CDC14 COORDINATES CYCLIN DESTRUCTION WITH THE ONSET OF CYTOKINESIS

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DEDICATION

I would like to thank my family and my wife, Kristy Bembenek.

CDC14 COORDINATES CYCLIN DESTRUCTION WITH THE ONSET OF CYTOKINESIS

by

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The Cdc14 family of protein phosphatases operate during the final stages of mitosis in various organisms. The Cdc14 phosphatases are downstream components of two homologous signaling pathways: the mitotic exit network (MEN) of *S. cerevisiae* and septation initiation network (SIN) of *S. pombe*. Studies of these pathways have revealed divergent roles of Cdc14. In the MEN pathway, Cdc14 is required for cyclin degradation by dephosphorylating Cdh1. The dephosphorylated form of Cdh1 binds to and activates a ubiquitin ligase known as the anaphase-promoting complex (APC/C), which then ubiquitinates mitotic cyclins, targeting them for degradation by the 26S proteosome. In

contrast, Cdc14 of the SIN is dispensable for cyclin degradation, but plays an important role during cytokinesis. Two Cdc14 homologues are found in vertebrates, hCdc14A and hCdc14B. I have investigated the regulation of Cdc14 phosphatases to obtain insights into the mechanisms of mitotic exit in higher eukaryotes. Biochemical studies demonstrate that recombinant hCdc14A and hCdc14B can dephosphorylate human Cdh1 and stimulate APC/C^{Cdh1} ligase activity *in vitro*. Since both the MEN and SIN pathways control Cdc14 localization, I have examined the regulation of the subcellular localization of hCdc14A, hCdc14B and the budding yeast Cdc14. In HeLa cells, hCdc14A localizes to the centrosome whereas hCdc14B is nucleolar during interphase. Both hCdc14 homologues localize to the centrosome and midbody during mitosis. In budding yeast, Cdc14p localizes to the nucleolus during most of the cell cycle and is released in late anaphase when it localizes to the centrosome and the bud neck. The subcellular localization the Cdc14 homologues in HeLa cells is regulated by a nuclear export signal. *S. cerevisiae* strains carrying only NES mutant *CDC14* alleles are capable of degrading mitotic cyclins and escaping mitosis. However, they exhibit a temperature-sensitive phenotype at 37°C because they fail to complete cytokinesis and lack centrosome and bud neck localization of Cdc14. This demonstrates that the Cdc14 phosphatases are regulated by nucleocytoplasmic shuttling. Collectively, my work strongly suggests that the Cdc14 phosphatases play a conserved role in coordinating the destruction of mitotic cyclins with the execution of cytokinesis.

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LIST OF DEFINITIONS

APC/C – Anaphase-Promoting Complex or Cyclosome

ATP – Adenosine 5'-Triphosphate

Cdk – Cyclin-Dependent Kinase

DTT – dithiothreitol

eMTOC – Equatorial Microtubule-Organizing Center

FEAR – Cdc Fourteen Early-Anaphase Release

GAP– GTPase-Activating Protein

GEF – Guanine Nucleotide Exchange Factor

GST – Glutathione S-transferase

GTP – Guanosine 5'-Triphosphate

MEN – Mitotic Exit Network

MTOC – Microtubule Organizing Center

NES – Nuclear Export Signal

Plk – Polo-Like Kinase

RanGAP1 – Ran GTPase-Activating Protein 1

RanBP1 – Ran Binding Protein 1

S. cerevisiae – *Saccharomyces cerevisiae* or Budding Yeast

S. pombe – *Schizosaccharomyces pombe* or Fission Yeast

SIN – Septation Initiation Network

SPB – Spindle Pole Body, yeast equivalent of the centrosome

Ub – Ubiquitin

CHAPTER ONE
Introduction

Regulation of the Cell Cycle

Overview of Cell Cycle Events

During the eukaryotic cell cycle, the cell accomplishes the proper segregation of its genome and the equal partition of all cellular components into the two daughter cells. The cyclin-dependent kinases (Cdks) are the master regulators that orchestrate the various complicated cellular processes of cell division (Figure 1-1). The Cdks are generally heterodimers containing a kinase subunit and a cyclin (1). Combinations of the different isoforms of kinase subunits and cyclins give rise to a large variety of functional Cdks that phosphorylate specific substrates and promote different stages of the cell cycle (2). The Cdc14 phosphatases can remove the Cdk-mediated phosphorylation of several key cell cycle regulators and antagonize the functions of Cdks. However, to understand how Cdc14 reverses the effect of Cdk1 and promotes mitotic exit, I will first examine the cellular events leading to the exit of mitosis and the molecular players involved in these events.

Several key events serve as major landmarks during progression through mitosis and provide a roadmap for understanding how regulators of mitotic exit operate. The first such event is the separation of sister-chromatids. After all sister chromatids are attached to microtubules emanating from the two opposing centrosomes (known as spindle pole bodies (SPBs) in yeast), the cohesin protein complex that maintains the sister-chromatid cohesion is removed by proteolysis (3). This allows the mitotic spindle to move chromatids poleward during anaphase. In telophase, a mature actin-based contractile ring and the equatorial microtubule-organizing center (eMTOC) are formed. Once the chromosomes have been properly segregated toward the opposite poles, the cell is partitioned into two daughter cells through the process known as cytokinesis (4). To

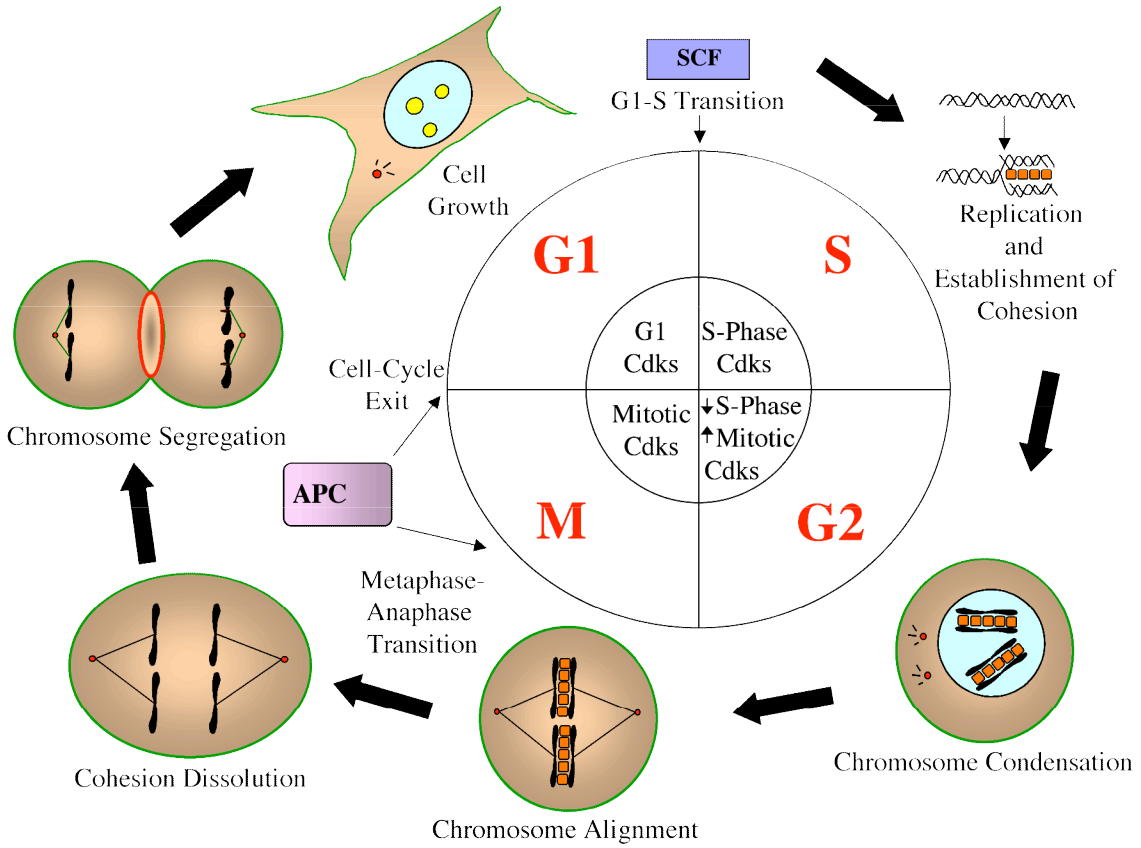


Figure 1-1. Schematic representation of the eukaryotic cell cycle.

complete mitosis, cells then disassemble the mitotic spindle, decondense chromosomes, and reform the nuclear envelope (in mammals). In the end, each daughter cell acquires a complete set of genomic DNA, one centrosome, and approximately half of the cellular organelles. Errors during mitosis, most notably chromosome mis-segregation, lead to genetic instability, which may contribute to cancer progression. To avoid this dire consequence, cells employ strict regulatory mechanisms to ensure the orderly execution and thus the fidelity of various mitotic processes.

Significant advances have recently been made toward the understanding of the molecular basis of mitotic exit. In essence, exit from mitosis requires the inactivation of the Cdk activity, and equally importantly, the reversal of some of the Cdk-mediated phosphorylation events. These dephosphorylation events in turn lead to changes in the localization, stability, and activity of a large set of cellular proteins. In general, factors that act to promote mitosis are inactivated, and those that are required for the final stages

of mitosis and the subsequent G1 phase are activated. Results from the various model organisms used to study this problem suggest that the Cdc14 family of phosphatases may play critical roles in both aspects of mitotic exit, i.e. the inactivation of Cdks and the reversal of Cdk-mediated phosphorylation.

Molecular Basis of Mitotic Exit

Cyclin Degradation and the Anaphase-Promoting Complex/Cyclosome (APC/C)

The B-type cyclins in eukaryotes form complexes with Cdk1 to promote entry into mitosis. The activities of these mitotic Cdks must then be lowered for the cell to complete the final events of mitosis and exit into the following G1 phase. Ubiquitin-mediated degradation of the mitotic cyclins ensures the complete and irreversible inactivation of the mitotic Cdks. The anaphase-promoting complex or the cyclosome (APC/C), a multi-subunit ubiquitin protein ligase (E3), is primarily responsible for the removal of B-type cyclins and other key mitotic regulators (5). The core APC/C has little intrinsic ubiquitin ligase activity and requires the association of either Cdc20 or Cdh1, two related WD40-repeat-containing co-activators, for full activity (6). Cdc20 and Cdh1 have recently been shown to recruit substrates to the APC/C and confer the substrate specificity of APC/C (7-13). Not surprisingly, binding of Cdc20 or Cdh1 to APC/C is controlled by elaborate signaling networks that act to prevent the degradation of the key mitotic regulators until the completion of certain cellular events (14). The pathways that regulate Cdc20 and Cdh1 therefore indirectly control the timing of cyclin degradation, thus influencing the Cdk oscillator that dictates the progression of the cell cycle (Figure 1-2) (15,16).

APC/C^{Cdc20} Initiates Chromosome Segregation

The Cdc20 protein (*Fizzy* in *Drosophila*, FZY-1 in *C. elegans*, p55Cdc or hCdc20

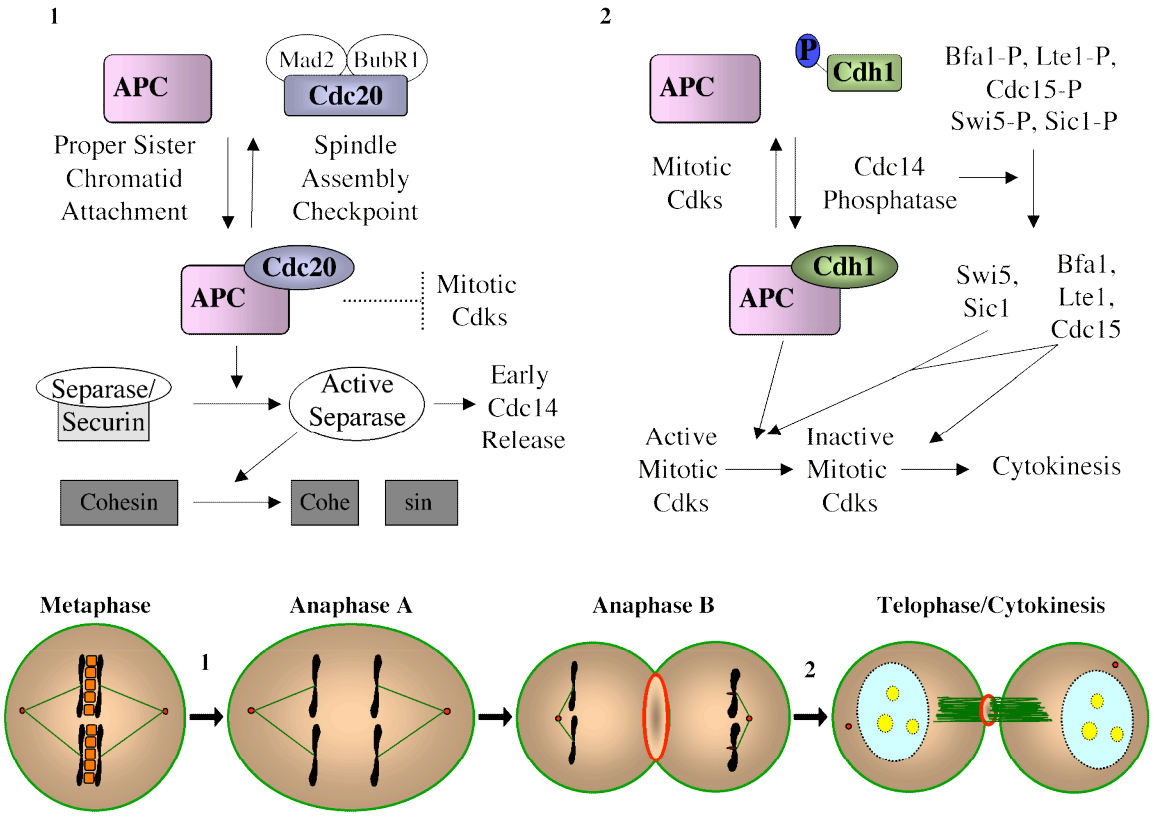


Figure 1-2. Outline of the signaling pathways that regulate APC/C activity during mitosis.

in humans, and Slp1 in *S. pombe*) binds to the APC/C and targets securin, an inhibitor of the separase, for ubiquitination (17-19). Separase released from securin then cleaves the Scc1 subunit of the cohesin complex that tethers sister-chromatids together during chromosome alignment on the metaphase plate (18). It is generally believed that cleavage of Scc1 by separase causes the loss of cohesion between sister-chromatids, which then migrate to opposing poles during anaphase. The spindle checkpoint senses tension and occupancy of kinetochores by microtubule attachments (20). Kinetochores without attachment and tension coalesce the spindle checkpoint proteins to transmit a diffusive signal that prevents Cdc20 from activating the APC/C (21,22). Once all kinetochores are correctly attached to microtubules, the spindle checkpoint is satisfied and Cdc20 is allowed to activate the APC/C, leading to ubiquitination of securin, activation of separase, cleavage of Scc1, and the onset of sister-chromatid separation (3,23). In addition to securin degradation, the APC/C^{Cdc20} complex also begins the

degradation of mitotic cyclins and possibly other important substrates (24).

Role of APC/C^{Cdh1} and Cdc14 in Mitotic Exit

In certain systems, such as the early embryonic cell divisions of *Drosophila* and *Xenopus* that lack G1 or G2 phases, APC/C^{Cdc20} is sufficient for mitotic exit by ubiquitinating enough cyclin to allow the inactivation of Cdk1. In fact, the Cdh1 protein is not even present in *Xenopus* egg extracts (25). However, during the somatic cell cycle that has a well-defined G1 phase, Cdh1 (also known as Hct1 in *S. cerevisiae*, *Fizzy-related* in *Drosophila*, FZR-1 in *C. elegans*, and Srw1/Ste9 in *S. pombe*) also mediates the degradation of mitotic cyclins and the inactivation of Cdk1, thus promoting mitotic exit (16,26). In addition to ubiquitinating mitotic cyclins, APC/C^{Cdh1} is also known to cause the degradation of many important mitotic regulatory proteins, including Cdc20 and the polo-like kinase (Plk Cdc5), thus resetting the cell cycle and establishing G1 (27,28). To delay their own demise, the mitotic Cdks phosphorylate Cdh1 and block its association with the APC/C (29). Several lines of evidence indicate that the Cdc14 phosphatase dephosphorylates Cdh1 and promotes its stimulatory activity toward the APC/C when the cell is prepared to complete mitosis and enter G1 (26,29-32).

Regulation of Cdc14 by MEN and SIN

Through genetic analysis, a complex network of genes in the budding yeast *Saccharomyces cerevisiae*, known as the Mitotic Exit Network (MEN), has been shown to control the Cdc14-dependent activation of APC/C^{Cdh1} (33). Similarly, in the fission yeast *Schizosaccharomyces pombe,* Cdc14 is regulated by a highly homologous signaling pathway, known as the Septation Initiation Network (SIN) (34,35). The SIN was identified by genetic analysis aimed at understanding the control of cytokinesis rather than cyclin degradation and mitotic exit. Surprisingly, the SIN does not appear to be required for the activation of APC/C^{Cdh1}. Thus, studies on the two genetic networks

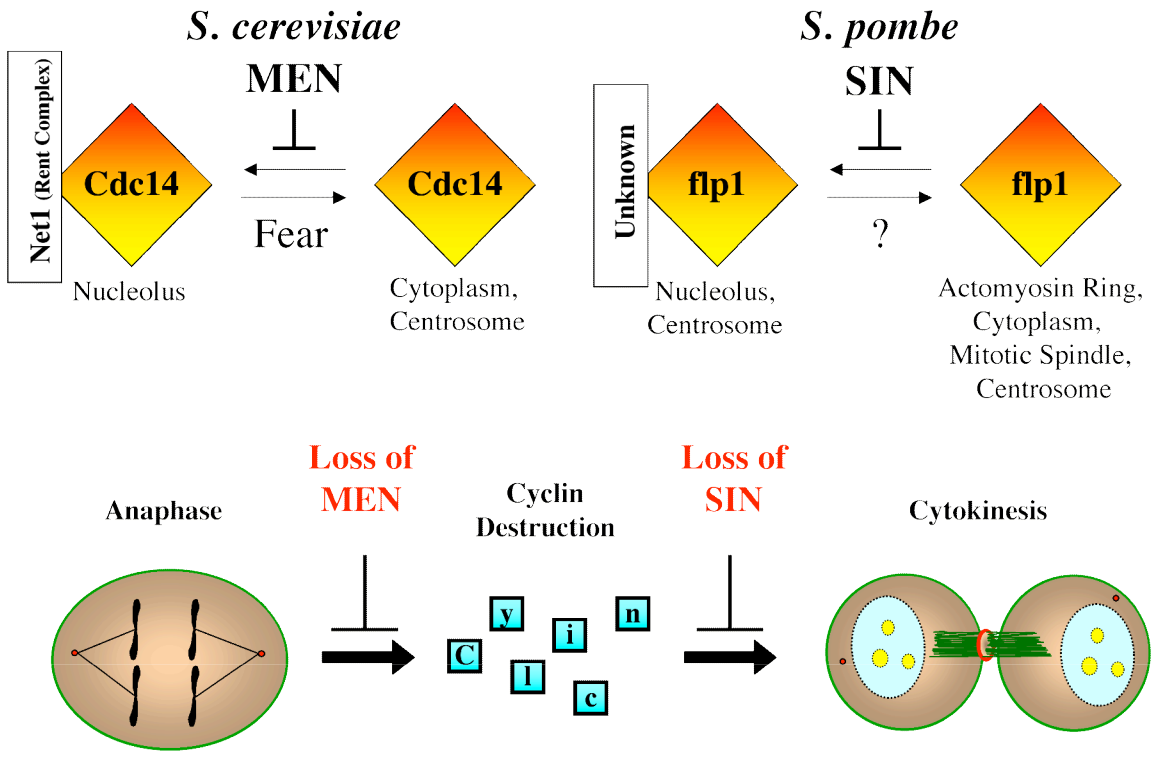


Figure 1-3. Comparisons of the MEN and SIN.

