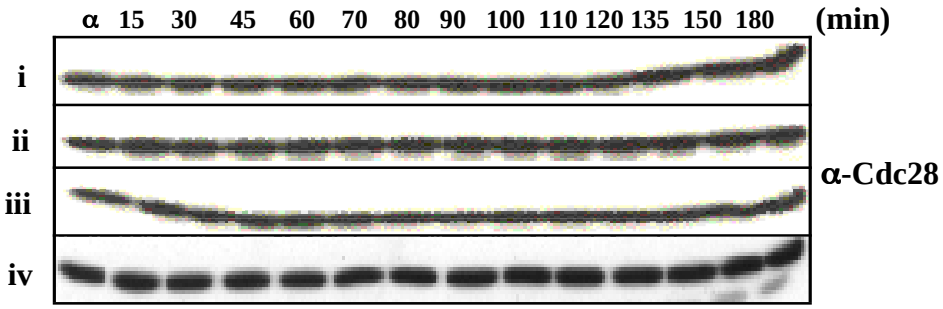


Figure II-11

Figure II-11: Cdc28 levels as loading controls.

(A) Cdc28 levels (α-Cdc28) were used as a loading controls for the indicated time points and strains for Figure 6A.

(B) Cdc28 levels (α-Cdc28) were used as a loading controls for the indicated time points for Figure 6B. Roman numerals refer to the following strain genotypes: (i) wild-type (RJD2617), (ii) *cdc15-2* (RJD2610), (iii) *cdc15-2 spo12Δ* (RJD2620), and (iv) *cdc15-2 slk19Δ* (RJD2621).

Table II-1

Peptide Recovered [a]	Net1 Sequence [b]	Mutated in (13m) [c]	Mutated in (6m) [c]	Mutated in (3Cdk) [c]	Mutated in (6Cdk) [c,f]
160-184 +1P	R>SKLNNG**S**PQ**S**WQPQQQIPSSSGVLR>I [d]	Y, Y	Y,Y	Y, N	Y, N
210-216 +1P	R>VS**T**PLAR>Q	Y	Y	Y	Y
226-241 +1P	K>IVSNN**S**DDEDEDIGER>S	Y	Y	N	N
242-256 +1P	R>SFLPPPTQPQ**S**PPIR>I	Y	Y	Y	Y
257-265 +1P 257-266 +1P	R>IS**S**GIDAGKK>I	Y	Y	N	N
292-307 +2P [e]	R>LLSG**T**PIMSTM**T**PNR>V	N, N	N, N	N, N	Y, Y
352-359 +1P	R>KPPV**T**TPR>I	Y	N	N	N
360-366 +1P	R>IT**S**GM(ox)LK>I	Y	N	N	N
381-393 +1P 381-393 +2P	K>EGP**SS**PASILPAK>A	Y,Y	N, N	N, N	N, N
495-505 +1P	R>KS**S**LETIVEK>K	Y	N	N	N
606-618 +1P	R>IVPQD**S**DSSSFPK>S	Y	N	N	N
670-678 +1P	R>NILPQR**T**PR>S	Y	N	N	N
742-749 +1P	K>DI**S**LHSLK>G	N	N	N	N
1013-1023 +1P	R>VVVN**T**PREPVR>S	N	N	N	N
1028-1038 +1P	K>IEAP**S**PSVNKK>I	N	N	N	N
1079-1092 +1P	K>VPR**S**LSSLSDLVSR>G	N	N	N	N

[a] '+xP' refers to the number of phosphate groups per peptide as determined by mass spectrometry.

[b] Larger-sized font indicates mapped phosphorylation sites. Carets (>) mark sites of trypsin cleavage that yielded the peptides shown. 'ox' refers to oxidized Methionine.

[c] 'Y' indicates that the codon for a given site was mutated to encode Alanine, whereas 'N' indicates that it was not mutated.

[d] Initial analysis was unable to determine which Serine was phosphorylated in the fragment spanning amino acids 160-184, but subsequent analysis with a phospho-specific antibody implicated Serine166. Phosphorylation status of Serine169 is unknown (underlined).

[e] Yielded a small amount of a species at +80 Da from the doubly-charged form at the same retention time in the HPLC analysis, but we could not detect any monophosphate in the precursor scans and it was later determined that this peptide was most likely phosphorylated on both Threonines as indicated.

[f] In the (6Cdk) mutant, Threonine 62 was also mutated to Alanine to completely eliminate all Cdk consensus sites from the first 341aa N-terminal fragment of Net1.

Table II-1: Net1 *in vivo* phosphorylation sites.