(MEN and SIN) operating in budding and fission yeast have led to the conclusion that, except for the regulation of Cdc14, the MEN and SIN networks have been established to perform very different tasks (Figure 1-3).

The genetic dissection of MEN and SIN has been invaluable in identifying a large set of factors responsible for the inactivation of mitotic Cdks and/or for the completion of cytokinesis. However, the apparent discrepancy between the MEN and SIN outputs has caused some distraction to the studies of this important cell cycle circuitry. It is possible that the different findings between the budding and fission yeast may ultimately be a result of specific features associated with the cell cycle of the two organisms. Pathways homologous to the MEN and SIN could in fact have very similar functions in controlling both mitotic exit and cytokinesis in all organisms. The work described here focuses on understanding the regulation of mitotic exit and cytokinesis in mammalian cells, with specific focus on the regulation of the human Cdc14 homologues.

I propose the following hypothesis to reconcile the difference between MEN and SIN in general and the difference between the roles of Cdc14 in MEN and SIN signaling in particular. A fact common to all organisms studied to date is that cytokinesis cannot occur prior to the inactivation of Cdk1 (Figure 1-3). It is thus possible that factors required for cytokinesis are inhibited by mitotic Cdks. The general model derived from the evidence currently available in several model systems is that the Cdc14 phophatase promotes both the exit from mitosis and cytokinesis in two ways. First, Cdc14 dephosphorylates several substrates, which then causes the global inactivation of mitotic Cdk activity. Second, Cdc14 removes Cdk-mediated phosphorylation on specific substrates required during cytokinesis to activate them. Although the Cdc14 homologue in *S. pombe*, cdc fourteen like protein 1 or cdc14 like protein 1 (flp1/clp1), is not required for cyclin degradation during SIN signaling (34,35), flp1/clp1 is very likely to be involved in this process during the normal cell cycle. The flp1/clp1-null *S. pombe* mutants may degrade cyclins and inactivate Cdk1 through alternative mechanisms (possibly through the actions of the APC/C^{Cdc20} complex), but display an elevated rate of cytokinesis defects compared to wild-type cells, highlighting the role of flp1/clp1 during cytokinesis (34,35). Unlike *S. pombe*, alternate mechanisms are unable to overcome the loss of Cdc14 function in *S. cerevisiae* for the degradation of mitotic cyclins. Inactivation of Cdc14 in budding yeast thus leads to cyclin stabilization, preventing both mitotic exit and cytokinesis. The failure to degrade mitotic cyclins in the MEN mutants of *S. cerevisiae* masks any potential cytokinesis phenotypes. Extensive reviews are available on the MEN and SIN (36-40). I will briefly describe these networks with emphasis on the most recent findings and discuss how they relate to my general hypothesis of the function of Cdc14 in both mitotic exit and cytokinesis. I will also attempt to highlight important issues that may resolve the discrepancies between the MEN and SIN, and then present my work indicating that Cdc14 homologues play a role in both processes during the final stages of mitosis in both mammalian and fungal cells.

The MEN of *S. cerevisiae*

Molecular Components of the MEN

Inactivation of the MEN in *S. cerevisiae* through temperature-sensitive mutants or deletion analysis causes cells to arrest in late anaphase with large buds and high mitotic Cdk activity. MEN activity is therefore required for proper cyclin degradation, and cyclin degradation is required for cytokinesis. MEN components include the Cdc14 dual-specificity phosphatase, the protein kinases Cdc5 (polo-like kinase family), Cdc15, and Dbf2/Dbf20 in complex with Mob1 (no known structural and functional motifs), the Nud1 scaffolding protein, the Tem1 GTPase, the Lte1 guanine nucleotide exchange factor (GEF), and the Bub2-Bfa1 complex that comprises a two-component GTPase-activating protein (GAP) (Figure 1-4).

The TEM1 GTPase Cycle

The Tem1 small GTPase is thought to act at or near the top of this signaling network, and is anchored at the SPB along with the rest of the MEN by the Nud1 scaffolding protein (41). The two-component GAP Bub2-Bfa1 negatively regulates Tem1 (42). Deletion of Bub2, however, does not have the same effect as most of the other MEN components. Bub2 is a spindle checkpoint gene and was identified in a screen for genes required for maintaining the mitotic arrest in response to microtubule depolymerization by drug treatment (43). The majority of spindle checkpoint genes identified in this genetic screen regulate the ubiquitin ligase activity of APC/C^{Cdc20} (44). On the other hand, Bub2 appears to be the only spindle checkpoint gene that sustains mitotic arrest by negatively regulating the MEN through Tem1 inactivation (45). It should be pointed out that Bub2 is dispensable for the normal cell cycle in the absence of spindle damaging agents, largely due to additional regulatory mechanisms on Tem1 (see

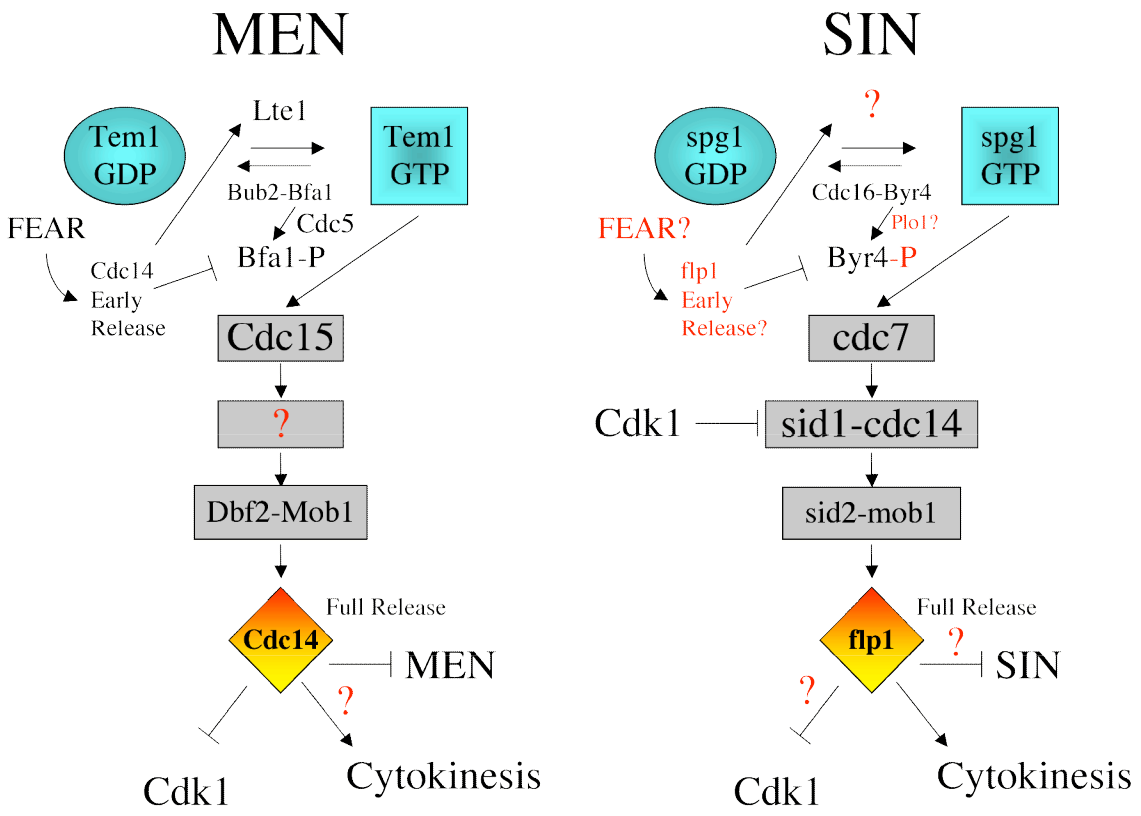


Figure 1-4. Schematic outline of the interactions between molecular players of the MEN and SIN.

below) and the timing of cell cycle events (i.e. the rate of spindle positioning into the daughter cell occurs faster than the execution of cytokinesis after MEN activation).

Mutations of Lte1 cause telophase arrest at low temperatures. Overexpression of Tem1 can bypass this arrest (46). This and other evidence support the role of Lte1 in activating Tem1 through exchange of GDP for GTP on Tem1. Presumably, the GEF activity of Lte1 is only required for Tem1 nucleotide exchange at lower temperatures, as Tem1 has high intrinsic exchange activity at higher temperatures (42). Interestingly, Tem1, Bub2 and Bfa1 localize preferentially to the SPB that repositions into the daughter cell, (47) whereas Lte1 is localized at the cortex of the daughter cell (47,48). This spatial regulation is thought to allow Tem1 to become activated only when the leading spindle pole has properly moved into the daughter cell. For this reason, the Bub2-regulated MEN pathway is also known as the spindle position checkpoint.

Role of Cdc15, Dbf2, Cdc5 during Mitotic Exit

Tem1 activation then leads to the recruitment the Cdc15 kinase to the SPB (49). The kinase activity of Cdc15 is constitutively active and is thought to be regulated only by the availability of substrates, which are localized at the SPB (50). Cdc15 localization to the SPB leads to the activation of the kinase activity of Dbf2–Mob1 complex (51,52). In late mitosis, both Cdc15 and Dbf2–Mob1 translocate from the SPB to the mother-bud junction during cytokinesis (53,54). The exact role of these components at the bud neck is not clear.

Cdc5 plays a complex role during several stages of mitosis. First, during prometaphase, Cdc5 is involved in the activation of APC/C^{Cdc20} through a yet unknown mechanism (28). Second, Cdc5 phosphorylates the cohesin subunit Scc1, which facilitates its cleavage by the separase, Esp1 (55). Third, Cdc5 phosphorylates Bfa1 and inhibits the GAP activity of Bub2–Bfa1 toward Tem1 (56). Fourth, Cdc5 phosphorylates Net1, which is thought to promote its dissociation from Cdc14 and the release of Cdc14 from sequestration (see below) (57,58). At the end of mitosis, Cdc5 also localizes to the bud neck with other MEN components, and plays a role in the completion of cytokinesis (59).

Release of Cdc14 from the Nucleolus

The activation of this GTPase/kinase intracellular signaling cascade eventually leads to the activation of Cdc14 and mitotic exit. In interphase cells, Cdc14 is sequestered in the nucleolus where it remains inactive by association with the Net1/Cfi1 component of the RENT (for Regulator of Nucleolar silencing and Telophase) complex (60,61). An active MEN signal is required for the sustained release of Cdc14 from the nucleolus. Because mutations of Cdc5, Cdc15, Dbf2, and Tem1 all lead to a telophase arrest with Cdc14 sequestered in the nucleolus and because overexpression of Cdc14 bypasses the lethality of most MEN mutants, Cdc14 is thought to be the most

downstream component of the MEN pathway (15).

Once Cdc14 is released from the nucleolus, it dephosphorylates many substrates, including Cdh1 (Hct1 in yeast), Swi5, Sic1, Cdc15, and Lte1 (50,62-64). These substrates fall into two general classes; Cdk inhibitors, and modulators of MEN activity. Dephosphorylation of Swi5 leads to the transcriptional up-regulation of Sic1, a Cyclin-dependent Kinase Inhibitor (CKI) (62). Moreover, dephosphorylation of the Sic1 protein is required for its stabilization and accumulation. Also, dephosphorylation of Cdh1/Hct1 activates the APC/C and leads to the degradation of Clb2 (30). Dephosphorylation of Lte1 and Cdc15 coincide with the activation of the MEN and are thought to promote the activity of these proteins. Therefore, Cdc14 is a key component of the MEN and triggers the exit from mitosis through multiple inter-connected mechanisms.

Transient Release of Cdc14 in Early Anaphase

Recently, several findings have refined the aforementioned model of mitotic exit in budding yeast. Earlier evidence had suggested an additional mechanism for Cdc14 release from the nucleolus independent of MEN activity (60). For some time, it had been known that, in addition to controlling the cleavage of the Scc1 subunit of cohesin during the metaphase-anaphase transition (see above), Securin (Pds1) and Separase (Esp1) play a role in the proper execution of mitotic exit (65,66). The mitotic exit function of Pds1 and Esp1 is dependent on several MEN components, including Cdc5. Overexpression of Cdc5 results in an active form of APC/C which does not promote the degradation of Pds1 in vivo, implicating active APC/C^{Cdh1} (28). Additionally, Spo12, a protein involved in the regulation of meiosis and the proper timing of mitosis, was shown to be a high-copy suppressor of MEN components (33). Interestingly, Spo12 localizes to the nucleolus, suggesting that a possible mechanism for suppression of MEN mutations by Spo12 might be through the delocalization of a small portion of Cdc14 from the nucleolus (67). These observations were tied together by several recent reports that made careful observations

of the dynamics of Cdc14 localization throughout anaphase. It was demonstrated that a transient MEN-independent release of Cdc14 occurs in early anaphase, prior to MEN activation (68-70). Several genes, collectively referred to as the Cdc Fourteen Early Anaphase Release (FEAR) network, were required for this early release of Cdc14, including Cdc5, Spo12, Esp1, and the Esp1 substrate, Slk19 (68).

Subsequently, Cdc5 was shown to affect Cdc14 release from the nucleolus in part by phosphorylating Net1, weakening the affinity of Net1 for Cdc14 (57,58). In an elegant study, it was determined that Esp1 activation promotes Cdc14 release from the nucleolus independent of its role in chromosome segregation. The physical interaction between Esp1 and its substrate Slk19, rather than the protease activity of Esp1, is required for the transient release of Cdc14 (71). Thus, progression through mitosis requires two distinct functions of Esp1. These results strongly suggest that the events of mitotic exit are mechanistically coupled to the proper completion of chromosome segregation. This appears to be accomplished by a bifurcation in the progression through mitosis by the dual-roles of separase in both cleaving Scc1 to allow the onset of anaphase and in promoting the initial release of Cdc14 from the nucleolus.

The Relationship between the MEN and FEAR

Cdc5 is the only common component of the MEN and FEAR. It is required for Cdc14 release both in early and late anaphase, which is consistent with its pleiotropic roles in mitosis, including the phosphorlyation of Bfa1, Net1, and Scc1. What, then, is the functional consequence of the early release of Cdc14? Not surprisingly, it is required for the full activation of the MEN. It turns out that the early released Cdc14 localizes to the SPB that moves into the daughter cell through the physical interaction with Bfa1 and Tem1 (69,70). This binding interaction may help block the GAP activity of Bub2-Bfa1 toward Tem1, thus allowing the loading of GTP onto Tem1 by Lte1 (69).

In addition to binding to Bfa1 and Tem1 at the SPB, the transient release of

Cdc14 in early anaphase may also stimulate the activity of the MEN by dephosphorylating Cdc15 and Lte1. The effect of Cdc15 dephosphorylation by Cdc14 is unknown, although it might affect the SPB localization of Cdc15, and thus Dbf2-Mob1 activation, or promote its translocation to the contractile ring (50). This will activate more players of MEN, and somehow enhance Cdc15 activity toward mitotic exit. Lte1 has recently been shown to localize to the bud in a manner dependent on actin, Cdc42, and the mitotic Cdks (63), demonstrating a role for cell polarity proteins in mitotic exit. The Rho-like GTPase, Cdc42, activates Cla4 and leads to Lte1 phosphorylation and localization to the cortical tip of the bud through binding to Kel1 (64,72). Cla4 also directly phosphorylates septin subunits to induce formation of the septin ring at the bud neck, a crucial structure for the cytokinesis (73). On the other hand, overexpression of Cdc14 in metaphase cells leads to the premature dephosphorylation and delocalization of Lte1 (63). The release of Cdc14 in early anaphase may positively influence the activity of Lte1 by releasing it from the cell cortex and allowing its access to Tem1 on the SPB. Thus, Cdc14 potentiates the signaling of the MEN through several parallel pathways.

Role of the MEN during Cytokinesis

Several lines of evidence indicate that the MEN in budding yeast might also be important for cytokinesis, similar to the SIN in fission yeast. For example, many MEN components localize to both the SPB and the bud neck. More interestingly, a Net1 mutant has been isolated that allows the release of Cdc14 in the absence of proper Tem1 function. This mutant degrades mitotic cyclins with the proper timing, but exhibits severe cytokinesis defects when the function of Tem1 is compromised (74). This strongly suggests that Tem1, which is at the top of the MEN signaling cascade, is required for certain aspects of cytokinesis in addition to its role during Cdc14 release. This function of Tem1 can only be revealed when the requirement for MEN in the inactivation of Cdk1 is bypassed, in this case, by suppressing the function of Net1 as a

tether for Cdc14. The Mob1 protein, which complexes with Dbf2, is required for actomyosin ring contraction and thus proper cytokinesis when its role in cyclin degradation is bypassed (75,76). Temperature sensitive mutations of Dbf2 cause the loss of the actomyosin ring and actin patches which are required for constriction of the bud neck in late mitosis (53). Additionally, certain mutations in Cdc15 allow rebudding to occur, indicative of Cdk1 inactivation, and are defective for cytokinesis (77). Furthermore, Cdc15 has recently been shown to relocate from the mother SPB to the daughter SPB only after the activation of Cdc14 and inactivation of Cdk1 (54). Cdc15 fragments defective in SPB localization are capable of forming an actomyosin ring, but undergo several rounds of DNA replication and form chains of cells without completing cytokinesis. Cdc5 mutations also cause growth of cells into chains by disruption of septin formation, and cytokinesis defects independent of the Bub2-Bfa1 pathway (78). Finally, overexpression of a C-terminal domain of Cdc5 inhibits cytokinesis (59).

Inactivation of MEN Signaling

All the available data now support the following order of events. First, Cdc14 dephosphorylates Lte1, both of which then associate with the SPB through interactions with Tem1 and Bfa1. This leads to the activation of the MEN. Cdc14 then dephosphorylates Cdc15, activating Dbf2-Mob1 and downstream MEN components at the SPB. Cdc15 and Dbf2-Mobl then relocate to the contractile ring and play an undetermined role in the execution of cytokinesis. Concurrent with these events, Cdc14 also promotes the destruction of mitotic cyclins through Cdh1. Cdc14 also dephosphorylates Swi5 and Sic1, leading to the accumulation of Sic1. This culminates in the global inactivation of Cdk1, allowing mitotic exit and cytokinesis. At telophase, Cdc14 inactivates the MEN signaling by reversing the Cdc5-dependent phosphorylation of Bfa1, which restores the GAP activity of Bub2-Bfa1 towards Tem1 (69).

Interestingly, despite their direct association, Cdc14 does not appear to

dephosphorylate Bfa1 in early anaphase (69). The mechanism by which Cdc14 is prevented from dephosphorylating Bfa1 in early anaphase is unknown. One possibility is that the fully activated MEN causes a full release of Cdc14 from the nucleolus, increasing the concentration of Cdc14 in the cytoplasm and elevating Cdc14-binding to the SPB. Only high enough concentrations of Cdc14 at the SPB in telophase could then lead to the dephosphorylation of Bfa1 and the inactivation of MEN. Alternatively, a yet unidentified mechanism actively prevents the dephosphorylation of Bfa1 by Cdc14, but not its binding to Cdc14, until the MEN activity is no longer required. Finally, a recently discovered protein, Amn1p, is required to inactivate MEN signaling in G1 in part by binding Tem1 and disrupting the Tem1-Cdc15 binding interaction (79).

The SIN of the Fission Yeast *S. pombe*

Molecular Components of the SIN

In sharp contrast to the MEN defects in *S. cerevisiae*, most loss-of-function SIN mutants in *S. pombe* properly degrade mitotic cyclins and exit mitosis. The primary defect of SIN mutants is the incomplete or improper execution of cytokinesis and septum formation, resulting in elongated and multinucleated cells with multiple septa that eventually undergo lysis. Despite these differences, the MEN and SIN are highly similar intracellular signaling networks with most MEN components having homologues in the SIN. In fact, it is highly likely that homologues of all known MEN components will eventually be found in the SIN, and vice versa (Figure 1-4).

The SIN consists of cdc11 (SPB scaffold), spg1 (a Ras-like GTPase), cdc16–byr4 (a two-component GAP), plo1 (a polo-like kinase), cdc7 (a protein kinase), sid2 (a protein kinase), mob1, and flp1/clp1 (dual-specificity phosphatase). These genes are homologues of Nud1, Tem1, Bub2-Bfa1, Cdc5, Cdc15, Dbf2, Mob1, and Cdc14 of the MEN in *S. cerevisiae*, respectively. There is currently no known GEF for spg1 in the

SIN that is homologous to Lte1 in the MEN, whereas the sid1-cdc14 (unrelated to the *S. cerevisiae* Cdc14) protein kinase complex has no known homologue in the MEN.

The cdc11 gene encodes a scaffold homologue of the *S. cerevisiae* nud1, and is required to localize SIN components at the SPB (80). The spg1 GTPase is an activator of SIN signaling, and is inhibited by the two-component GAP, cdc16-byr4 (81,82). Overexpression of spg1 leads to multiple rounds of septum formation without cytokinesis (83). Likewise, deletion of either cdc16 or byr4 causes the same phenotype due to hyperactive SIN function (84,85). Additionally, cdc16-byr4 function is required, in a similar fashion to Bub2-Bfa1, to maintain high mitotic Cdk activity in the presence of a metaphase arrest caused by an active spindle checkpoint (84). The role of flp1/clp1 in this premature exit from mitosis has not been directly tested and could be an important experiment toward resolving the discrepancies between the MEN and SIN networks. The cdc7 kinase, the *S. cerevisiae* Cdc15 homologue, is recruited to the spindle in an spg1-GTP-dependent manner, and activates the sid2-mob1 kinase (Dbf2-Mob1 *S. cerevisiae* homologue) complex through the sid1-cdc14 kinase (86). The active sid1-cdc14 complex is recruited to the SPB only after the inactivation of Cdk1. This step may be one of the key links between the inactivation of mitotic Cdks and the initiation of cytokinesis.

The plo1 polo-like kinase, similar to Cdc5 of the MEN, has a complex role in SIN signaling. It is required for the formation of the equatorial microtubule organizing center, the medial ring and thus septation (87,88). Overexpression of plo1 causes the recruitment of cdc7 to the SPB and activation of the SIN (89). The plo1 kinase was also shown to activate spg1 and act at a step upstream of cdc16-byr4 (90). This indicates that plo1 may function in a similar fashion to the Cdc5 of the MEN network. It will be interesting to examine whether plo1 directly phosphorylates and inhibits byr4, causing the activation of spg1 and the recruitment of cdc7 to the SPB and if it influences the localization of flp1/clp1, the *S. pombe* Cdc14 homologue (see below). Likewise, there is currently no experimental evidence to support direct interactions between flp1/clp1 and

spg1 or cdc16 as predicted based upon analysis of the MEN.

Differences Between the MEN and SIN

The SIN mutants were able to degrade mitotic cyclins and undergo mitotic exit. Consistent with this, flp1/clp1 is not essential for viability in *S. pombe* (34,35). Instead of arresting in telophase with elevated cyclins, the flp1/clp1 deletion mutants degrade mitotic cyclins normally (34,35). Dephosphorylation of the rum1 and ste9/srw1 proteins (homologues of sic1 and Hct1 of *S. cerevisiae* respectively) also occurs normally in these cells (34,35). Consequently, the flp1/clp1 mutant cells exit mitosis, albeit with a slight increase (about 8% of the population) in cytokinesis failures (34,35). Unexpectedly, the flp1/clp1 mutants exhibit a mild so-called *wee* phenotype first found in the wee1 mutants, i.e. these cells are small in size due to premature entry into mitosis. Furthermore, although overexpression of flp1/clp1 does not overcome the phenotypes of other SIN mutants, it does produce a dramatic delay in mitotic entry. This delay in mitotic entry correlates with the dephosphorylation of the cdc25 phosphatase, an activator of Cdk1 (34,35). Recent work has shown that Cdc14 directly influences Cdk1 inactivation by directly dephosphorylating Cdc25, which leads to its inactivation and contributes to Cdk1 inactivation (91). These data are consistent with flp1/clp1 playing a role in restraining mitotic entry by inhibiting cdc25 and thus delaying the activation of Cdk1. Despite the failure to rescue the cytokinesis phenotype of several SIN mutants, there are strong genetic interactions between flp1/clp1 and the SIN. Certain SIN mutant cells with flp1/clp1 deleted lose viability and have a more pronounced septation defect (34,35). This strongly suggests that flp1/clp1 might have a role in potentiating SIN signaling, similar to that of Cdc14 in further activating other MEN components in the budding yeast. Interestingly, loss of flp1/clp1 strongly attenuated the recruitment of the sid1p kinase to the SPB, a step that is dependent upon Cdk1 inactivation. This indicates that flp1/clp1 negatively regulates Cdk1 during mitotic exit, despite not being strictly required

for cyclin degradation and exit from mitosis (34,35).

Another apparent discrepancy between flp1/clp1 and the budding yeast Cdc14 lies in their localization patterns. In interphase cells, flp1/clp1 localizes largely to the nucleolus, but a small portion of flp1/clp1 is also found at the SPB (34,35). This observation casts doubt on the generality of the hypothesis that nucleolar sequestration by the RENT complex completely inactivates the Cdc14 family of phosphatases, as first suggested by results from *S. cerevisiae*. This localization pattern of flp1/clp1 is also consistent with it playing a role during interphase, such as the regulation of cdc25 and consequently Cdk1. During mitosis, flp1/clp1 is released from the nucleolus into the cytoplasm and the nucleus. In addition to the diffuse localization, flp1/clp1 released from the nucleolus also specifically localizes to the spindle, the eMTOC, and the central actomyosin ring. Surprisingly, the SIN network is not required to release flp1/clp1 from the nucleolus, but is necessary for maintaining flp1/clp1 in a released state (34,35). This observation is consistent with the discovery of the FEAR network and how Cdc14 release is regulated in *S. cerevisiae*. The prominent differences between MEN and SIN led to the conclusion that, despite many homologous features, they function in very different fashions and control distinct cellular processes.

Requirement for flp1/clp1 in Mitotic Exit Under Special Circumstances

The dma1 gene was identified as a multicopy suppressor of null phenotype of cdc16, a spindle checkpoint gene (92,93). The dma1 protein contains an FHA domain (a putative phosphopeptide-binding module) and a RING domain (a domain commonly found in E3 ubiquitin ligases) (92). Like cdc16 and byr4, dma1 is not an essential gene. However, similar to cdc16 and byr4, deletion of dma1 abrogates the ability of the spindle checkpoint to maintain a metaphase arrest in the presence of spindle damaging agents (92). In a study to further analyze the relationship of dma1 with the SIN, Guertin *et al.* showed that dma1 prevents plo1 from localizing to the SPB. Although dma1 has yet to

be demonstrated to be an E3 ubiquitin ligase, its mammalian homologue, CHFR, has been shown to directly ubiquitinate Plk1 in vitro (94). It is thus possible that dma1 directly ubiquitinates plo1 and regulates its localization or turnover at the SPB, thus preventing the inactivation of cdc16-byr4 by plo1 and the untimely activation of spg1 in the presence of an active spindle checkpoint (95).

As mentioned above, the dma1 deletion mutant cells exhibit a defective spindle checkpoint and undergo mitotic exit in the presence of spindle damaging agents, such as nocodazole (95). Interestingly, flp1/clp1 is required for the inactivation of mitotic Cdks and the mitotic exit of the dma1-null cells in the presence of an active spindle checkpoint (and thus an inactive APC/C^{Cdc20} complex) (95). Normal mechanisms for the inactivation of active Cdk1 in *S. pombe* again involve the stabilization and increased expression of the CKI rum1 and the activation of the Cdh1 homologue, ste9/srw1 (96-100). Although the fission yeast cells can overcome the loss of flp1/clp1 and exit mitosis, the failure of the flp1/clp1 and dma1 double mutants to undergo mitotic exit in the presence of spindle damage (which leads to APC/C^{Cdc20} inhibition) indicates that flp1/clp1 is required for cyclin degradation under certain adverse conditions (95). This strongly suggests that flp1/clp1 might contribute to the activation of APC/$C^{ste9/srw1}$ and/or the stabilization of rum1 during the normal cell cycle. It will be interesting to test whether cdc16 mutants that have a defective spindle checkpoint also require the function of flp1/clp1 for abnormal Cdk1 inactivation and mitotic exit, which would be expected based upon the genetic studies of the homologous Bub2 gene in budding yeast. Along this line, it will also be interesting to test the ability of flp1/clp1 to directly dephosphorylate ste9/srw1 and rum1 proteins *in vitro*. In summary, despite the differences between the MEN and SIN, my hypothesis is that these networks have been established to coordinate the destruction of mitotic cyclins with the onset of cytokinesis to ensure fidelity during the final stages of mitosis. This hypothesis has provided a theoretical framework from which I have approached the problem of understanding the regulatory pathways of human

Cdc14.

Mitotic Exit Signaling Networks in Higher Eukaryotes

Homologues of the MEN and SIN in Higher Eukaryotes

It is very likely that higher eukaryotes utilize signaling networks similar to MEN and SIN to monitor late mitotic events such as spindle position and sister chromatid segregation. Database searches have identified several homologues of the MEN and SIN networks in mammals and other higher eukaryotes. A centrosomal component known as centriolin shares identity with Nud1 of budding yeast and cdc11 of fission yeast and may serve as a scaffold for downstream signaling components (101). Also, the depletion of centriolin by RNAi induces defects in the completion of cytokinesis, implicating that it has a similar role to its Nud1 and cdc11 homologues (101). GAPCENA has homology to Bub2 (*S. cerevisiae*) and cdc16 (*S. pombe*), and localizes to the centrosomes (102). The small GTPase RhoA has been shown to be required for the proper execution of cytokinesis in mammalian cells (103). Apparent homologues of Dbf2/Sid2, Mob1, Cdc5/Plo1 have been identified in mammals (104-108). Likewise, two human homologues of Cdc14, hCdc14A and hCdc14B, have been identified (109). As expected, these homologous human proteins have been shown to play various roles in mitosis and some of them are shown to localize to mitotic structures including the centrosomes, similar to their yeast counterparts. However, these various factors have not yet been assembled into a signaling network that responds to spindle position cues and controls exit from mitosis in mammals. My thesis work has focused on elucidating the role of Cdc14 during the final stages of mitosis with the ultimate goal of delineating a signaling pathway responsible for the proper execution of the final events in mitosis.

Several lines of evidence suggest the existence of a signaling pathway that responds to spindle position and demonstrates a link between the centrosomes and

cytokinesis in mammals. First, micro-manipulation studies showed that anaphase was delayed in mammalian cells with mis-oriented spindles until spindle repositioning occurred (110). Laser ablation of the centrosome in vertebrate cells leads to an increase in cytokinesis defects and prevents subsequent entry into the subsequent cell cycle prior to S-phase (111). Similarly, microsurgical removal of the centrosomes in mammalian cells caused defects in cytokinesis, and live imaging studies of cells stably expressing GFP-Centrin to label the centrosomes demonstrated that the mother centriole migrates to the midbody immediately prior to abscission (112). Taken together, these data suggest that centriolin and hCdc14A at the centrosome play an integral part in carrying out the proper execution of cytokinesis, although the exact mechanism remains to be established. The crucial role of centrosomes in mammalian cytokinesis is consistent with the functions of the SPBs in yeast, which act as hubs for organizing and assembling active complexes of the MEN and SIN molecules involved with mitotic exit and cytokinesis, indicating that an analogous signaling network might exist in mammalian cells.

Cdc14 in Higher Eukaryotes

The human genome encodes two homologues of Cdc14, hCdc14A and hCdc14B. Studies concurrent with the work presented here demonstrated that hCdc14A localizes to the centrosomes throughout the cell cycle whereas hCdc14B localizes to the nucleolus during interphase (113). Studies have also demonstrated that overexpression of hCdc14A in mammalian tissue culture cells promotes human APC/C^{Cdh1} formation even in the presence of non-degradable cyclin B, leads to premature centriole splitting in S-phase, and aberrant, multi-polar mitotic spindles (32,114). Depletion of hCdc14A from HeLa cells by RNA interference (RNAi) also led to multiple defects in mitosis, including failure to undergo cytokinesis (114). These data establish that human Cdc14 homologues contribute to both the destruction of mitotic cyclins and the execution of cytokinesis. In chapter 2, I describe the work demonstrating that Cdc14 homologues directly activate the

APC/C^{Cdh1} *in vitro*. In chapter 3, I describe a characterization of the regulation of Cdc14 subcellular localization in both mammalian and budding yeast cells.

CHAPTER TWO

Biochemical Analysis of Cdc14

Regulation of the APC/C by the Dual-Specificity Phosphatases hCdc14A and hCdc14B

INTRODUCTION

Orderly progression through the cell cycle relies on the activity of a large ubiquitin protein ligase, the anaphase-promoting complex (APC/C) or cyclosome (15,115-117). APC/C requires two-related WD40 repeat-containing cofactors, Cdc20 and Cdh1, for the recruitment and selection of various substrates at different stages of the cell cycle (7,12,26,118,119). During the metaphase-to-anaphase transition, APC/C^{Cdc20} mediates the ubiquitination and degradation of the securin protein, leading to the activation of the separase, dissolution of the cohesin complex, and chromatid separation (117). In late anaphase, APC/C^{Cdh1} is turned on and ubiquitinates cyclin B, thus inactivating the cyclin B/cdc2 mitotic kinase and triggering the exit from mitosis (29,45,115,117). The substrates of APC/C^{Cdc20} contain the destruction box (D-box) motif whereas APC/C^{Cdh1} has a broader substrate specificity (7). In addition to ubiquitinating D-box-containing substrates, APC/C^{Cdh1} promotes the ubiquitination of additional mitotic regulatory proteins containing the KEN box motif, thus resetting the cell cycle (9). To ensure that the APC/C^{Cdh1} substrates are degraded after chromatid separation, APC/C^{Cdh1} has to be activated at a later time as compared to APC/C^{Cdc20} (115). Therefore, the conversion from the APC/C^{Cdc20} complex to APC/C^{Cdh1} is tightly regulated during mitosis (45,65).

The mechanism of the activation of APC/C^{Cdh1} and exit from mitosis is best characterized in budding yeast (29). Mutations of a set of yeast genes, including Tem1, Lte1, Dbf2, Dbf20, Cdc5, Cdc14, Cdc15, and Mob1, stabilize mitotic cyclin Clb2p and cause late anaphase arrest, indicating that they are required for the exit from mitosis (28,29,60,61). These genes are collectively referred to as the mitotic exit network (MEN) (60). Among the MEN genes, Tem1 is a small GTP-binding protein that belongs to the Rab subfamily (15,46). Lte1 is a guanine nucleotide exchange factor for Tem1 and presumably regulates its activity positively (15,120). Lte1 and Tem1 remain spatially segregated until late anaphase, at which time Lte1 and Tem1 co-localize to the bud (48). The activation of Tem1 then triggers the dissociation of the Cdc14 dual-specificity protein phosphatase from the RENT (for Regulator of Nucleolar silencing and Telophase) complex, resulting in the release of Cdc14 from the nucleolus (60,61,121). In early mitosis, the yeast Cdh1 is phosphorylated by the Clb2/Cdc28 mitotic kinase, and phosphorylation of Cdh1 prevents its interaction with APC/C (30). In late anaphase, the Cdc14 protein liberated from the nucleolus dephosphorylates Cdh1 and activates APC/C^{Cdh1} (29). Therefore, Cdc14 may be the most downstream component of the MEN proteins.