Strain name	Strain Genotype	Strain comments
RJD 2603	*net1::mTn3/URA3*	clone V13E1 with insertion point at aa(1104)*
RJD 2604	*net1::mTn3/URA3*	clone V39B3 with insertion point at aa(835)*
RJD 2605	*net1::mTn3/URA3*	clone V130G6 with insertion point at aa(754)*
RJD 2606	*net1::mTn3/URA3*	clone V30E10 with insertion point at aa(738)*
RJD 2607	*net1::mTn3/URA3*	clone V148D2 with insertion point at aa(259)*
RJD 2608	*net1::mTn3/URA3*	clone V66G4 with insertion point at aa(197)*
RJD 2609	*net1::mTn3/URA3*	clone V109B1 with insertion point at aa(189)*
RJD 1783	*net1::net1(1-621)-HA::KanMX6*	**
RJD 2610	*net1Δ::his5, NET1-TEV-myc9::TRP1, cdc15-2 MATa*	
RJD 2611	*net1Δ::his5, net1-13m-TEV-myc9::TRP1, cdc15-2, MATa*	
RJD 2612	*net1Δ::his5, net1-6m-TEV-myc9::TRP1, cdc15-2, MATa*	
RJD 2613	*net1Δ::his5, net1-3Cdk-TEV-myc9::TRP1, cdc15-2, MATa*	
RJD 2614	*net1Δ::his5, net1-6Cdk-TEV-myc9::TRP1, cdc15-2, MATa*	
RJD 2615	*net1Δ::his5, NET1-TEV-myc9::TRP1, cdc14-1, MATa*	
RJD 2616	*net1Δ::his5, net1-3Cdk-TEV-myc9::TRP1, cdc14-1, MATa*	
RJD 2617	*net1Δ::his5, NET1-TEV-myc9::TRP1, MATa*	
RJD 2618	*net1Δ::his5, NET1-TEV-Myc9::TRP1, slk19Δ::KanMX6, MATa*	
RJD 2619	*net1Δ::his5, NET1-TEV-Myc9::TRP1, spo12Δ::URA3, MATa*	
RJD 2620	*net1Δ::his5, NET1-TEV-myc9::TRP1, cdc15-2, spo12Δ::URA3, MATa*	
RJD 2621	*net1Δ::his5, NET1-TEV-myc9::TRP1, cdc15-2, slk19Δ::KanMX6, MATa*	
RJD 2622	*clb2Δ::LEU2, cdc15-2, MATa*	
RJD 2623	*net1Δ::his5, NET1-TEV-myc9::TRP1, clb2Δ::LEU2, MATa*	
RJD 2624	*net1Δ::his5 , NET1-TEV-myc9::TRP1, clb1Δ::URA3, clb2Δ::LEU2, GAL1p-CLB2::URA3, MATa*	
RJD 2625	*net1Δ::his5, NET1-TEV-myc9::TRP1, dbf2-2, MATa*	
RJD 2626	*net1Δ::his5, net1-3Cdk-myc9::TRP1, dbf2-2,MATa*	
RJD 2627	*net1Δ::his5, Net1-WT-myc9::TRP1, dbf2-2, YCH111 [CLB2 (C2Dk100)HA3, URA3], MATa*	
RJD 1349	*NET1-TEV-myc9::his5+, bar1::hisG, MATalpha*	
RJD 1417	*NET1-TEV-myc9::his5+, bar1::hisG, cdc5-1, MATa*	
RJD 1408	*NET1-TEV-myc9::his5+, bar1::LEU2, cdc14-1, MATalpha*	
RJD 2628	*NET1-TEV-myc9::his5+, cdc5-1, cdc14-1, bar1::hisG? pep4::TRP1?, MATa*	
RJD 2629	*tetO112 ::URA3x3, 3tetR-GFP::HIS3, esp1-1, MATa*	
RJD 2633	*tetO112 ::URA3x3, 3tetR-GFP::HIS3, cdc15-2, MATa*	
RJD 2631	*tetO112 ::URA3x3, 3tetR-GFP::HIS3, cdc15-2, clb2Δ::LEU2, MATa*	
RJD 602	*cdc15-2, MATa*	
RJD 2632	*net1Δ::his5, NET1-TEV-myc9::TRP1, cdc28-as1, MATa*	
RJD 2633	*net1Δ::his5, net1-6Cdk-TEV-myc9::TRP1, MATa*	
RJD 2634	*myc9-NET1::LEU2, bar1::hisG, GAL1p-CDC5(ΔDB)-HA::URA3, MAT a*	
RJD 2635	*myc9-NET1::LEU2, pep4::TRP1, MAT a*	

*Y800 background (leu2-98, cry1(R), ade2-10,his3-200, ura3-52, lys2-801, can1(R), trp1-1, cyh2(R))

**BJ5459 background (ura3-52, trp1, lys2-801, leu2D1, his3D200, pep4::HIS3, prb1D1.6R can1)

Table II-2

Table II-2: Strains used in this study.

Chapter III- New Components of the RENT Complex

Introduction

Given the ability of the RENT complex to regulate mitotic exit via controlling the release and activation of Cdc14 (Shou et al., 1999; Visintin et al., 1999), we sought to identify more components that might govern this unique interaction of a mitotic control protein with its nucleolar inhibitor Net1. The RENT complex is composed of three identified components: Net1 a nucleolar protein responsible for maintaining nucleolar integrity and regulating the activation and release of Cdc14 (the protein phosphatase responsible for reversal of Cdk phosphorylation at the end of mitosis) (Visintin et al., 1998), and Sir2 (the histone deactylase responsible for maintaining transcriptional silencing at the rDNA, mating type, and telomeric loci) (Gottschling, 2000; Shou et al., 1999; Straight et al., 1999). The RENT was initially identified using a unique approach known as Sequential Epitope tagging/immunoAffinity chromatography/Mass spectrometry (SEAM) (Shou et al., 1999). This new technique enabled us to identify new protein interactors that co-immunoprecipitated with tagged subunits of the RENT complex, specifically Net1. The first interactor to be identified was RPC40, a subunit of RNA polymerase I holoenzyme which resides in the nucleolus and is responsible for ribosomal RNA transcription. The second interactor identified was a catalytic subunit of Casein Kinase II (CKII), a pleiotropic, multi-ubiquitous serine or threonine protein kinase conserved throughout eukaryotes and implicated in a diverse range of cellular processes

including ionic sensing (Glover, 1998), nuclear matrix regulation, cellular growth (Ahmed, 1999; Ahmed et al., 2000), calmodulin regulation (Benaim and Villalobo, 2002), DNA damage, transcriptional regulation (Schultz, 2003), and cell-cycle progression (Glover, 1998; Glover et al., 1994).