Homologues of several MEN proteins, such as Cdc14, Cdc5, and Mob1 have been identified in vertebrates (107,109,122). In particular, the polo-like kinases, the vertebrate homologs of Cdc5, have been implicated in the activation and maintenance of APC/C activity (123). Moreover, phosphorylation of human Cdh1 (hCdh1) blocks its ability to activate APC/C (31,124). These findings suggest that similar mechanisms for the regulation of APC/C^{Cdh1} and the exit from mitosis may exist in mammalian cells.

However, little is known about whether and how the MEN genes might regulate APC/C^{Cdh1} in organisms other than the budding yeast.

Here I report the biochemical characterization of the human homologues of Cdc14, hCdc14A and hCdc14B. Phosphorylation of hCdh1 by cyclin B1/cdc2 alters the conformation of hCdh1 and blocks its ability to stimulate the ligase activity of APC/C. The purified hCdc14A and hCdc14B proteins dephosphorylate hCdh1 and restore its ability to activate APC/C. Expectedly, hCdc14A does not affect the activity of APC/C^{Cdc20}. Moreover, hCdc14A exists as part of a 500 kD complex and is an abundant phosphatase of hCdh1 in HeLa cells. Finally, hCdc14A localizes to centrosomes throughout the cell cycle, whereas hCdc14B localizes to the nucleolus during interphase and can be detected on centrosomes during mitosis. Because several yeast MEN proteins have been shown to localize to spindle pole bodies, the centrosome localization of hCdc14A and hCdc14B is consistent with their playing an important role in the regulation of APC/C in mammalian cells. Therefore, my data suggest that human Cdc14 homologues activate APC/C^{Cdh1} in late anaphase, and are members of the potential MEN and SIN signaling pathway in mammals.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

For the production of human Cdc14, the phosphatase-dead mutant of hCdc14 with its active site cysteine mutated to a serine (hCdc14-PD), hCdc20, and hCdh1 proteins, recombinant baculoviruses encoding these proteins fused at the N-termini with the His_6-

tag were constructed using the Bac-to-Bac system (Gibco). Sf9 cells were infected with the appropriate viruses for 50 hrs and lysed with a buffer containing 20 mM Tris (pH 7.7), 150 mM NaCl, and 0.1% Triton X-100. The proteins were incubated with Ni^{2+}-NTA beads (Qiagen) and eluted with a step gradient of imidazole. To obtain phosphorylated hCdh1 protein, the Sf9 cells were treated with 0.5 μM of okadaic acid for 4 hrs prior to harvesting.

To obtain active cyclin B1/cdc2 kinase, I constructed baculoviruses encoding human cyclin B1 and an AF point-mutant of human cdc2. The cdc2-AF mutant changes T14 and Y15 of human cdc2 to alanine (A) and phenylalanine (F), respectively, thereby eliminating the potential inhibitory phosphorylation at these two sites by Wee1 and Myt1. The recombinant cyclin B1/cdc2 complex was then purified through the GST-moiety on cdc2 after co-infecting Sf9 cells with the cyclin B and cdc2 baculoviruses.

Kinase and Phosphatase Assays

For the kinase assay, cyclin B1/cdc2 was incubated with hCdh1N and γ-^{32}P-ATP in the kinase buffer (20mM HEPES, pH 7.7, 50 mM NaCl, 10 mM $MgCl_2$, 2 mM EDTA, and 0.02% Triton-X 100). Reactions were incubated for 1 hr at room temperature, and were either quenched with SDS sample buffer or used for subsequent phosphatase assays. For the phosphatase assay, purified His_6-hCdc14A, His_6-hCdc14B, (wild-type or phosphatase-dead) or hCdc14A-containing column fractions of HeLa cell lysates were added to the kinase reaction mixture and incubated for 1 hr and then quenched with SDS sample buffer. The samples were then separated on SDS-PAGE followed by autoradiography.

APC/C Ubiquitination Assay

To purify interphase APC/C, the α-APC/C3 (Cdc27) beads were incubated with 10 volumes of interphase *Xenopus* egg extracts for 2 hrs at 4 ˚C and washed five times with XB containing 500 mM KCl and 0.5% NP-40 and twice with XB. The interphase APC/C beads were then incubated for 1 hr at room temperature with hCdc20 or hCdh1 proteins in the presence or absence of cyclin B1/cdc2, hCdc14A, and hCdc14B. After incubation, the APC/C beads were washed twice with XB, and assayed for cyclin ubiquitination activity. Each ubiquitination assay was performed in a volume of 5 μl. The reaction mixture contained an energy-regenerating system, 150 μM of bovine ubiquitin, 5 μM of the Myc-tagged N-terminal fragment of human cyclin B1, 5 μM of human E1, 2 μM of UbcH10, and 2 μl of the APC/C beads. To inhibit cyclin B1/cdc2 kinase activity during the APC/C activity assay, I used the roscovitine Cdk inhibitor at a concentration of 30 μM. The reconstituted ubiquitination reactions were incubated at room temperature for 1 hr, quenched with SDS sample buffer, and analyzed by SDS-PAGE followed by immunoblotting with α-Myc.

Cell Culture

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum in the presence of 2 mM thymidine (Sigma) for 18 hrs to arrest the cell cycle at the G1/S boundary, washed with PBS, and grown in fresh medium without thymidine for 8 hrs. Cells were then incubated with 2 mM thymidine for another 18 hours, transferred into fresh medium, and harvested at various time points. The cells were lysed with the NP-40

lysis buffer. HeLa lysates were then fractionated on a superose 6 column and blotted with α-hCdc14A.

Immunofluorescence

The α-hCdc14A antibody was made against a GST-fusion protein containing a C-terminal fragment (residues 330-580) of hCdc14A (splice variant 2), and the α-hCdc14B antibody was made using the C-terminal fragment (residues 379-459) of hCdc14B (isoform 1). The fusion proteins were expressed in bacteria and purified with glutathione-agarose beads and used to immunize rabbits at Zymed Laboratories, Inc. For immunostaining, HeLa cells grown to 50-70% confluency were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton-X100 in PBS, and incubated with 1 μg/ml of affinity-purified polyclonal α-hCdc14A antibody or 1:1000 dilution of α-hCdc14B rabbit serum and 1:2000 dilution of α-tubulin monoclonal antibody. After washing, fluorescent secondary antibodies against rabbit and mouse IgG were added at 1:500 dilution (molecular probes). The cells were again washed three times in PBS, and viewed using a 63X objective on a Bio-Rad confocal microscope for hCdc14A or slides were mounted with Aqua Poly/Mount (Polysciences, Inc.) and visualized with a 63X objective on a Zeiss axiovert 200M fluorescence microscope for hCdc14B. The images were acquired with a CCD camera using the Intelligent Imaging software and further processed with Adobe Photoshop.

RESULTS

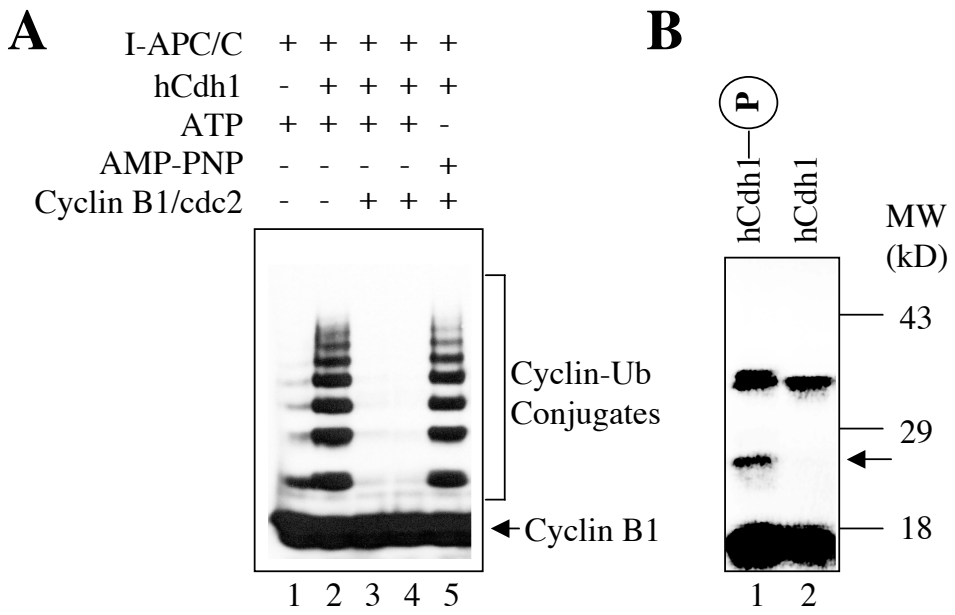


Figure 2-1. Human cyclin B1/cdc2 inactivates APC/C^{Cdh1} in an ATP-dependent manner. A, Interphase APC/C (I-APC/C) from interphase Xenopus egg extracts exhibited basal level ubiquitination activity (lane 1). Addition of hCdh1 greatly stimulated the ligase activity of APC/C (lane 2). Treatment of hCdh1 with cyclin B1/cdc2 inhibited its ability to activate APC/C (lanes 3-4). The reaction in lane 4 contained twice as much cyclin B1/cdc2 as that of lane 3. In the presence of AMP-PNP, cyclin B1/cdc2 failed to inhibit APC/C^{Cdh1} (lane 5). B, in vitro translated ^{35}S-labeled hCdh1 protein was treated in the presence (lane 1) or absence (lane 2) of cyclin B1/cdc2. Chymotrypsin was added to the reaction mixture at a final concentration of 1 μg/ml for 10 min. The reactions were quenched with SDS sample buffer and analyzed by SDS-PAGE followed by autoradiography. The extra 25 kD proteolytic fragment of phosphorylated hCdh1 is marked by the arrow.

Phosphorylation of hCdh1 by Cyclin B1/cdc2 Blocks its Ability to Activate APC/C

Because Clb2/Cdc28 inactivates Cdh1 through phosphorylation during mitosis in yeast (30), I expected that cyclin B1/cdc2 might act similarly in vertebrates. I therefore tested whether purified cyclin B1/cdc2 inhibited the activity of APC/C^{Cdh1} in ubiquitination assays. As shown in Figure 2-1A, incubation of the interphase APC/C with purified hCdh1 protein produced in Sf9 cells greatly stimulated its ubiquitination activity (compare lanes 1 and 2). Addition of cyclin B1/cdc2 in the presence of ATP significantly reduced the ability of hCdh1 to activate APC/C (lanes 3 & 4). Because the

cyclin B1 subunit of the recombinant cyclin B1/cdc2 kinase is a substrate of APC/C, it is conceivable that cyclin B/cdc2 might inhibit APC/C through substrate competition rather than phosphorylation of Cdh1. To rule out this possibility, I performed the same assay in the presence of an ATP analogue, AMP-PNP. Because the ubiquitin-activating enzyme (E1) hydrolyzes the α-β bond of ATP (125), AMP-PNP is fully capable of supporting the ubiquitination reaction. However, AMP-PNP cannot support the phosphorylation reaction as kinases transfer the γ-phosphate to their protein substrates. As expected, incubation of cyclin B1/cdc2 with hCdh1 in the presence of AMP-PNP did not inactivate APC/C^{Cdh1} (lane 5), indicating that the inhibition of hCdh1 by cyclin B1/cdc2 was due to the kinase activity, not substrate competition.

It has been previously shown that phosphorylated hCdh1 has a decreased affinity toward APC/C (31,124), which could be caused by electrostatic repulsion between APC/C and the additional phosphate groups on hCdh1. Alternatively, phosphorylation might also cause a conformational change of hCdh1, preventing it from binding to APC/C. To test the latter hypothesis, I performed limited protease digestion of hCdh1 phosphorylated by cyclin B1/cdc2 and the unphosphorylated hCdh1 (Figure 2-1B). Limited protease digestion of the unphosphorylated hCdh1 protein by chymotrypsin yielded two major proteolytic products of 16 and 35 kD, which presumably corresponded to the N-terminal domain and the C-terminal WD40 domain of hCdh1, respectively (Figure 2-1B). In addition to the two major proteolytic fragments, digestion of the phosphorylated hCdh1 protein with chymotrypsin resulted in the appearance of an additional band at 25 kD (Figure 2-1B). This indicated that phosphorylation indeed induced a conformational change in hCdh1.

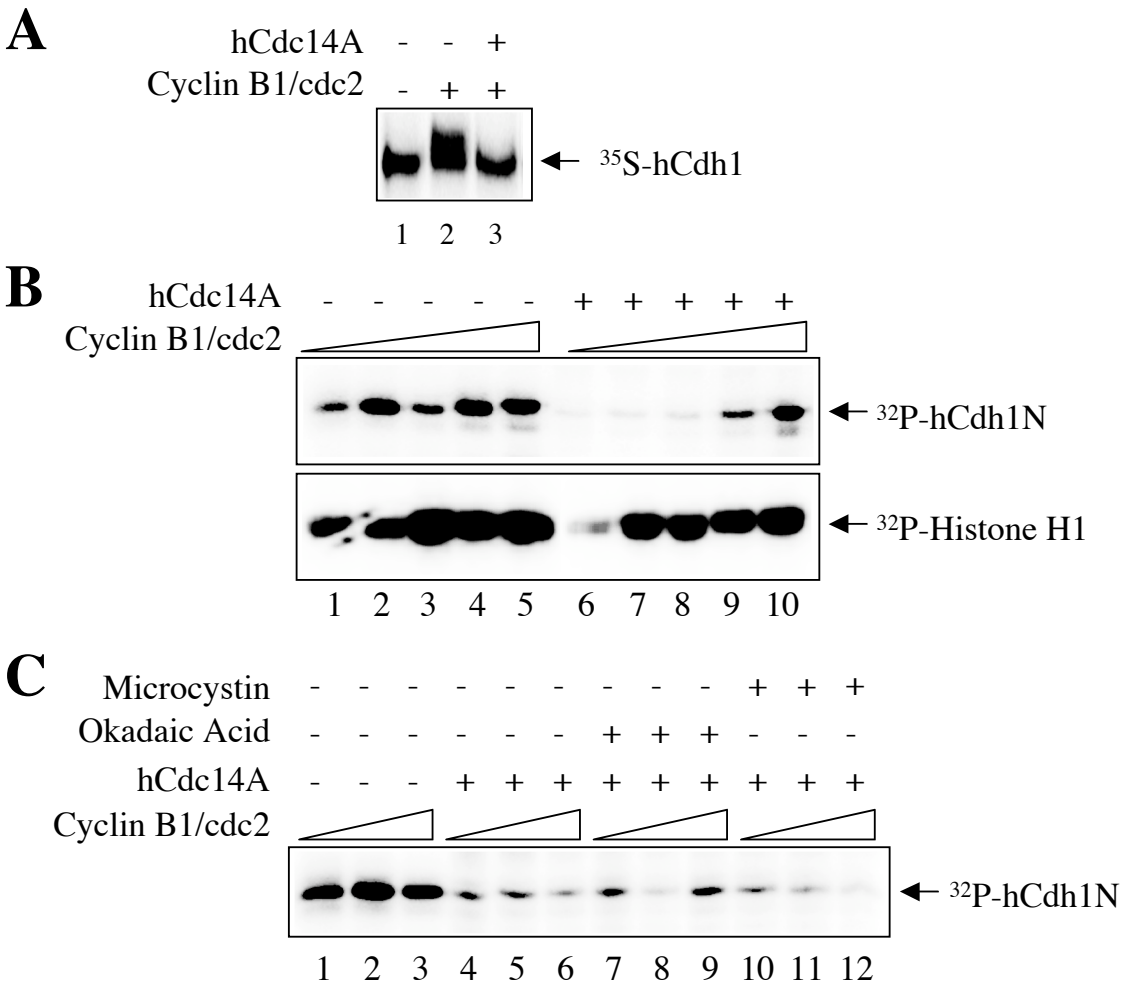


Figure 2-2. The hCdh1protein is a substrate of hCdc14A. A, phosphorylation of hCdh1 by cyclinB1/cdc2 resulted in the appearance of slower migrating hCdh1 species (compare lane 1 and 2), which was removed by the addition of hCdc14A (lane 3). B, an N-terminal fragment of hCdh1 (hCdh1N) (lanes 1-5, top panel) and histone H1 (lanes 1-5, bottom panel) were efficiently phosphorylated by cyclin B1/cdc2, as revealed by ^{32}P-PO_4 incorporation. Incubation of the kinase reaction mixture with hCdc14A led to efficient dephosphorylation of hCdh1N (lanes 6-10, top panel), but not histone H1 (lanes 6-10, bottom panel). C, hCdh1N was phosphorylated by cyclin B1/cdc2 (lanes 1-3). The hCdc14A efficiently dephosphorylated hCdh1N (lanes 4-6). The phosphatase activity of hCdc14A towards hCdh1N was not inhibited by okadaic acid (lanes 7-9) or microcystin (lanes 10-12).

The hCdc14A and hCdc14B proteins are Functional Phosphatases for hCdh1

Li et al. identified two human homologues of the yeast Cdc14, hCdc14A and hCdc14B, on the basis of sequence similarity (109). Overexpression of both human

Cdc14 homologues rescued the temperature-sensitive phenotype of cdc14-1^{ts} in budding yeast (109). These data suggest that hCdc14A and hCdc14B might be functionally related to yeast Cdc14. To determine whether hCdc14A is a functional phosphatase for hCdh1, purified recombinant hCdc14A protein was added to kinase assays containing cyclin B1/cdc2 and ^{35}S-labeled hCdh1. Incubation of hCdh1 with cyclin B1/cdc2 generated slower migrating phosphorylated species of hCdh1 (Figure 2-2A; lane 2). Addition of hCdc14A reversed the mobility shift of hCdh1, indicating that hCdc14A dephosphorylated hCdh1 (Figure 2-2A; lane 3).