RNA Polymerase I (Pol I)

In *Saccharomyces cerevisiae*, the nucleolus contains 150-180 tandem repeats of a 9.1 kilobase segment of DNA known as the rDNA on the arm of Chromosome XII (Petes and Botstein, 1977). The rDNA transcription product codes for a single 35S rRNA precursor which is post-transcriptionally processed to yield a 25S and 5.8S RNA species incorporated in the 60S ribosomal subunit and an 18S RNA species found in the 40S ribosomal subunit (Warner et al., 1973). Transcription of the ribosomal DNA repeats is carried out by RNA Polymerase I (Pol I), a holoenzyme composed of 14 proteins (Carles et al., 1991; Huet et al., 1975) and accounts for at least 60% of total cellular transcript. Pol I specificity for rDNA transcription is achieved through promoter specificity but little else in known about the sites of initiation and termination. Transcription of rDNA by Pol I has been linked to an interesting phenomenon known as gene silencing in which Pol II normally transcribed-genes are transcriptionally repressed when inserted into the rDNA tandem array (Smith and Boeke, 1997). Regulation of nucleolar silencing requires a functional RENT complex as well as a functional Pol I as demonstrated by a recent finding that silencing in addition to requiring a functional Sir2 protein also required transcription by RNA polymerase I (Buck et al., 2002; Shou et al., 2001). The direction of spreading was surprisingly controlled by the direction of Pol I transcription (Buck et

al., 2002). An intact nucleolus is also required for maintenance of proper silencing as demonstrated by *net1-1* and rDNA deletion mutants (Oakes et al., 1998; Shou et al., 2001). Thus, Pol I transcription and proper nucleolar silencing play an important role in maintaining cellular function and preventing senescence through the formation of rDNA circles as has been demonstrated for silencing mutants (Defossez et al., 1998; Gershon and Gershon, 2000; Shore, 1998).

Casein Kinase II (CKII)

Casein kinase II (CKII) is one of the most highly conserved Serine/Threonine kinases from yeast to man (Guerra and Issinger, 1999). It consists of a tetrameric holoenzyme ($\alpha_2\beta_2$) made up of two regulatory subunits (β_2) (Ckb1, Ckb2) and the more evolutionally conserved two catalytic subunits (α_2) (Cka1, Cka2). To date, more than 160 potential substrates have been identified for CKII (Pinna and Meggio, 1997) highlighting the pleiotropic, yet surprisingly essential role for CKII is cell survival. Notably, Nucleolin, a protein found in the nucleolus of mammalian cells has proved to be one of the best substrates for CKII (Caizergues-Ferrer et al., 1987; Schneider and Issinger, 1988). In *Saccharomyces cerevisiae*, CKII's function is essential as deletion of both the α-catalytic subunits is lethal yet, again surprisingly; deletion of one or both of the regulatory β-subunits displays no obvious phenotype (Glover, 1998). The most obvious phenotype arises when *ckb1Δ ckb2Δ* mutant cells are challenged on minimal media, and any combination of *cka1Δ* or *cka2Δ* with either of the *ckb1Δ* or *ckb2Δ* display slow growth and flocculation (Glover, 1998). Over-expression analysis of either the catalytic or regulatory subunits from Drosophila in *Saccharomyces cerevisiae* has had little impact

on our understanding of CKII's function (Rethinaswamy et al., 1998) even though CKII kinase consensus motif [(S/t)-X-X-(E/d)] has been determined and studied on a large variety of its *in vitro* substrates (Guerra and Issinger, 1999). Interestingly, temperature-sensitive alleles of Cka1 and Cka2 arrest in a mixed population of unbudded and elongated-budded cells at the G2/M stage of the cell cycle (Glover, 1998). CKII mutants in *S. cerevisiae* display another interesting ability in that they are required for cells to undergo "adaptation" in response to DNA damage; a process by which cells continue with the cell cycle after a prolonged delay at the DNA-damage checkpoint (Toczyski et al., 1997). Subcellular localization of CKII has been shown to play an important part in regulation in addition to post-translational modification (Faust and Montenarh, 2000; Jans and Hubner, 1996; Tawfic et al., 2001). Thus, even though CKII was one of the first kinases identified; preciously little is know about its molecular functions given its pleiotropic nature.

Results

RNA Polymerase I and Casein Kinase II Interact with Net1

To look for new components of the RENT complex, we performed a series of immuno-precipitation experiments with a myc9- immuno epitope tag on Net1 in various MEN mutant backgrounds so as to find a MEN-dependent component to the RENT complex. The epitope tag was additionally modified to include an engineered TEV protease Cleavage site, thus facilitating a subsequent purification of Net1 complexes from immuno-affinity beads (Figure 1). Immuno-affinity purification was also performed on

other components of the Mitotic Exit Network (MEN) to try to identify new factors for that pathway (Figure 1). Samples were then run on a 4-15% gradient gel with along with the untagged control and banding patterns were observed via silver staining and subsequently compared to identify new bands (Figure 1). In-gel sequencing analysis of Net1 samples revealed no major differences in protein complexes from various MEN mutants (R. Azzam, A. Shevchenko, and J. Graumann, unpublished observations). Two new components were identified that associated with the RENT complex. The RNA polymerase I subunit *RPC40*, and the Casein kinase II subunit *CKA1*. These results were latter confirmed after performing Mud-PIT analysis with on the samples obtained from the same MEN mutants and identifying all subunits of Casein Kinase II (CKII) (*CKA1, CKA2, CKB1, CKB2*) as associating with the RENT complex (R. Azzam and J. Graumann, unpublished observations).

To verify that the interaction we observed with RNA Polymerase I and Casein Kinase II is reproducible, we performed co-immunoprecipitation experiments with Net1. Purified Pol I holoenzyme with the A135 tagged-subunit interacts with Baculo-purified Net1 *in vitro* as judged by Western blotting (Figure 2A) in an almost 1:1 ratio. Immuno-tagging all four subunits of CKII and immuno-precipitating them also brings down Net1 (Figure 2B). Thus, both Pol I and CKII interact with Net1 as judged by mass spectrometry and immunoprecipitation experiments from *S. cerevisiae* and in a semi-purified system. Furthermore, Net1 has been definitively implicated in regulating nucleolar structure and Pol I transcription (Shou et al., 2001).

Casein Kinase II Mutants Display Synthetic Interactions with MEN Mutants

Casein Kinase II mutants arrest as a mixture of budded and unbudded cells in G2/M (Glover, 1998). Thus, to further analyze the role of Casein Kinase II in regulating mitotic exit, we sought to investigate how temperature sensitive mutants of CKII (specifically mutants in CKA1 catalytic subunit) interact with MEN mutants. Double mutants *cka2-8 tem1-3, cka2-8 cdc15-2, cka2-8 cdc14-1,* and *cka2-8 dbf2-2* showed a reduction in restrictive temperature compared to single mutants (Figure 3). Interestingly, *cka2-8 cdc5-1* double mutants appear to improve the temperature sensitivity of *cdc5-1* cells (Figure 3).