I also performed kinase and phosphatase assays in the presence of γ-^{32}P-ATP (Figure 2-2B). Cyclin B1/cdc2 phosphorylated both histone H1 and an N-terminal fragment of hCdh1 (hCdh1N) (Figure 2-2B; lanes 1-5). Inclusion of hCdc14A in the kinase assays greatly reduced hCdh1N phosphorylation while it dephosphorylated histone H1 to a much lesser extent (Figure 2-2B; lanes 6-10). Therefore, hCdh1 is a substrate of the hCdc14A phosphatase. Because recombinant hCdc14 was purified from Sf9 cells, it is possible that there was trace amount of other phosphatase contamination in my preparation of hCdc14A. To rule out this possibility, high concentrations of microcystin or okadaic acid were added to the hCdc14A reactions. The two most abundant phosphatases in cells are PP1 and PP2A, both of which can be inhibited by microcystin or okadaic acid. In contrast, hCdc14A belongs to the family of dual-specificity phosphatases, which use distinct mechanisms for catalysis. As expected, the phosphatase activity of hCdc14A was not inhibited by either microcystin or okadaic acid (Figure 2-2C). I also compared the dephosphorylation of hCdh1N by hCdc14A, hCdc14A-PD, hCdc14B, and hCdc14B-PD (Figure 2-8A). Again, radiolabeled phosphate is removed

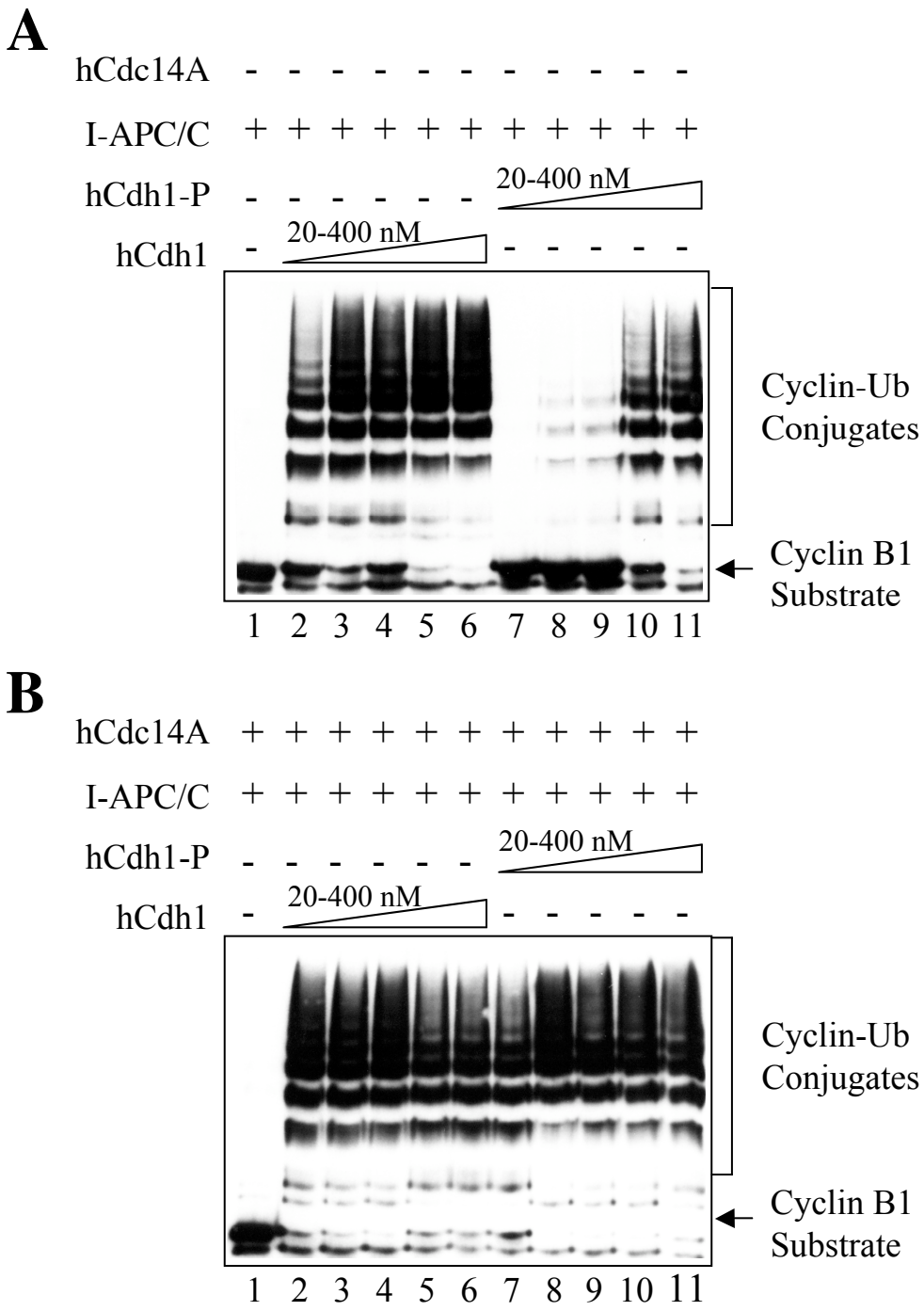


Figure 2-3. hCdc14A Stimulates APC/C^{Cdh1}. A, addition of unphosphorylated hCdh1 protein stimulated the ligase activity of Xenopus interphase APC/C (I-APC/C) (compare lane 1 and lanes 2-6). A Phosphorylated form of hCdh1 greatly reduced its ability to stimulate the APC activity (lanes 7-11). B, same as in A, except that hCdc14a was included in lanes 2-11.

from hCdh1N in the presence of wild-type phosphatase (lanes 1, 4), but not in the

presence of phosphatase-dead hCdc14 (lanes 2, 5) or upon addition of sodium tungstate, which inhibits dual-specificity phosphatases (lanes 3, 6).

Both hCdc14A and hCdc14B Activate APC/C^{Cdh1}

I next tested whether dephosphorylation of hCdh1 by hCdc14A or hCdc14B restored the ability of hCdh1 to activate APC/C. To obtain phosphorylated hCdh1 protein in large quantities, I treated Sf9 cells infected by hCdh1-encoding baculoviruses with okadaic acid, which inhibited abundant phosphatases and promoted a mitotic-like state in these cells (31). The hCdh1 protein purified from okadaic acid treated cells was hyperphosphorylated. Consistent with earlier report (31), phosphorylated hCdh1 protein (hCdh1-P) was much less active in stimulating the APC/C activity, as compared to hCdh1 purified from untreated Sf9 cells (Figure 2-3A). Addition of hCdc14A dramatically increased the ability of phosphorylated hCdh1 to stimulate the ligase activity of APC/C (Figure 2-3B; lanes 7-11). Interestingly, hCdc14A also slightly enhanced the activity of hCdh1 purified from Sf9 cells not treated with okadaic acid (Figure 2-3B; lanes 2-7). It is possible that a portion of Cdh1 protein purified from Sf9 cells was phosphorylated even in the absence of okadaic acid treatment. To estimate the E3 ligase activity enhancement of APC/C^{Cdh1} by hCdc14A, I repeated the experiment with much less hCdh1 added to the reactions (Figure 2-4A). The activity of hCdc14A-treated APC/C^{Cdh1} at 20 nM (Figure 2-4A) was similar to that of APC/C activated with 400 nM of phosphorylated hCdh1 (Figure 2-3A). Therefore, hCdc14A enhanced the activity of phosphorylated APC/C^{Cdh1} by approximately 20 fold. To determine whether the phosphatase activity of hCdc14A was required for this stimulation, I constructed,

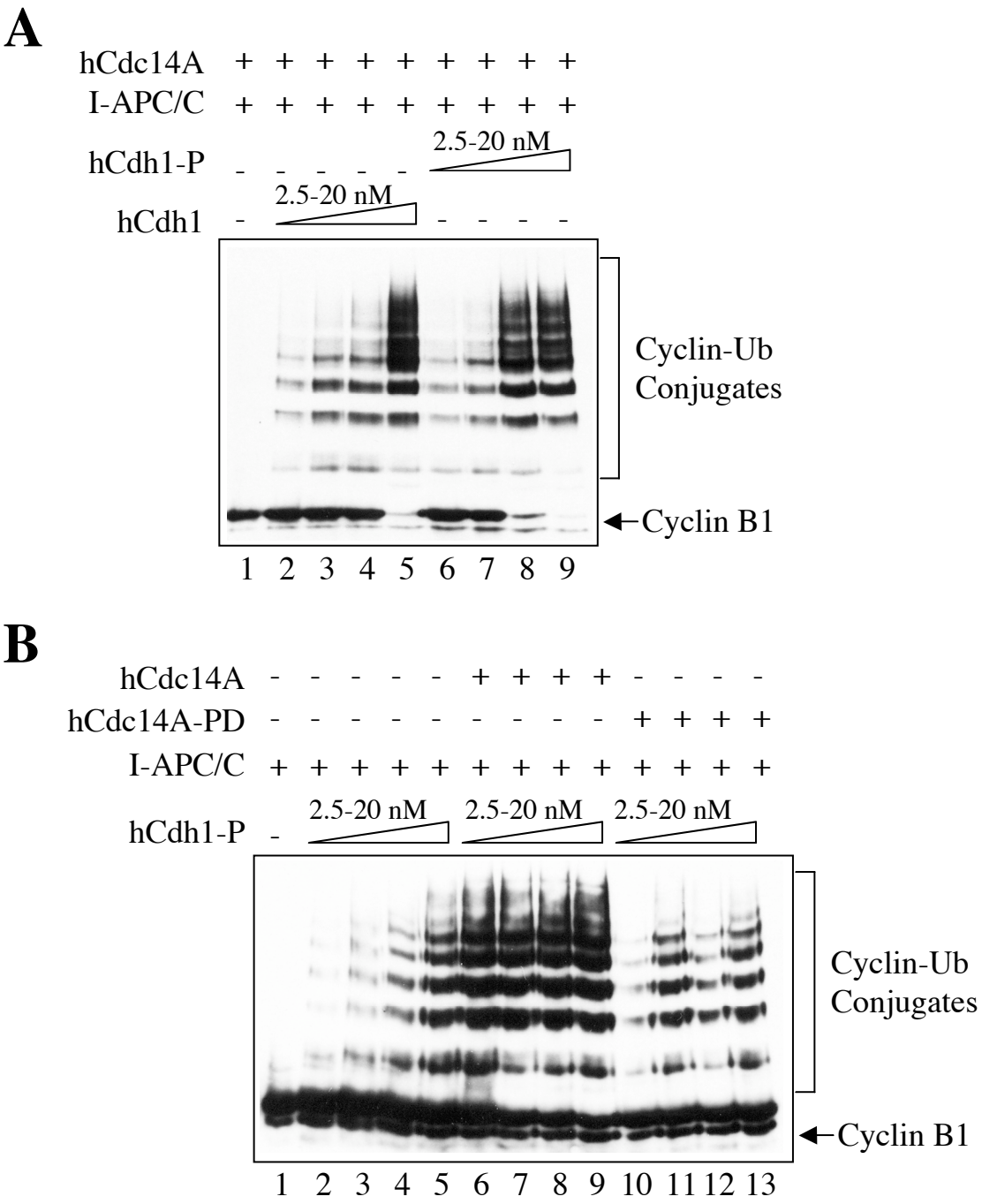


Figure 2-4. APC/C^{Cdh1} activation by hCdc14A wild-type and phosphatase-dead (PD). A, same as Figure 2-3B, except that less hCdh1 protein was used in the reactions. B, stimulation of APC/C^{Cdh1} depends on the phosphatase activity of hCdc14A. The hCdc14A mutant protein that does not possess phosphatase activity (hCdc14A-PD) was much less efficient in stimulating APC/C^{Cdh1} (lanes 6-9), as compared to the wild-type hCdc14A (lanes 10-13).

expressed, and purified a phosphatase-inactive mutant of hCdc14A by replacing its active

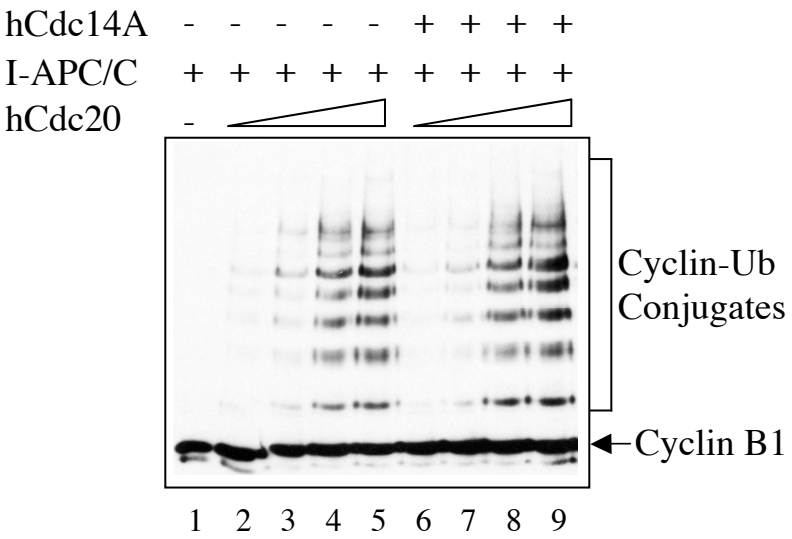


Figure 2-5. HCdc14A does not affect the activity of APC/C^{Cdc20}. interphase Xenopus APC/C was incubated with hCdc20, in the absence (lanes 2-5) or presence (lanes 6-9) of hCdc1A, and assayed for ubiquitination activity.

site cysteine with serine. Addition of the phosphatase-dead mutant of hCdc14A (hCdc14A-PD) only stimulated the activity of APC/C^{Cdh1} slightly (Figure 2-4B). Therefore, the phosphatase activity of hCdc14A is required for its ability to activate APC/C^{Cdh1}.

To ascertain that the stimulatory effect of hCdc14A was specific to Cdh1, I tested the effect of hCdc14A addition on the activity of the APC/C^{Cdc20} complex. Expectedly, hCdc14A did not affect the activity of APC/C^{Cdc20} (Figure 2-5), indicating that any potential dephosphorylation of APC/C subunits by recombinant hCdc14A does not affect its ligase activity. Therefore, dephosphorylation of hCdh1 by hCdc14A is most likely responsible for the observed stimulatory effect of hCdc14A. I also wanted to show that hCdc14B, like hCdc14A, could activate APC/C^{Cdh1} *in vitro*. We confirmed that hCdc14B, like hCdc14A, could stimulate the APC/C^{Cdh1} ligase using the two different purified forms of hCdh1 as described above (data not shown). We also wanted to

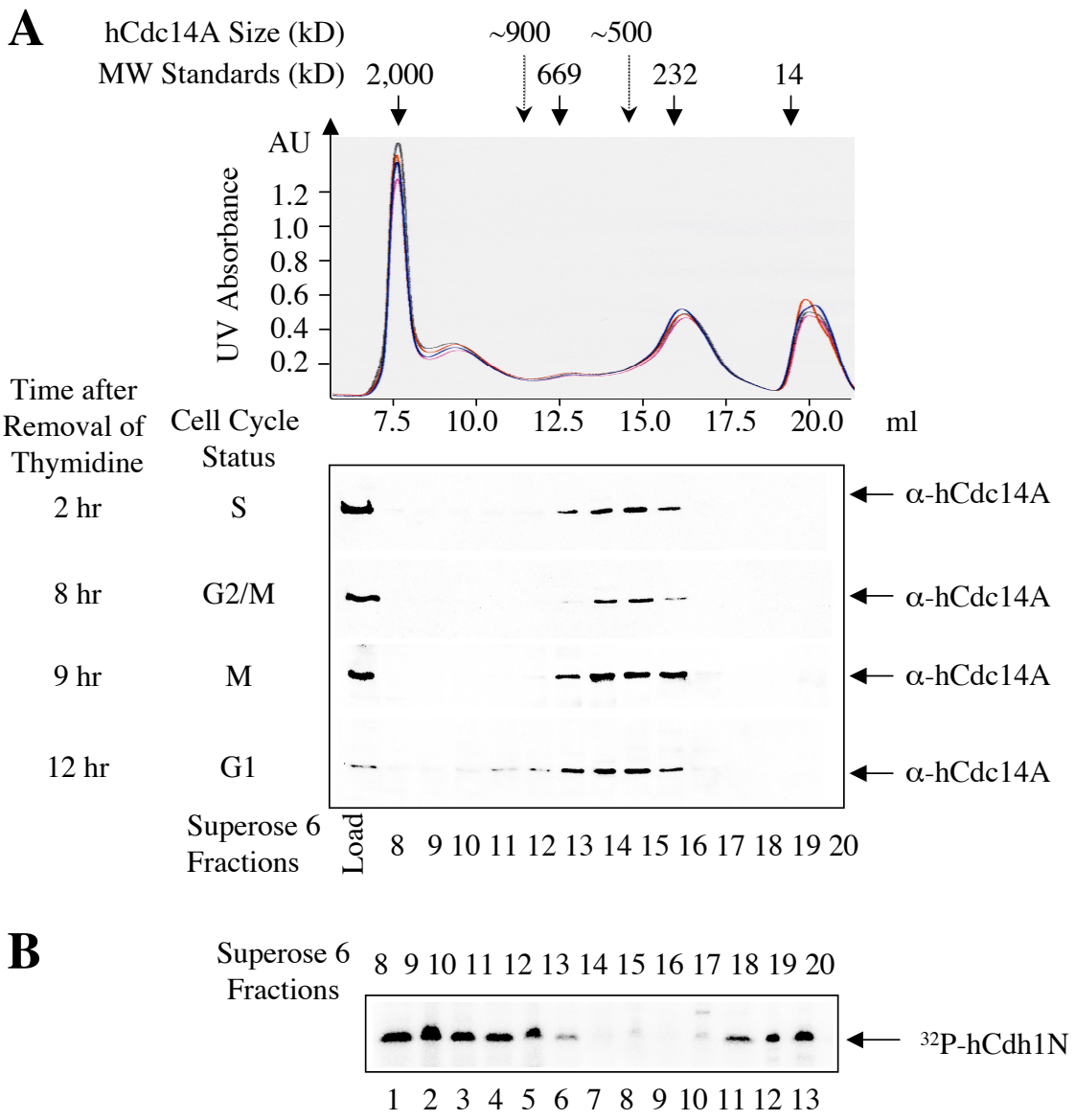


Figure 2-6. Endogenous hCdc14A exists as a part of a 500 kD complex in HeLa cells. A, The lysate of HeLa cells synchronized with a double-thymidine block was fractionated on a 26 ml superose 6 column. The upper panel shows the UV traces of all four column runs in different colors. All four traces were virtually identical, indicating that there was little variation between the column runs. The lower panel shows the α-hCdc14A immunoblots of column fractions. The estimated molecular weight of the peak fractions was indicated. B, the fractions of the lysate made from cells 12 hrs after thymidine removal were concentrated and assayed for their ability to dephosphorylate hCdh1N in the presence of microcystin.

perform the APC/C assay with cyclin B1/cdc2 kinase-mediated phosphorylation of hCdh1 instead of using hCdh1 purified from okadaic acid-treated Sf9 cells. We incubated the cyclin B1/cdc2 kinase with or without the kinase inhibitor roscovitine with

hCdh1, and then tested the ability of the human Cdc14 homologues to reactivate hCdh1. If roscovitine is incubated with cyclin B1/cdc2 for the duration of the kinase reaction with hCdh1, APC/C^{Cdh1} complex forms and is active (Figure 2-8B; lanes 2-4). When hCdh1 is incubated with the cyclin B1/cdc2 kinase in the absence of inhibitor for 1 hour, and then subsequently inhibited with roscovitin, the Cdh1 protein is much less efficient toward stimulating the APC/C ligase activity (lanes 5-7). As expected, addition of wild-type hCdc14A (lanes 15-17) or hCdc14B (lanes 8-10), but not hCdc14A-PD (lanes 18-20) or hCdc14B-PD (lanes 11-13) can reverse the inhibitory effect of cyclin B1/cdc2 kinase on hCdh1.