Casein Kinase Mutants Arrest in Anaphase with Unsegregated rDNA

If Casein Kinase II activity is involved in late mitotic events, then CKII mutants should display late mitotic phenotypes. A modified strain containing an integrated GFP chromosomal marker was used to visualize chromosomal segregation in *cka2-8, esp1-1,* and *top2-1* mutants (Figure 4). Whereas *esp1-1* and *top2-1* mutants arrest with one visible GFP dot indicating failure of chromosomal DNA segregation, *cka2-8* mutants arrest with two visible GFP dots at the opposite ends of the DAPI mass indicating proper segregation of chromosomal DNA (Figure 4). Thus, *cka2-8* mutants arrest in the anaphase stage of the cell cycle, similar to MEN mutants.

It has been previously demonstrated that *cdc14-1* mutants arrest in late anaphase with unsegregated rDNA which differs from other MEN mutants (Granot and Snyder, 1991). To examine rDNA segregation in various mutant backgrounds, we chromosomally tagged Net1- which is known to residue in the nucleolus for the entire duration of the cell cycle (Shou et al., 1999)-with GFP in various strain backgrounds. Whereas *cdc15-2* cells

arrest with segregated nucleoli, *cdc14-1* mutants arrested with an unsegregated nucleolus, similar to that observed for *cka2-8* cells, as judged by Net1-GFP immuno-fluorescence (Figure 5A). Also, *cka2-8* cells arrest with Cdc14 in the nucleolus in early anaphase as judged by spindle length (Figure 5B). Thus, CKII activity appears to be required for proper rDNA segregation similar to Cdc14.

Conclusions

The RENT complex, and especially Net1, appears to play a crucial role not only in the regulation of mitotic exit and transcriptional silencing through the activation and release of Cdc14 and sir2, respectively; but also regulation of nucleolar architecture and RNA polymerase I transcription. The role of RNA polymerase I and its link to Net1 has been clearly established through the subsequent studies by Shou and colleagues (Shou et al., 1999). The role of Casein Kinase II is less clear but appears to a) interact with Net1, b) display a genetic interaction with MEN mutants, and c) be involved in proper segregation of rDNA. Interestingly, the arrest of *cka2-8* mutants appears to be RAD9 dependent implicating a role for the DNA damage checkpoint (data not shown) in concert with a previously identified role for CKII in the “adaptation” response to DNA damage (Toczyski et al., 1997).

Given the recently reported roles for Cdc14 and the FEAR pathway in regulating proper nucleolar division during meiosis (Buonomo et al., 2003), and the fact that a CKII consensus phosphorylation site has been mapped *in vivo* on Net1; it would be interesting to further examine how CKII might be able to regulate Net1 function to insure proper nucleolar division.

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Experimental Procedures

Cell Growth Procedures

Cells were grown in yeast extract-peptone (YP) or in yeast minimal (YM) media containing 2% glucose (YPD,YMD), 2% raffinose (YPR,YMR) or 2% galactose (YPG,YMG) as carbon source. Where appropriate, minimal media were supplemented with leucine, histidine, tryptophan, uracil, and adenine to complement auxotrophies.

Cell Extract Preparation and Western Blotting

Cells were grown to an $O.D._{600}$ of 1.0, and for every time point 2 ml of culture was collected and TCA added to a final concentration of 20%. Cells were collected by centrifugation and washed with 2 ml of Tris-HCl (pH 7.5). SDS loading buffer [70 µl of 100 mM Tris-HCl (pH 7.5), 20% glycerol, 4% SDS, 2 M Urea, 200 mM DTT] was added, tubes were boiled for 3 minutes, and 100µl of acid-washed glass beads (500 µm) were added to each tube followed by boiling for an additional 2-minutes. Tubes were

vortexed for 45 sec for 15 times with 1-minute cooling intervals. Tubes were boiled again for 2-minutes and 5 μl of sample was fractionated on a 10% SDS-PAGE gel followed by transfer to a nitrocellulose membrane. Western blot analysis was performed with the following primary antibodies at the indicated dilutions: Anti-myc (9E10) (1:5000), anti-Net1 (1:250), and anti-HA (1:5000).

Immunoprecipitation and Clb2–Cdk release/kinase assay.

To prepare extracts for immunoprecipitation, 10 $O.D._{600}$ units of a log phase cell culture was harvested and washed with 2 ml of Tris-HCl (pH 7.5). Cells were re-suspended in 500μl lysis buffer [25 mM HEPES/KOH (pH7.5), 150 mM NaCl, 1 mM DTT, 0.2% Triton, 1 mM EDTA, 1 mM PMSF, 1 mM Benzamidine, 1x Protease Inhibitor Cocktail (Aprotinin, Chymostatin, Leupeptin, and PepstatinA all at 5 μg/ml in 90% DMSO)], transferred to a flat-bottom 2 ml tube and supplemented with 100 μl of acid-washed glass beads (500 μm). Samples were vortexed 15 times for 45 seconds each with 1-minute cooling intervals. Tubes were then centrifuged for 5 minutes at 14,000 rpm and the supernatant was collected. Clarified extract (400 μl) was incubated with 60 μl of 9E10-coupled protein A beads for 1 hour on a rotator at 4°C. Beads were collected and washed ten times in wash buffer [25 mM HEPES/KOH (pH7.5), 150 mM NaCl, 1 mM DTT, 0.2% Triton], and divided to approximately 50 μl beads per reaction condition. Myc9-Net1 (purified from insect cells infected with a recombinant baculovirus) was used as indicated (Shou et al., 1999).