The hCdc14A is a Major Phosphatase of hCdh1 in HeLa Cells

I next determined the native size of hCdc14A in HeLa cell lysates synchronized at different cell cycle stages. HeLa cells were synchronized at the G1/S boundary by a double thymidine block and released into fresh medium. Samples were taken at various time points. Based on FACS analysis and cyclin B1 immunoblotting, cells were effectively synchronized (data not shown). The cell lysates were fractionated on a gel filtration column and the resulting fractions were then blotted with the α-hCdc14A antibody (Figure 2-6A). In all cell cycle stages, the majority of hCdc14A eluted in fractions 14 and 15, which corresponded to a molecular mass of about 500 kD. I then concentrated the fractions of the G1-phase lysate (12 hrs after the release of the thymidine block) and assayed their ability to dephosphorylate hCdh1N. A final concentration of 2 μM of microcystin was added to inhibit PP1 and PP2A in these fractions. The microcystin-insensitive phosphatase activity that dephosphorylates

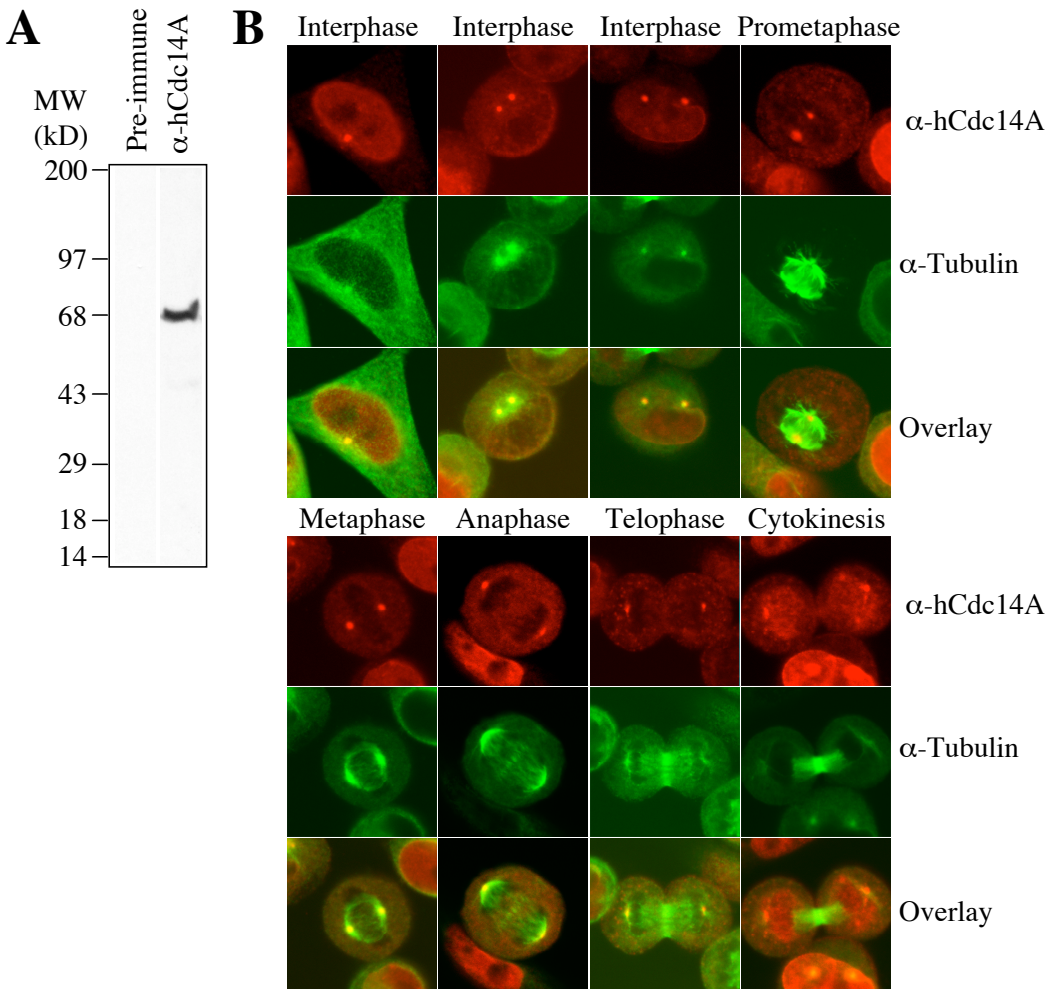


Figure 2-7. Endogenous hCdc14A localizes to centrosomes in HeLa cells. A, HeLa cell lysate was separated by SDS-PAGE and blotted with either the pre-immune serum or the affinity-purified α-hCdc14A antibody. B, HeLa cells at various cell cycle stages were double-stained with α-hCdc14A (in red) and α-tubulin (in green) antibodies.

hCdh1N co-eluted with hCdc14A, with the exception of fraction 17 (Figure 2-6B). Therefore, hCdc14A co-elutes with a major hCdh1 phosphatase in HeLa cell lysates.

Subcellular Localization of hCdc14A and hCdc14B

The yeast Cdc14 protein is sequestered by Net1/Cfi1 in the nucleolus (60,61,121). The release of Cdc14 from the nucleolus at late anaphase then activates APC/C^{Cdh1}, leading to the exit from mitosis (60,61). To determine whether a similar regulatory mechanism exists in mammals, I examined the localization of the human Cdc14

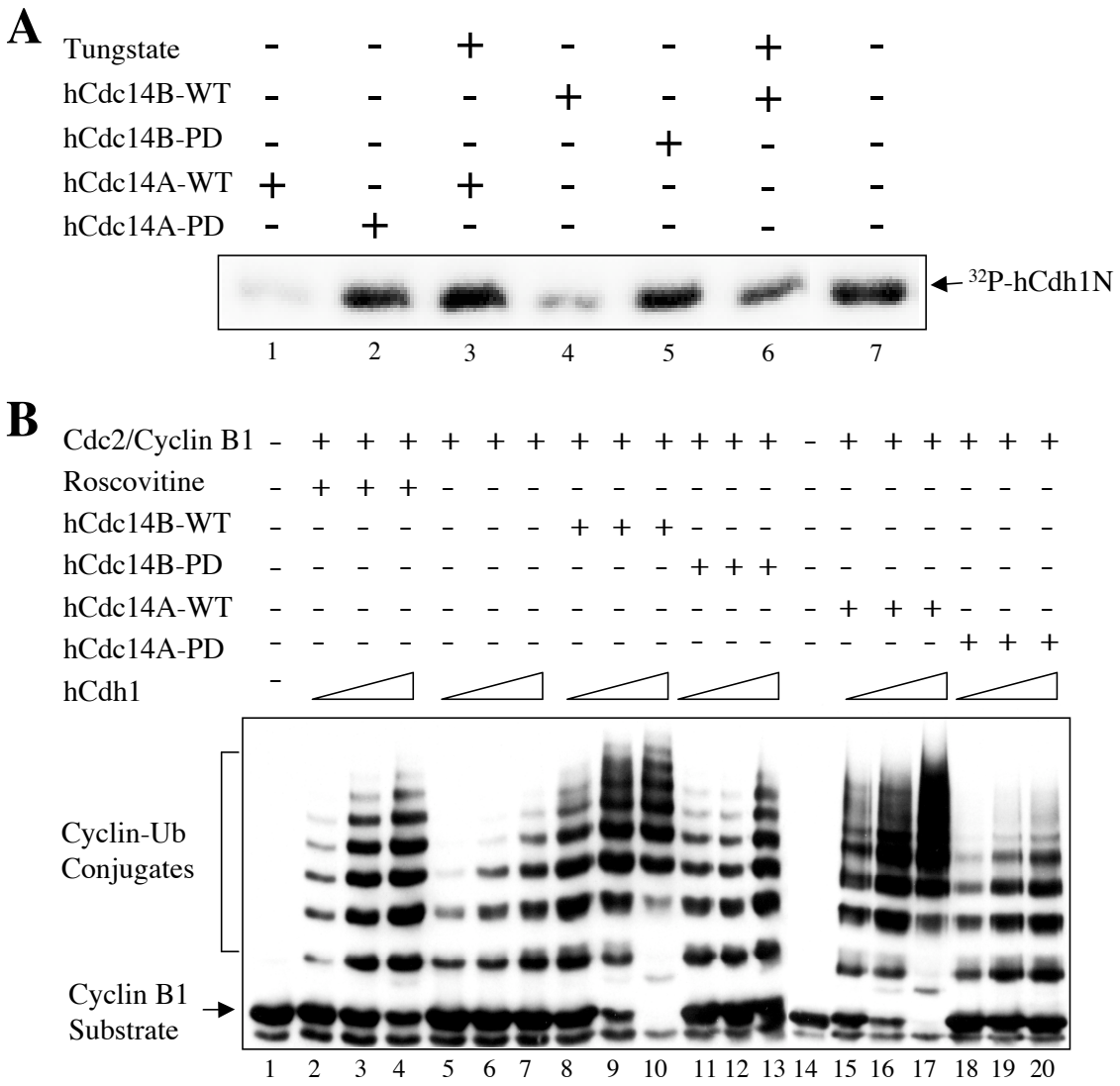


Figure 2-8. The hCdh1 is a substrate of both hCdc14A and hCdc14B. A, an N-terminal fragment of hCdh1 (hCdh1N) were efficiently phosphorylated by cyclin B1/cdc2, as revealed by $^{32}P\text{-}PO_4$ incorporation (lane 7). Incubation of the kinase reaction mixture with hCdc14A or hCdc14B led to efficient dephosphorylation of hCdh1N (lanes 1, 4), but not with hCdc14A-PD or hCdc14B-PD (lanes 2, 5). Addition of sodium tungstate, a dual-specificity phosphatase inhibitor, prevented Cdc14-mediated dephosphorylation of substrate (lanes 3, 6). B, The cdc2/cyclin B1 kinase inhibits APC/C^{Cdh1} (lanes 5-7) but not in the presence of 30 μM roscovitine(lanes 2-4). The inhibition of APC/C^{Cdh1} is reversed in the presence of hCdc14A (lanes 15-17) or hCdc14B (lanes 8-10), but not hCdc14A-PD (18-20) or hCdc14B-PD (11-13).

homologues in HeLa cells by indirect immunofluorescence. The affinity-purified polyclonal α-hCdc14A antibody recognized a predominant band of 68 kD in HeLa cell lysate (Figure 2-7A), whereas the α-hCdc14B antibody detects several bands, including one at the predicted size (data not shown). The predicted molecular weight of hCdc14A

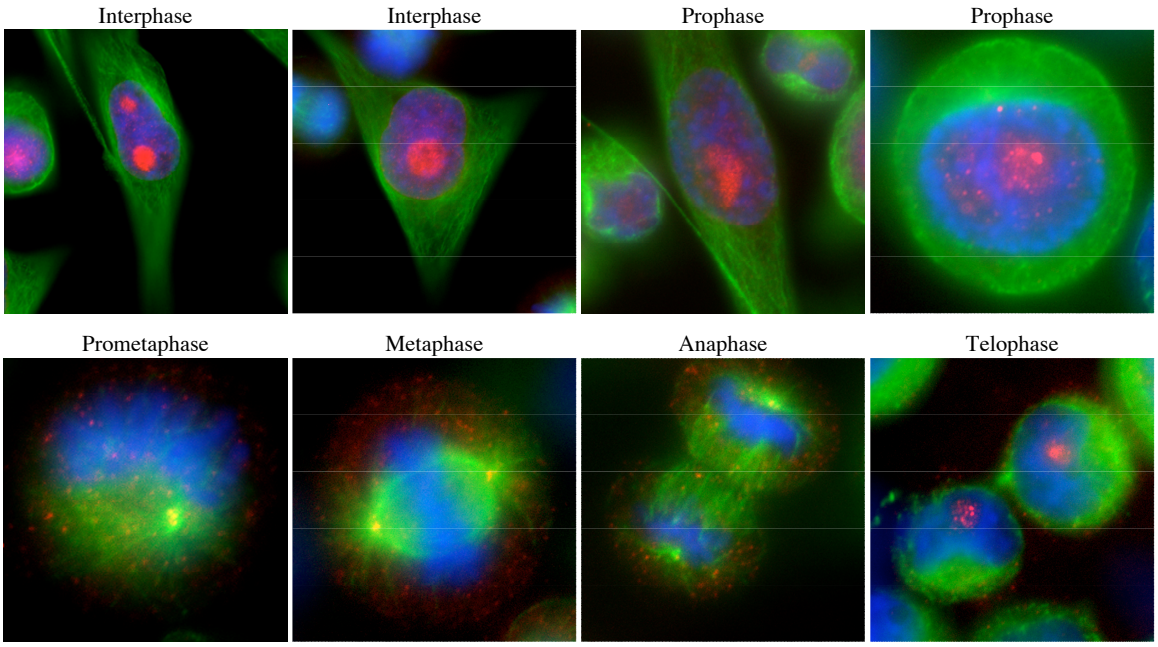


Figure 2-9. Endogenous hCdc14B localizes to nucleoli in HeLa cells. HeLa cells at various cell cycle stages were stained with α-hCdc14B (in red), α-tubulin (in green) and DAPI (in blue).

is 69.6 kD. Therefore, I originally concluded that the α-hCdc14A antibody specifically detects hCdc14A in HeLa cell lysate. Subsequent experiments determined that RNAi depletion of endogenous hCdc14A or hCdc14B could not deplete the major bands detected by western blot analysis by either antibody (data not shown). However, the fact that immunofluorescence of human Cdc14 homologues generates the same localization pattern as GFP fusion proteins (see chapter 3) gives us confidence of the localization of these proteins. HeLa cells were fixed with paraformaldehyde, permeablized, and stained with the α-hCdc14A antibody, or fixed with methanol and stained with antiserum against hCdc14B. In addition to the nuclear staining (which may be cross-reactive since the GFP fusion proteins do not localize to the nucleus under normal conditions), hCdc14A also specifically localized to the centrosomes in interphase cells (Figure 2-7B). The centrosomal localization of hCdc14A persisted in all phases of mitosis (Figure 2-7B). I did not observe clear nucleolus staining of hCdc14A at any stages of the cell cycle. In

contrast, hCdc14B is mostly nucleolar during interphase and is not detected at the centrosome. However, in mitosis the hCdc14B signal is diffuse throughout the cell and can be seen at centrosomes (Figure 2-9). I cannot rule out that the hCdc14B antibody cross reacts with endogenous hCdc14A since the C-terminal regions used for antibody production of both homologues share low sequence identity. However, the centrosomal hCdc14B staining may be valid because the GFP fusion protein can be detected on the centrosome (see Chapter 3).

DISCUSSION

Conversion from APC/C^{Cdc20} to APC/C^{Cdh1} in Late Anaphase

APC/C controls two critical transitions during the cell cycle: sister-chromatid separation and the exit from mitosis by mediating the sequential degradation of securin and mitotic cyclin. The degradation of securin should always proceed that of cyclin B and other APC/C substrates, such as Cdc5/Plk (7,27,28) and Cdc20 itself (9), so that sister-chromatid separation will occur prior to exit from mitosis and cytokinesis. Therefore, the timing of degradation of these APC/C substrates has to be carefully orchestrated. The timely degradation of the APC/C substrates depends on the proper regulation of the APC/C activity, especially the conversion from APC/C^{Cdc20} to APC/C^{Cdh1} in late anaphase. Phosphorylation of APC/C and Cdh1 plays an important role in controlling this conversion. In mitosis, phosphorylation of several APC/C subunits enhances the activity of APC/C^{Cdc20} (7,31). On the other hand, phosphorylation of hCdh1 inhibits its ability to stimulate APC/C. This is essential for keeping APC/C^{Cdh1}

off until cyclin B/cdc2 has completed its functions in mitosis. My results reported herein indicate that, similar to the regulation of APC/C^{Cdh1} in budding yeast, dephosphorylation of hCdh1 by hCdc14A and hCdc14B leads to the activation of APC/C^{Cdh1} at late anaphase in mammalian cells. In addition to phosphorylation, both APC/C^{Cdc20} and APC/C^{Cdh1} are also regulated by Mad2 and its related protein Mad2b, in response to the mitotic checkpoint signals (7,10,126). It remains to be determined whether and how phosphorylation of hCdh1 and Mad2b-binding cooperate to inhibit APC/C^{Cdh1} in late anaphase.

Localization and Regulation of hCdc14 Homologues

In yeast, a major mechanism for regulating the function of Cdc14 is to control its subcellular localization (60,61). Prior to late anaphase, Cdc14 is anchored in the nucleolus by the RENT protein complex containing Net1/Cfi1. It is the release of Cdc14 from the nucleolus that allows Cdc14 to encounter its substrate Cdh1 and activate APC/C^{Cdh1}. However, hCdc14A does not appear to localize to the nucleolus in HeLa cells. Instead, aside from the diffusive nuclear distribution, hCdc14A localizes to the centrosomes. Spatial separation between hCdc14A and its substrate might not be the mechanism of regulation for hCdc14A in mammalian cells. The hCdc14B homologue does localize to the nucleoli during interphase and may represent a reservoir of Cdc14 phosphatase that is utilized during mitosis to promote mitotic exit. Interestingly, the two mammalian Cdc14 homologues mirror the localization pattern of the sole Cdc14 homologue in *S. pombe*, which localizes to both the SPB and nucleolus during interphase. Regardless, nucleolar sequestration is not expected to be the only mechanism for the

regulation of the vertebrate Cdc14, simply because the nucleoli in vertebrate cells dissolve in pro-metaphase long before the activation of APC/C^{Cdh1}. It is also worth noting that no Net1/Cfi1 homologs have been identified in vertebrates so far.