Immunofluorescence

Immunofluorescence was performed as previously described in Chapter II with direct immuno-fluorescence being performed on live cells after they have been arrested at 37°C for approximately 3 hours. Indirect-immunofluorescence was performed as previously described (Shou et al., 2002; Shou et al., 1999). Rabbit anti-Cdc14 (1/3000) and rat anti-tubulin monoclonal antibody YL1/34 (1/1000) were used at the indicated dilutions. Images of cells were collected on a Zeiss Axioskop or Axiovert 200M microscope.

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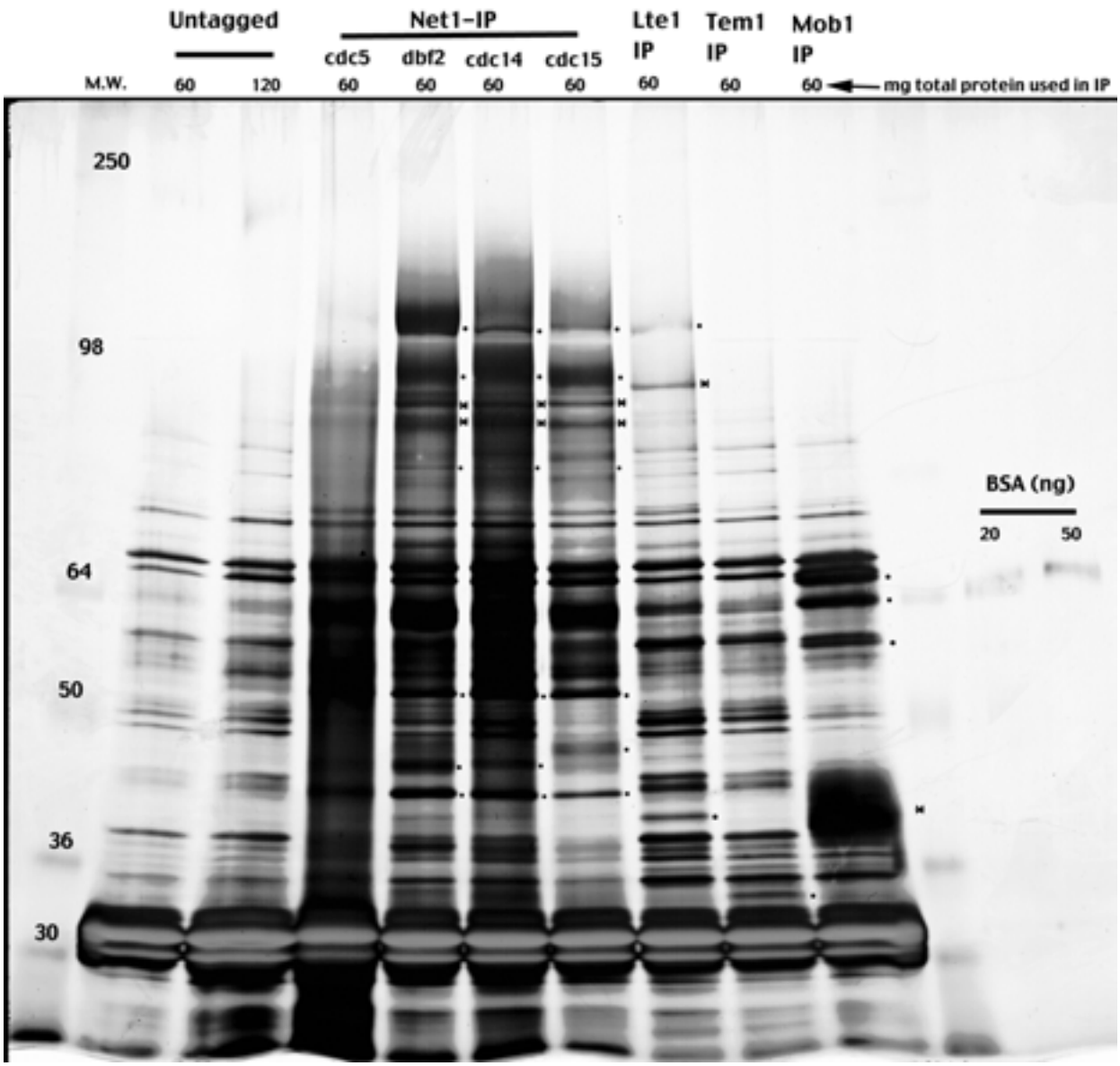
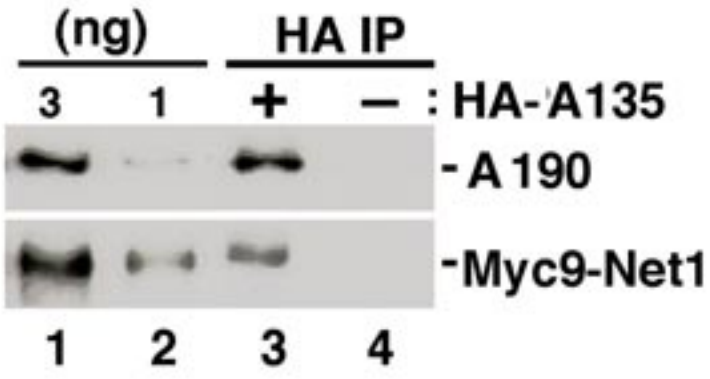

Untagged
Net1-IP
cdc5
dbf2
cdc14
cdc15
Lte1 IP
Tem1 IP
Mob1 IP
M.W.
60
120
60
60
60
60
60
60
60
mg total protein used in IP
250
98
64
50
36
30
BSA (ng)
20
50

Figure III-1

Figure III-1: Immunoprecipitation of Net1 and MEN components.

Silver stained 5-14% SDS-PAGE gel with Indicated amounts of extract used with 50μl of 9E10 coupled-agrose beads to immuno-precipitate (IP) Net1 from (*cdc5-1, dbf2-2, cdc15-2,* and *cdc14-1*). IP's of other MEN components (*LTE1, TEM1*, and *MOB1*) were also preformed to look for associated proteins. (*) indicates the tagged protein and its potential breakdown products, (•) indicate new bands in the IP lane that are not found in the control untagged lanes. The intensely staining band found in all lanes around 30kD is TEV protease used for the elution of the complex off the beads. 20 and 50 ng of Bovine Serum Albumin (BSA) are included for comparison.