The localization pattern of hCdc14A is consistent with its role in regulating APC/C^{Cdh1} *in vivo* for several reasons. In *Drosophila*, the degradation of cyclin B is spatially regulated: the spindle-associated cyclin B is degraded first at the spindle poles and the degradation then spreads toward the equator while the cytosolic cyclin B was degraded at an even later time (127). In mammalian cells, cyclin B1 degradation also occurs first at the spindle poles and chromosomes (128). Several APC/C subunits are enriched at centrosomes (129). Therefore, the localization of hCdc14A and hCdc14B, activators of the APC/C^{Cdh1}, at the centrosomes provides a potential explanation for the initiation of cyclin B degradation at the spindle poles. In addition, several MEN proteins, such as Dbf2 and Cdc15, and the negative regulator of the MEN pathway, Bub2, localize to spindle poles in budding yeast (47,53,54,130). The centrosome localization of hCdc14A suggests that the MEN proteins in mammalian cells might also be enriched at the centrosomes. The lack of nucleolar sequestration of hCdc14A in HeLa cells suggests that, despite the conservation of some of the molecular players of the mitotic exit network, the MEN proteins might utilize distinct mechanisms to regulate hCdc14A in mammalian cells. The hCdc14B homologue may be sequestered in the nucleolus by similar mechanisms and to this end it will be very interesting to determine what tethers it in the nucleolus during interphase.

CHAPTER THREE

Analysis of Cdc14 Localization

Crm1-Dependent Nuclear Export of Cdc14 Is Required For Cytokinesis in Budding Yeast

INTRODUCTION

Extensive genetic and biochemical studies in several model organisms have generated a template for understanding progression through the eukaryotic cell cycle. These studies demonstrate that mitotic Cdks phosphorylate a large subset of cellular proteins, which then commit the cell to mitosis and orchestrate the physical process of faithfully dividing the genome and cellular components into two cells. Once the cell has accurately segregated sister chromatids, mitotic Cdks are inactivated through the ubiquitin-mediated proteolysis of the cyclin subunit. The task of coordinating the inactivation of mitotic Cdks with the execution of the terminal events of mitosis is essential to accurate progression through the cell cycle. Two homologous signaling networks, the mitotic exit network (MEN) in budding yeast and the septation initiation network (SIN) of fission yeast, regulate the final stages of mitosis, reviewed in (36,37,131). Mutations disrupting MEN function lead to a late mitotic arrest where cells undergo chromosome segregation and have elongated spindles, yet maintain elevated mitotic Cdk activity and arrest in late telophase (15). In contrast, SIN mutants complete cyclin degradation and exit mitosis, but exhibit defects in cytokinesis. The difference in phenotypes between the MEN and SIN mutants has led to the hypothesis that these signaling networks have been established to perform different tasks at the end of mitosis

in different organisms, despite the close homology shared between them. Cdc14 homologues are critical downstream components of both the MEN and SIN and are highly conserved in many species. The Cdc14 family of phosphatases are logically appealing candidates for playing a central role during the exit from mitosis because they are capable of reversing Cdk-dependent phosphorylation events. However, similar to other MEN and SIN components, deletion of Cdc14 homologues generate different phenotypes and these homologues appear to perform unrelated tasks in various organisms based upon genetic and biochemical analysis.

The MEN and SIN are essentially small GTPase/ kinase signaling cascades that are activated at the spindle pole body (SBP, the equivalent of the mammalian centrosome) during the final stages of mitosis. When cells are near the completion of mitosis, inhibition of the small GTPase by a two-component GAP is prevented, leading to GTP loading. The active GTP-bound form of the small GTPase then recruits and activates several downstream kinases at the SPB. In both systems, several components have been shown to translocate to the bud neck after recruitment to the SPB. The active MEN and SIN signal is required to maintain the full release of downstream Cdc14 homologues (Cdc14p and flp1/clp1, respectively) from the nucleolus. Released Cdc14p has been observed on the SPB, while flp1/clp1 localizes to the SPB, spindle, and actomyosin ring. The localization of Cdc14 is thought to facilitate substrate access, ultimately leading to cyclin destruction in *S. cerevisiae* and contributing to the proper execution of cytokinesis in *S. pombe*. The precise role of the MEN during the final stages of mitosis has been the subject of intense study. In budding yeast, Cdc14p is kept inactive by remaining tethered to the nucleolus by the Net1p component of the RENT

complex during most of the cell cycle (60). The MEN is required for cyclin degradation due to its role in maintaining the full release of Cdc14p from the nucleolus in late anaphase; mutations of MEN components arrest cells with inactive Cdc14p sequestered in the nucleolus (33,60,61). The precise mechanism for Cdc14p release from the nucleolus into the nucleoplasm and cytoplasm by the MEN is not fully understood. Recent studies have shown that the FEAR network, consisting of Cdc5, Spo12, Esp1, and Slk19, cooperate to initiate the first wave of Cdc14p release during early anaphase partly by promoting the dissociation of Cdc14p from Net1p (68,70). Thus two separate phases of Cdc14p release contribute to the proper execution of mitotic events in budding yeast. Despite the emphasis on cyclin degradation, genetic manipulations that bypass mitotic Cdk inactivation have revealed roles for various MEN components during cytokinesis similar to the SIN, reviewed in (38). Previous reports have implicated a direct role of Cdc14p during cytokinesis in budding yeast that is not well understood. Growth at semi-permissive temperatures of the *cdc14-3* mutant exhibits defective cytokinesis (132), and overexpression of Sic1p in the *cdc14-1* mutant bypasses mitotic exit defects and causes cells to grow in chains (133).

Homologues of the MEN and SIN have been identified in higher eukaryotes, but their organization into clear signaling networks that regulate the end of mitosis has been difficult. The *C. elegans* Cdc14 has been observed on the equatorial microtubules during anaphase, and generates cytokinesis defects when depleted by RNAi. The human genome encodes two Cdc14 homologues, hCdc14A and hCdc14B. The hCdc14A protein resides on the centrosome throughout the cell cycle, dependent upon a NES within the C-terminal domain (114). RNAi of hCdc14A leads to multiple defects including failures in

cytokinesis, and its overexpression induces abnormal spindle and centrosome regulation (113,114). In contrast, hCdc14B resides in the nucleolus during interphase and its function is not well characterized. Both human Cdc14 homologues are capable of dephosphorylating hCdh1 and activating APC/C^{Cdh1} in vitro, unpublished observations, (134). Overexpression of hCdc14A induces the formation of APC/C^{Cdh1}, even in the presence of overexpressed non-degradable cyclin B (32). Therefore, Cdc14 homologues have been implicated in both mitotic exit and cytokinesis in various organisms, however a conserved function has not been clearly established. A question remaining in this field is how the mechanisms that regulate Cdc14 in the yeast model systems relate to those utilized in mammalian cells. This will become an important consideration in future analysis since precise understanding of mitotic exit has implications for treating human diseases like cancer.

The Ran GTPase cycle is a crucial system for the trafficking of cellular proteins and RNAs. Ran is a small GTPase that regulates cargo binding to karyopherins based upon its GDP or GTP bound state (reviewed in (135). During interphase, a Ran gradient established across the nuclear membrane modulates the loading and release of karyopherin-bound cargo to accomplish transport across the nuclear pore complex. Interphase cells establish a gradient of elevated RanGTP inside the nucleus and RanGDP in the cytoplasm by the action of the nuclear GTP exchange factor, RCC1, and the cytoplasmic Ran GTPase-activating protein (RanGAP), respectively. Import factors bind cargo in the cytoplasm, translocate across the nuclear pore, and release them in the presence of nuclear RanGTP. Export factors, such as Crm1, bind RanGTP and a nuclear export signal (NES) of the substrate in the nucleus and translocate them into the

cytoplasm where RanGAP induces cargo unloading by catalyzing GTP hydrolysis on Ran. The Ran cycle is also utilized for several crucial functions during mitosis, reviewed in (136). RCC1 is a GEF for Ran and has been established as a key regulator of chromatin dynamics during mitosis. RCC1 remains associated with chromatin throughout the cell cycle, maintaining elevated RanGTP concentrations in the proximity of chromatin. During mitosis, this provides a spatial cue for the release of the spindle-activating factors TPX2 and NUMA from importins near sister chromatids, contributing to the formation of a functional bipolar spindle (137-139). The Ran cycle is also important for proper spindle checkpoint function during metaphase (140). Subpopulations of Ran, Crm1, and RanBP1 have been observed at the centrosome in mammalian cells, and play an important role in the proper regulation of centrosomal structure and function (141,142). These provocative findings indicate that the RanGTPase cycle is crucial for proper execution of several key events during mitosis and can regulate important mitotic regulators during an open mitosis. Elucidating further mechanisms of how the ran cycle is utilized for the proper execution of mitotic events should provide useful insight into how cells ensure fidelity during division.

In this study, I investigate the regulation of the dynamic subcellular localization of Cdc14 homologues. I demonstrate that both human Cdc14 homologues can localize to the centrosome and midbody during the late stages of mitosis in HeLa cells. In budding yeast, Cdc14p is nucleolar during most of the cell cycle and is released during late anaphase. I show that upon release, Cdc14p also specifically localizes to the bud neck in addition to the previously reported centrosomal localization. Truncational mutagenesis of GFP fusion proteins demonstrate that the N-terminus is required for nuclear and

nucleolar localization of Cdc14 homologues in mammalian cells, while the C-terminus is required for retention in the cytoplasm. Sequence analysis shows a predicted NES within the C-terminal region not only of hCdc14A, but also the hCdc14B and budding yeast Cdc14 homologues. Cytoplasmic retention of hCdc14A and Cdc14p in HeLa cells requires a functional NES and Crm1 activity. The nucleolar hCdc14B protein also depends upon Crm1 and a NES to shuttle from a human nucleus into a mouse nucleus in a heterokaryon cell-fusion assay. To determine what role nuclear export of Cdc14 may play during cell cycle regulation, I compared budding yeast strains that express only a NES mutant allele of Cdc14 (Cdc14ΔNES) with wild-type and the previously characterized *cdc14-1* temperature sensitive strain. The NES mutant Cdc14 strains display temperature sensitive growth at 37°C. The Cdc14ΔNESp does not efficiently localize to the centrosome or bud neck at the restrictive temperature. Cells carrying only the Cdc14ΔNES allele arrest with 2N DNA content, like the Cdc14-1 strain, however they bypass a late mitotic arrest, fail to complete cytokinesis, and eventually lyse. My data reveals a role of Cdc14 during cytokinesis in budding yeast which appears to be conserved across species and is regulated by Crm1-dependent nucleocytoplasmic transport.

EXPERIMENTAL PROCEDURES

Expression Plasmids

For mammalian expression plasmids, the open reading frames (ORF) of hCdc14A, hCdc14B, and the budding yeast *CDC14* were amplified by PCR from human thymus cDNA (Clontech) and yeast genomic DNA, respectively, and cloned into the pCS2-GFP

mammalian expression vectors. Mutations of the nuclear export signal (NES) motifs of the Cdc14 proteins were introduced into these mammalian expression vectors using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) per manufacturer's protocols. For expression of CDC14 in budding yeast, the entire coding regions of either wild-type or NES mutants of Cdc14p-GFP were amplified from the pCS2 vectors and subcloned into pRS416 (NEB). The promoter region of *CDC14*, 1 kb of genomic DNA upstream of the *CDC14* ORF, was cloned into pRS416-CDC14-GFP between the SacI and XbaI sites. A transcriptional terminator was also inserted into these vectors between the XhoI and KpnI sites. To generate Cdc14p-YFP yeast expression plasmids for plasmid shuffling, the *YFP* ORF was inserted between the HindIII and XhoI sites of the pRS415 vector containing the *CDC14* promoter and terminator. The *CDC14* ORFs (no stop codons) with and without NES mutations were then inserted into the pRS415-YFP vector between the XbaI and HindIII sites.

Mammalian Cell Culture, Heterokaryon Assay, and Immunofluorescence

Human HeLa Tet-On (Clontech) and mouse L929 (ATCC) cells were grown in Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, and 100 μg/ml each of penicillin and streptomycin. HeLa cells were plated at 50% confluency on chambered glass slides (Nalge Nunc International) and transfected with mammalian expression vectors encoding hCdc14B-GFP using the Effectene Transfection Reagent (Qiagen) per manufacturer's protocols. The transfected HeLa cells were grown for 12-24 hrs to allow for the expression of hCdc14B-GFP. To inhibit Crm1, these cells were treated with 300 μM of Leptomycin B (LC Laboratories) for 5 hrs. The

hCdc14B-expressing HeLa cells were treated with cycloheximide to block protein synthesis and fused with mouse L929 cells as described previously(143).

For immunofluorescence, the fused cells were fixed with ice-cold methanol for 5 min and permeabilized with PBS containing 0.1% Triton-X100 for 5 min. Following three washes with PBS containing 0.1% Triton-X 100 for 5 min, DNA was stained with DAPI for 1 minute, followed by three additional washes with PBS. Slides were mounted with Aqua Poly/Mount (Polysciences, Inc.) and visualized with a 63X objective on a Zeiss Axiovert 200M fluorescence microscope. The images were acquired with a CCD camera using the Intelligent Imaging software and further processed with Adobe Photoshop.

Construction of Yeast Strains

For yeast transformations, yeast cultures grow overnight were diluted 1:30 and grown to a density of 5 X 10^7 cells/ml. After washing with sterile water, cells were resuspended in a buffer containing 40% PEG3350, 0.1 M lithium acetate, 1 M Tris (pH 7.5), and 0.1 M EDTA at a density of 4 X 10^8 cells/ml. Plasmid DNA (200 ng) was added to 0.5 ml yeast cell suspension along with sonicated, boiled and cooled carrier DNA (100 μg). The transformation mixture was incubated at 30˚C for 30 min, followed by incubation at 42˚C for 15 min. Aliquots of the suspension were then plated on auxotrophic synthetic dropout media (SD; 6.7 g/l yeast nitrogen base without amino acids (DIFCO) supplemented with the appropriate amino acids) to select for the transformed cells.

The heterozygous BY4743 *CDC14* (YFR028C) deletion diploid strain was obtained from Research Genetics. This strain was transformed with the wild-type

pRS416-CDC14-GFP vector with the uracil selection marker to allow for subsequent selection with 5-fluorouracil-6-carboxylic acid monohydrate (5-FOA). Transformed diploid cells were sporulated and dissected. The *cdc14Δ* haploid spores were identified by growth on YPD media supplemented with 50 μg/ml geneticin to select for the resistance gene used for the initial targeted disruption of *CDC14* ORF. The haploid *cdc14Δ* strain maintained by the wild-type pRS416-CDC14-GFP plasmid was then transformed with the pRS415 (with a leucine auxotrophic marker) plasmids containing the coding sequences of either wild-type or NES mutants of Cdc14p-YFP fusion proteins. Colonies that grew on SD-Leu plates were then plated onto SD-Leu plates with 1 mg/ ml 5-FOA to select for cells that had lost the wild-type pRS416-CDC14-GFP plasmid. To test temperature sensitivity, the strains were grown for two days on SD-Leu plates at 26˚C, 34˚C, or 37˚C.

Microscopy and Flow Cytometry of Yeast Cells

The morphology of the log phase yeast cells grown at 37˚C for 3 hours was analyzed on slides with the 100¥ objective on a Zeiss axiovert 200M fluorescence microscope. For time lapse video microscopy, yeast cells were mounted on an agarose gel pad containing SD-Leu media as described (144), and incubated at either room temperature or 37°C with a heated stage. The images were acquired with a CCD camera using the Intelligent Imaging software and further processed with Adobe Photoshop.

To perform FACS analysis, yeast cells were grown at 37˚C for 3 hours, harvested and sonicated briefly in 3 ml of sterile water. The cells were then fixed with the addition of 7 ml 95% ethanol while vortexing. After incubation at 4˚C overnight, the samples

were resuspended in a buffer containing 50 mM sodium citrate (pH 7.4) and 0.25 mg/ml boiled RNAse A. Following incubation at 50˚C for 1 hr, 1 mg of Proteinase K (USB) was added. The samples were incubated for another hour, mixed with 1.6 µg/ml propidium iodide in 50 mM sodium citrate (pH 7.4) for 30 min and analyzed by flow cytometry (FACS). For cell cycle synchronization, cells were arrested at G1 by the addition of 10 µg/ ml α-factor to log phase cultures for 3 hours and then released by washing twice with media supplemented with 0.1 mg/ml Pronase E (Sigma).