A)



B)

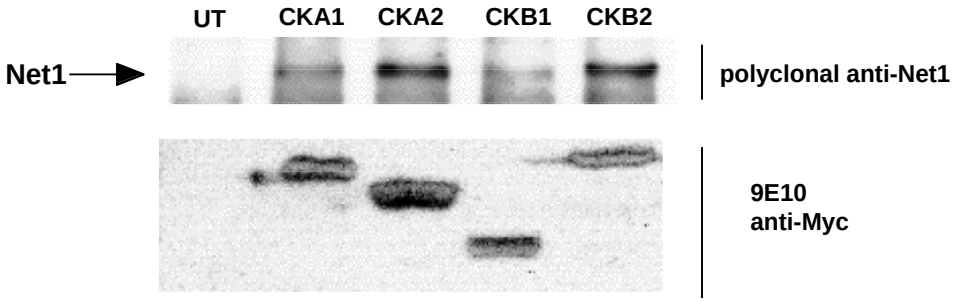


Figure III-2

Figure III-2: RNA Polymerase I and Casein Kinase II interact with Net1 *in vitro*.

(A) Purified Pol I and Myc9-Net1 interact *in vitro*. Purified Pol I (150 ng = 0.26 pmol; (Keener et al., 1998)) with its A135 subunit tagged with HA (+) or untagged (-) was immunoprecipitated with 12CA5 antibodies (against the HA epitope). The antibody beads were subsequently incubated with Myc9-Net1 (150 ng = 0.83 pmol) purified from insect cells, and proteins bound to the beads (lanes 3 and 4) were immunoblotted with antibodies against A190 and Myc9-Net1. To estimate the relative amount of Net1 complexed to Pol I, 3 ng (lane 1) and 1 ng (lane 2) of Pol I (top panel) and Myc9-Net1 (bottom panel) were immunoblotted with anti-A190 and 9E10 antibodies, respectively. We estimate that almost all Pol I molecules bound Net1 in this assay.

(B) Net1 co-immunoprecipitates with all four tagged subunits of CKII. Myc9-tagged subunits Cka1, Cka2, Ckb1, and Ckb2 are able to bring down varying amounts of Net1 compared to untagged (UT) control. Immunoprecipitation was performed as described in experimental procedures and western blots were immunoblotted with polyclonal anti-Net1 (gift from D. Moazed) and 9E10 antibodies to detect Net1 and the subunits of Casein Kinase II.

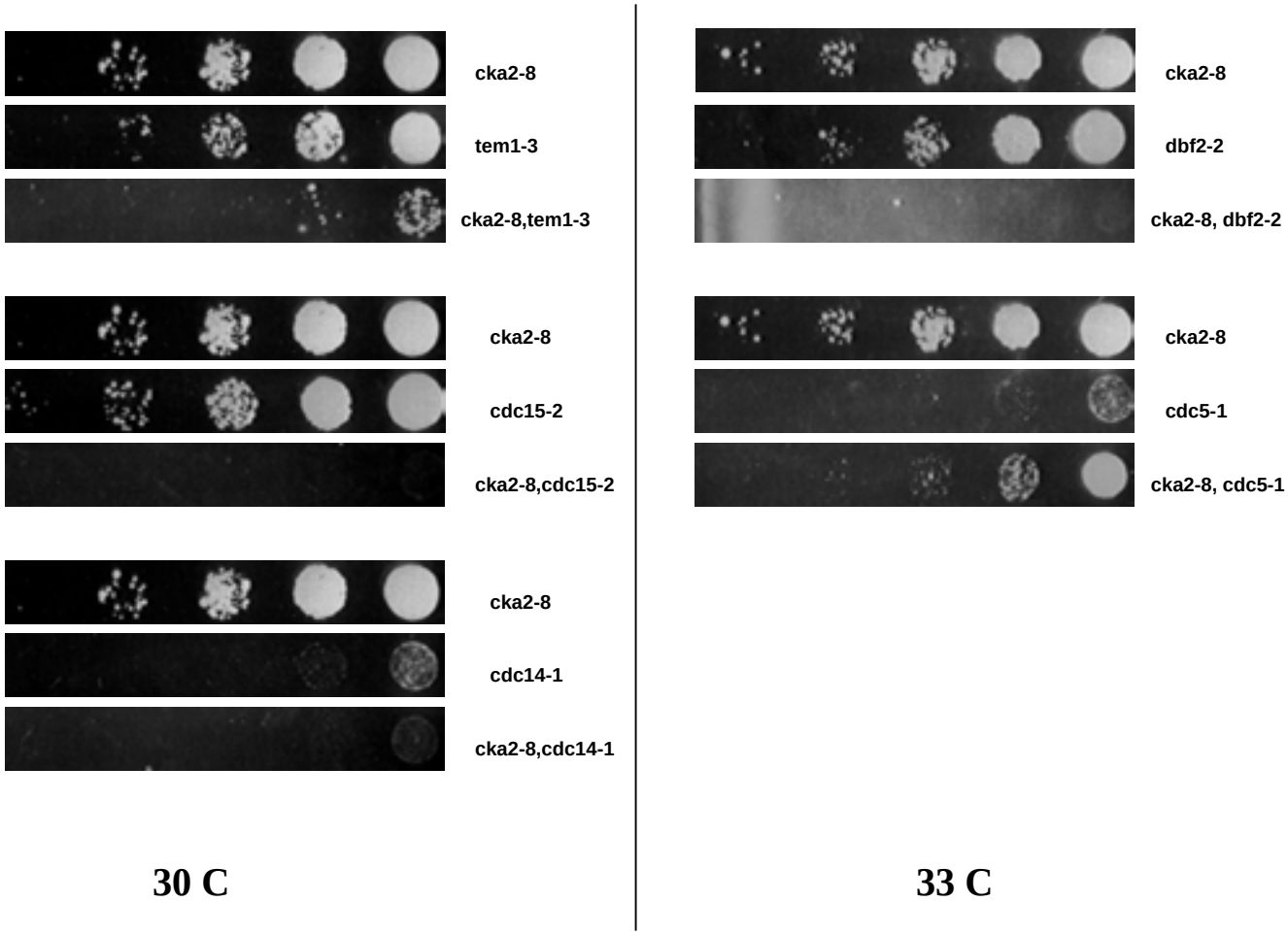

cka2-8
tem1-3
cka2-8,tem1-3
cka2-8
cdc15-2
cka2-8,cdc15-2
cka2-8
cdc14-1
cka2-8,cdc14-1
30 C
cka2-8
dbf2-2
cka2-8, dbf2-2
cka2-8
cdc5-1
cka2-8, cdc5-1
33 C

Figure III-3

Figure III-3: Casein Kinase II mutants genetically interact with MEN mutants.

Starting with 3000 cells, 3-fold serial dilutions of each the indicated strain (*cka2-8*) alone or in combination with various MEN mutants (*tem1-3, cdc15-2, cdc14-1*, and *cdc5-1*) were spotted on YPD plates from right to left, and incubated at the indicated temperature for 2-3 days before the picture was taken.

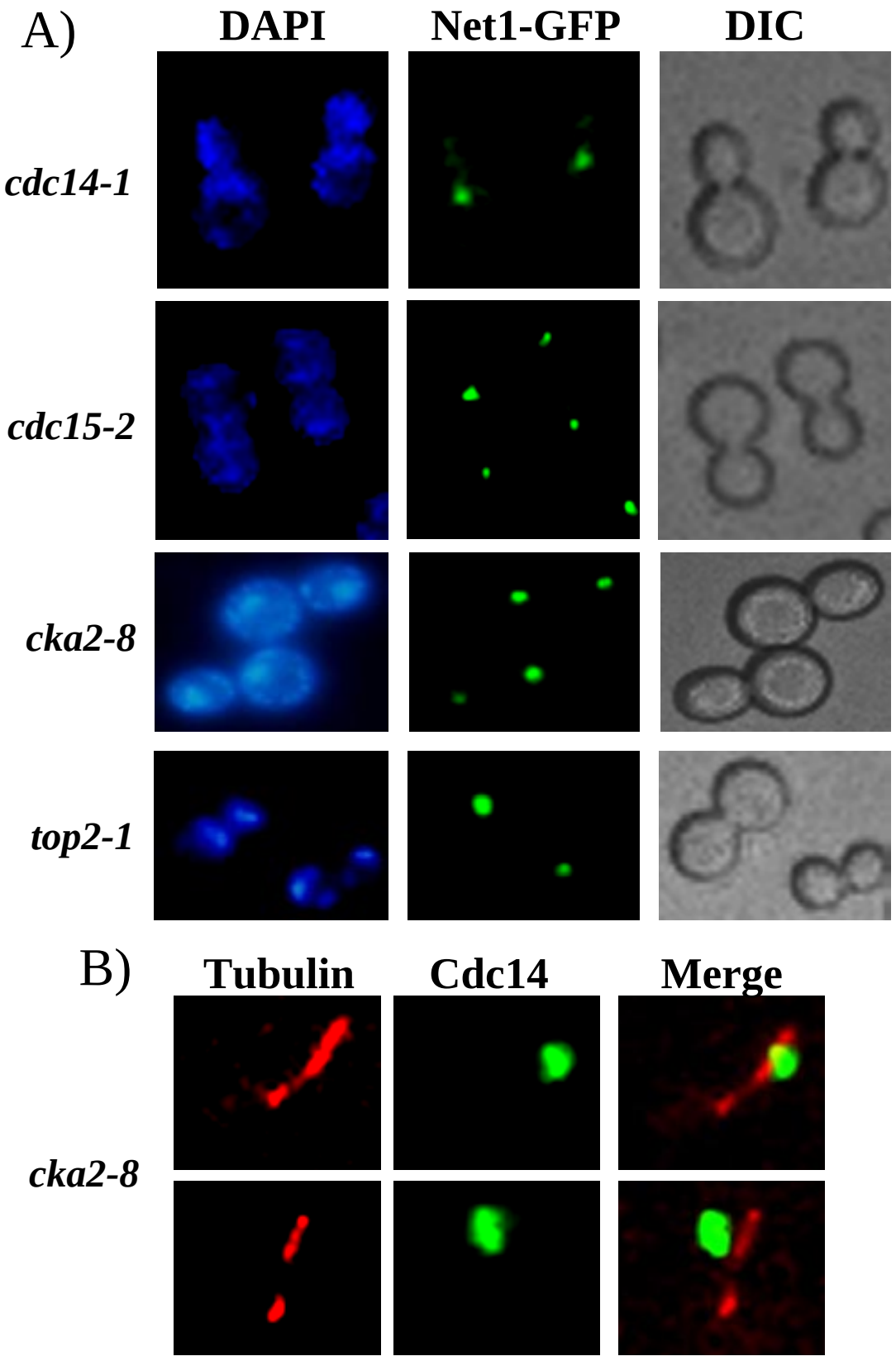

A)
DAPI
Net1-GFP
DIC
cdc14-1
cdc15-2
cka2-8
top2-1
B)
Tubulin
Cdc14
Merge
cka2-8

Figure III-4

Figure III-4: Casein Kinase II mutants arrest in early anaphase.

Mutant *esp1-1*, *top2-1*, and *cka2-8* cells containing a *lacO* array at the *LEU2* locus and expressing LacI-GFP from the *HIS3* locus were arrested in YP 2% glucose at 37°C. Samples were taken and analyzed after the arrest to determine number of cells with unsegregated chromosomes (one GFP dot) compared to cells that have segregated their chromosomes (two GFP dots). DAPI staining was performed to determine nuclear DNA content at the arrest point.

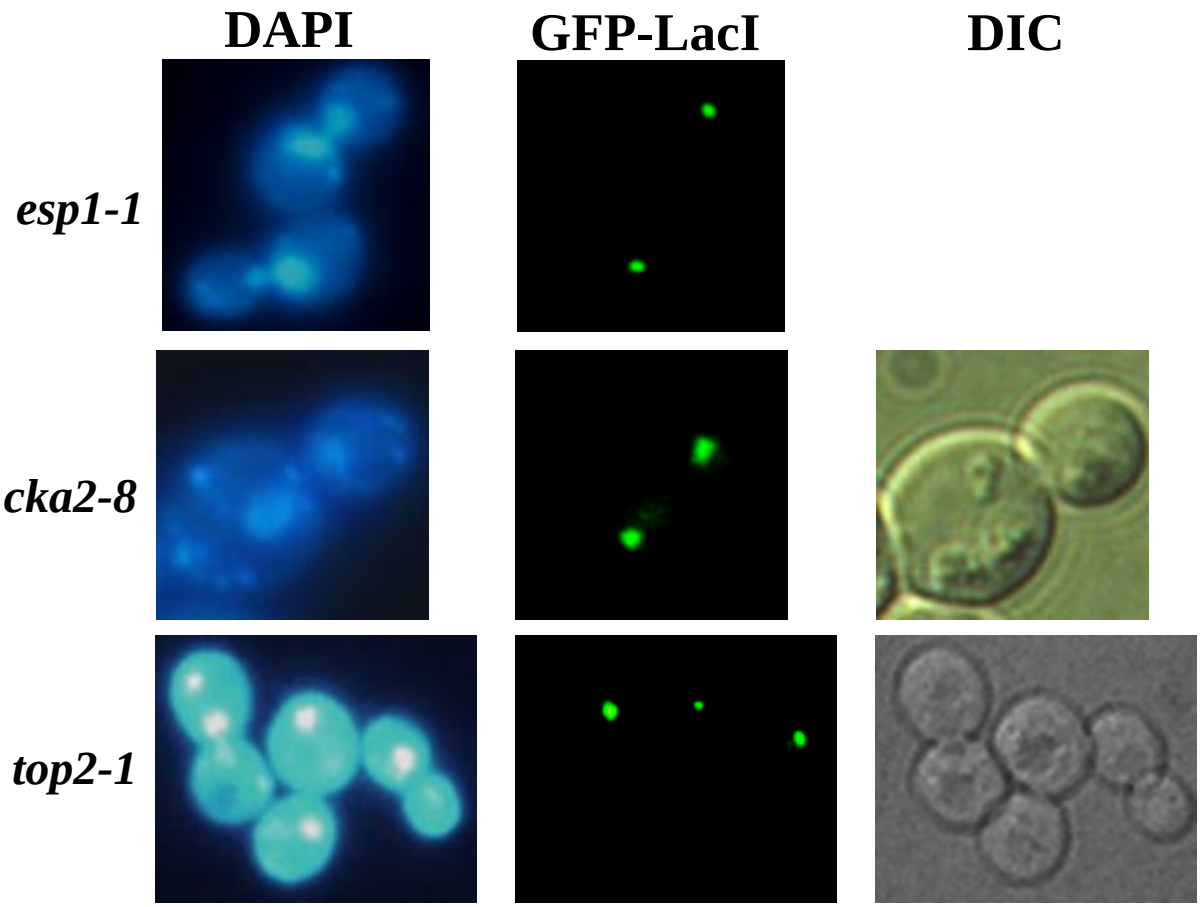


Figure III-5

Figure III-5: Casein Kinase II mutants arrest with unsegregated rDNA and Cdc14 in the nucleolus.

(A) Mutant *cdc14-1*, *cdc15-2, cka2-8,* and *top2-1* cells containing a Net1-GFP construct were arrested in YP 2% glucose at 37°C. Samples were taken and analyzed by immuno- for determination of nucleolar segregation and position. DAPI staining was performed to determine nuclear DNA content at the arrest point.

(B) Mutant *cka2-8* cells were arrested in YP 2% glucose at 37°C. Samples were taken and analyzed by indirect immuno-fluorescence with DAPI, rabbit anti-Cdc14 (1/3000), and rat anti-tubulin monoclonal antibody YL1/34 (1/1000) for determination of nuclear position, Cdc14 localization, and microtubule spindle length, respectively.

Chapter IV- Future Directions

Summary

The nucleolus can no longer be thought of as just an assembly factory for ribosomal proteins. Nucleolar sequestration now offers a new modality of regulation for important cellular proteins. Understanding the regulation of Cdc14 release and activation from Net1 gives us insight into the global role phosphorylation plays in mediating protein-protein interactions. Identification of *bone fide* kinase substrates for such kinases as Clb-Cdk, Cdc5, and Casein Kinase II will aid us in further understanding how kinases through modification of their substrates play a crucial role in regulating cellular survival and function.

Future Questions

Some of the unresolved questions still remain to be addressed. I list them for future reference:

1) How is regulation of Cdc14 activity achieved in higher and multi-cellular eukaryotes achieved?

2) What is the molecular function of the Mitotic Exit Network (MEN)? Does nucleo-cytoplasmic shuttling play a role in Cdc14 activation via the MEN?

3) How is the re-sequestration of Cdc14 achieved? What is the phosphatase that reverses Clb-Cdk phosphorylation to allow Cdc14 to re-bind to Net1?

4) What is the function of Clb-Cdk phosphorylation on other substrates in the pathway (i.e., Spo12)? How is this phosphorylation regulated?

5) Does Net1 phosphorylation target the RENT complex to specific regions of rDNA? Do they overlap with Fob1, Sir2, Cdc14?

6) Will phosphorylation of Net1 be sufficient to trigger release of Cdc14 in *fob1Δ* cells in various stages of the cell cycle?

7) Are there other components to the RENT complex that bind to Net1 or Cdc14 when it's activated and released?