RESULTS

Domain Analysis of Cdc14 Homologues

Previous studies in several systems clearly show that the subcellular localization of Cdc14 is central to its regulation. The two mammalian Cdc14 homologues share high sequence similarity, but display remarkably different subcellular localization patterns. During interphase, overexpressed hCdc14A-GFP is mostly cytoplasmic and localizes to the centrosome (Figure 3-1B), whereas hCdc14B-GFP resides in the nucleolus (Figure 3-1C). I analyzed transfected HeLa cells in mitosis to determine if human Cdc14 homologues are also capable of localizing to the cytokinesis furrow, since both the *S. pombe* and *C. elegans* Cdc14 homologues localize there. I examined GFP fusion hCdc14 proteins in cells in mitosis and observed that both homologues can localize to the centrosome and midbody in late mitosis, providing further evidence for a role during cytokinesis (Figure 3-1D). I analyzed various truncated human Cdc14-GFP fusion constructs to examine what domains control the subcellular localization of Cdc14 homologues in HeLa cells. Alignment of several Cdc14 sequences reveals a highly

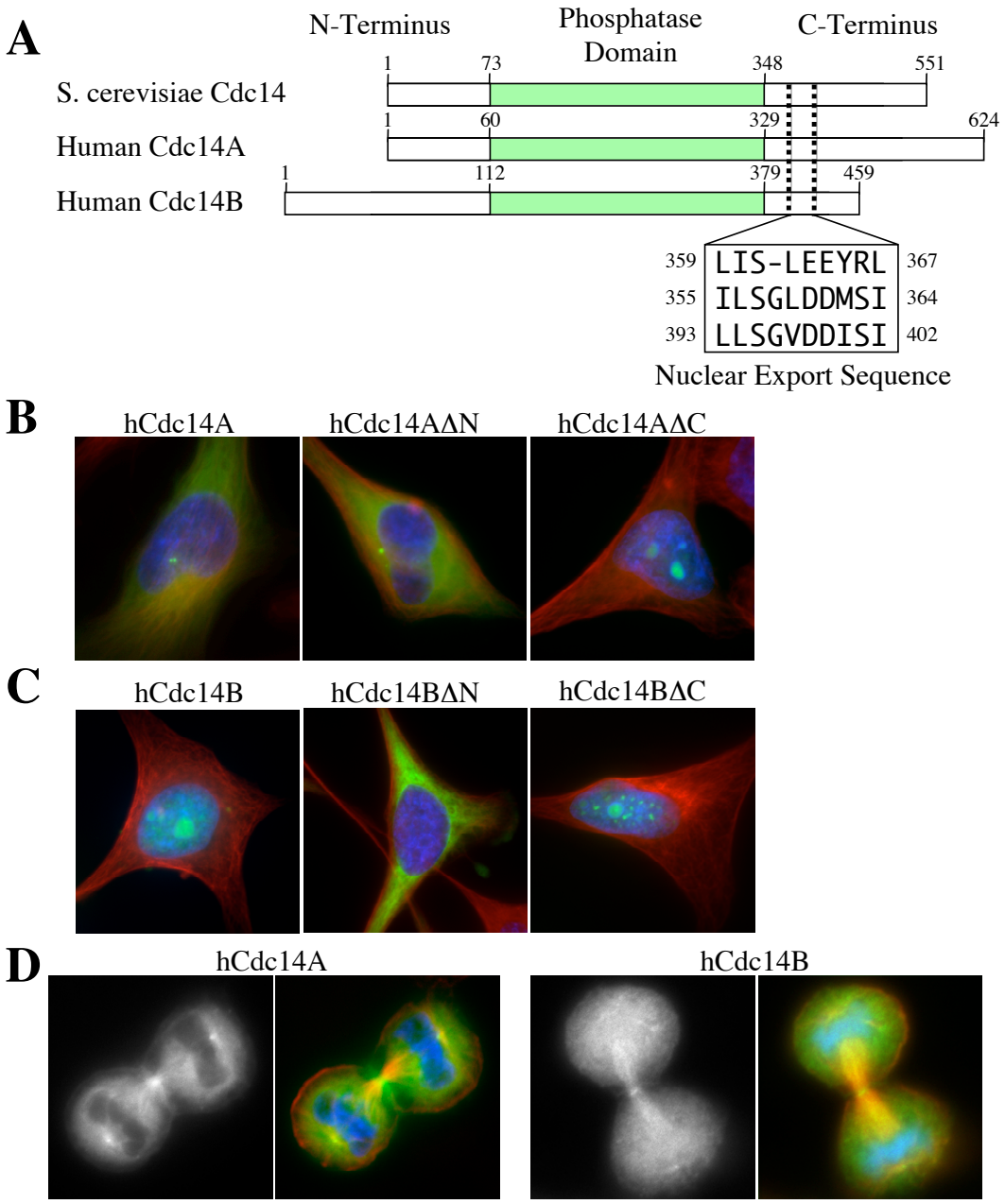


Figure 3-1. Analysis of Cdc14 Domain Organization. A, Cdc14 sequence alignment with NES shown in box. B-D, Cdc14-GFP fusion proteins were expressed in HeLa cells and costained with α-tubulin and DAPI. B, The hCdc14A full-length and 60 amino acid N-terminal truncations are cytoplasmic and centrosomal, however the 295 amino acid C-terminal truncation accumulates in the nucleus and nucleolus. C, The hCdc14B full length and 80 amino acid C-terminal deletion fusion proteins are nuclear, whereas the 112 amino acid N-terminal truncation mutant remains cytoplasmic. D, HeLa cells in telophase expressing hCdc14A and hCdc14B wild-type GFP fusion proteins show midbody and centrosomal localization.

conserved phosphatase domain, and N- and C-termini that are more divergent (Figure 3-1A). The hCdc14A homologue remains cytoplasmic when truncated 60 residues at the

N-terminus (Figure 3-1B), and expression of the N-terminal 83 residues alone are sufficient for localization in the nucleolus (Figure 3-2A). The hCdc14A protein accumulates in the nucleolus when 280 residues at the C-terminus of hCdc14A are truncated (Figure 3-1B). Similarly, the N-terminal 103 amino acids of hCdc14B are required for nucleolar localization (Figure 3-1C), and these 103 residues alone can localize to the nucleolus (Figure 3-2B). I deleted 80 residues C-terminal to the consensus phosphatase domain and found no effect on hCdc14B localization to the nucleolus (Figure 3-1C). I have also observed that overexpression of several hCdc14-GFP constructs causes several defects consistent with failures during cytokinesis (data not shown,) as previously reported (113). In summary, the N-terminal residues contain a nuclear and nucleolar localization signal, whereas the C-terminus appears to be required for nuclear export and centrosomal localization. This data is consistent with previous truncational analysis studies of hCdc14 (113).

Cdc14 Homologues are Regulated by Crm1-Dependent Nuclear Export

I then analyzed the N- and C- termini for sequence elements that mediate the subcellular localization of Cdc14 homologues. By sequence homology, the hCdc14A, hCdc14B and budding yeast Cdc14 (scCdc14p) proteins contain a putative NES (Figure 3-1A). To test whether this sequence element functions as a NES, I assayed the localization of various GFP fusion proteins in HeLa cells. Both hCdc14A-GFP and scCdc14p-GFP fusion proteins are mostly cytoplasmic when transfected into HeLa cells (Figure 3-3; left panels). Incubating cells with the Crm1 inhibitor leptomycin B for 4 hours causes both of these proteins to accumulate in the nucleus (Figure 3-3; second

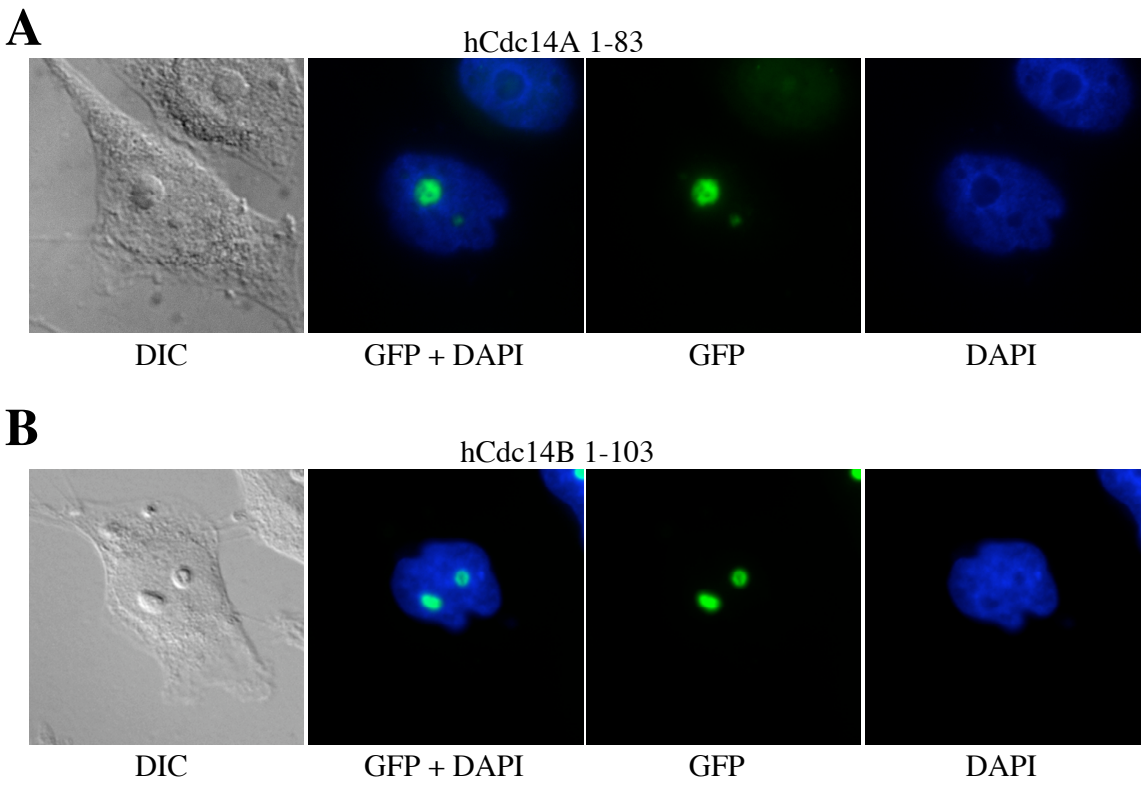


Figure 3-2. The N-terminus of human Cdc14 localizes to the nucleolus. Cdc14-GFP fusion proteins were expressed in HeLa cells and costained with DAPI. A, The hCdc14A 83 N-terminal residues localize to the nucleolus. B, The first 103 amino acids of hCdc14B are also sufficient to localize to the nucleolus.

panels). I also introduced several mutations that disrupt the NES in hCdc14A and Cdc14p and assayed their localization. The *CDC14-AAA* allele is a L359A/I360A/L362A point mutant, and the *CDC14ΔNES* mutation is a deletion of amino acid residues 359-367. I also did double and triple point mutations in critical hydrophobic residues of the NES according to my alignment (Figure 1A) in hCdc14A (hCdc14-AAA is I355A/L356A/L359A, and AA is M362A/I364A). Mutations within the NES lead to the accumulation of the hCdc14A and Cdc14p -GFP fusion proteins in the HeLa nucleus (Figure 3-3; right panels). The hCdc14A protein also becomes enriched in the nucleolus when nuclear export is inhibited (Figure 3-3A). Thus cytoplasmic retention of both the hCdc14A and Cdc14p homologues in HeLa cells requires a Crm1-dependent NES.

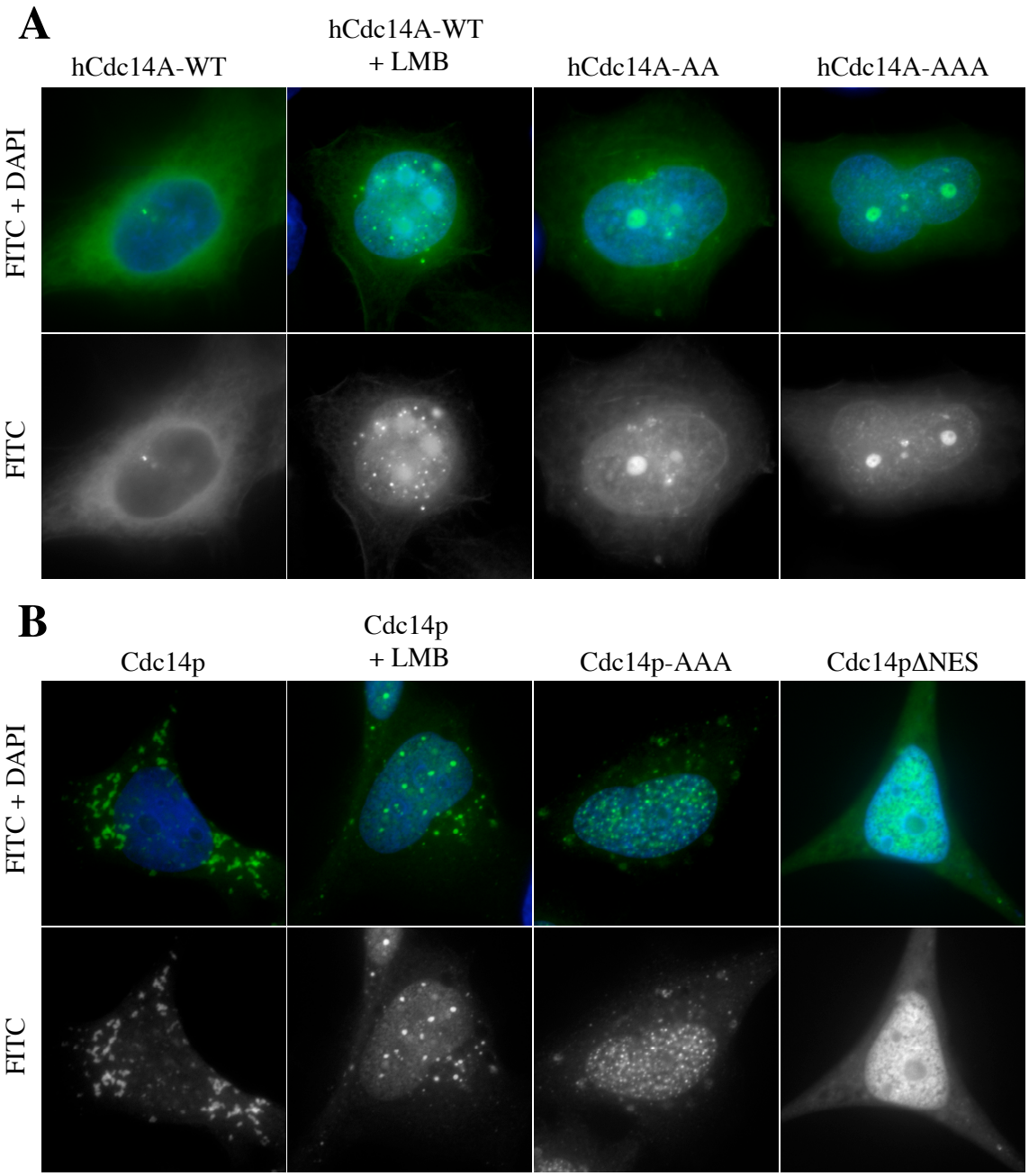


Figure 3-3. Cdc14 homologues undergo Crm1-dependent nucleocytoplasmic shuttling. A, The hCdc14A-GFP is cytosolic and centrosomal when overexpressed in HeLa cells. Incubation with Leptomycin B induces nuclear accumulation of hCdc14A, similar to mutations within the NES. B,Budding yeast Cdc14p-GFP expresses as cytoplasmic aggregates in HeLa cells that accumulate in the nucleus upon addition of Leptomycin B. Similarly, mutations within the putative NES induce nuclear accumulation of budding yeast Cdc14p in HeLa cells.

Interestingly, the hCdc14B protein also appears to have a NES despite being maintained in the nucleolus during interphase. In budding yeast, Cdc14p is thought to be sequestered in the nucleolus to keep it inactivated. Cdc14p is released during mitosis to

perform several tasks during the final stages of mitosis. In mammalian cells, the nuclear envelope is broken down and the nucleolus is dispersed during mitosis, which could cause a release of hCdc14B independent of nuclear export. I hypothesized that hCdc14B may have a NES to allow it to perform a function when the nuclear envelope is intact during interphase. To address this question, I tested the ability of the hCdc14B-GFP fusion protein to undergo nucleocytoplasmic shuttling during interphase by employing a heterokaryon cell fusion assay, as previously described (143). HeLa cells were transfected overnight with hCdc14B-GFP expression constructs, followed by treatment with cycloheximide to prevent further protein synthesis. The HeLa cells were then fused with an untransfected mouse cell and fixed after 4 hours to detect movement of the fusion protein into the mouse nucleus. In this assay, hCdc14B-GFP is able to shuttle from the human nucleus into the mouse nucleus during interphase (Figure 3-4). If the heterokaryon is treated with leptomycin B during the 4-hour incubation, hCdc14B shuttling into the mouse nucleus is inhibited (Figure 3-4). I analyzed NES mutants of hCdc14B (hCdc14B-AAA is L393A/L394A/V397A, and hCdc14B-AA is I400A/I402A) and found that movement of hCdc14B-GFP fusion proteins into the mouse nucleus is greatly reduced by the NES mutations (Figure 3-4). Nucleophosmin is also a resident of the nucleolus during interphase, however it is not capable of shuttling into the mouse nucleus this assay (Figure 3-4). Thus, despite being sequestered in the nucleolus during interphase, hCdc14B-GFP is capable of shuttling into a mouse nucleus in the heterokaryon cell fusion assay. I have thus defined a functional Crm1-dependent NES that plays a key role in regulating the subcellular localization of these three Cdc14 homologues in mammalian cells.

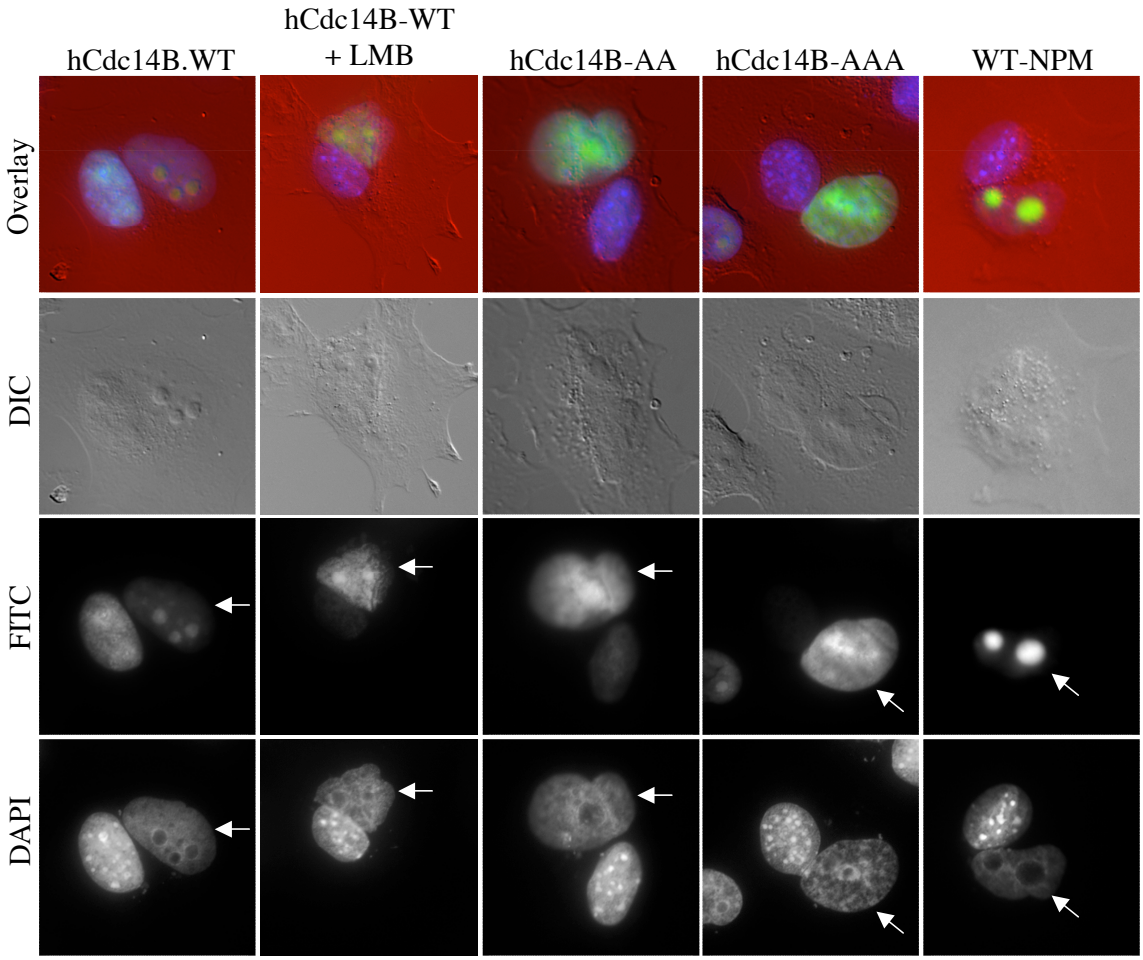


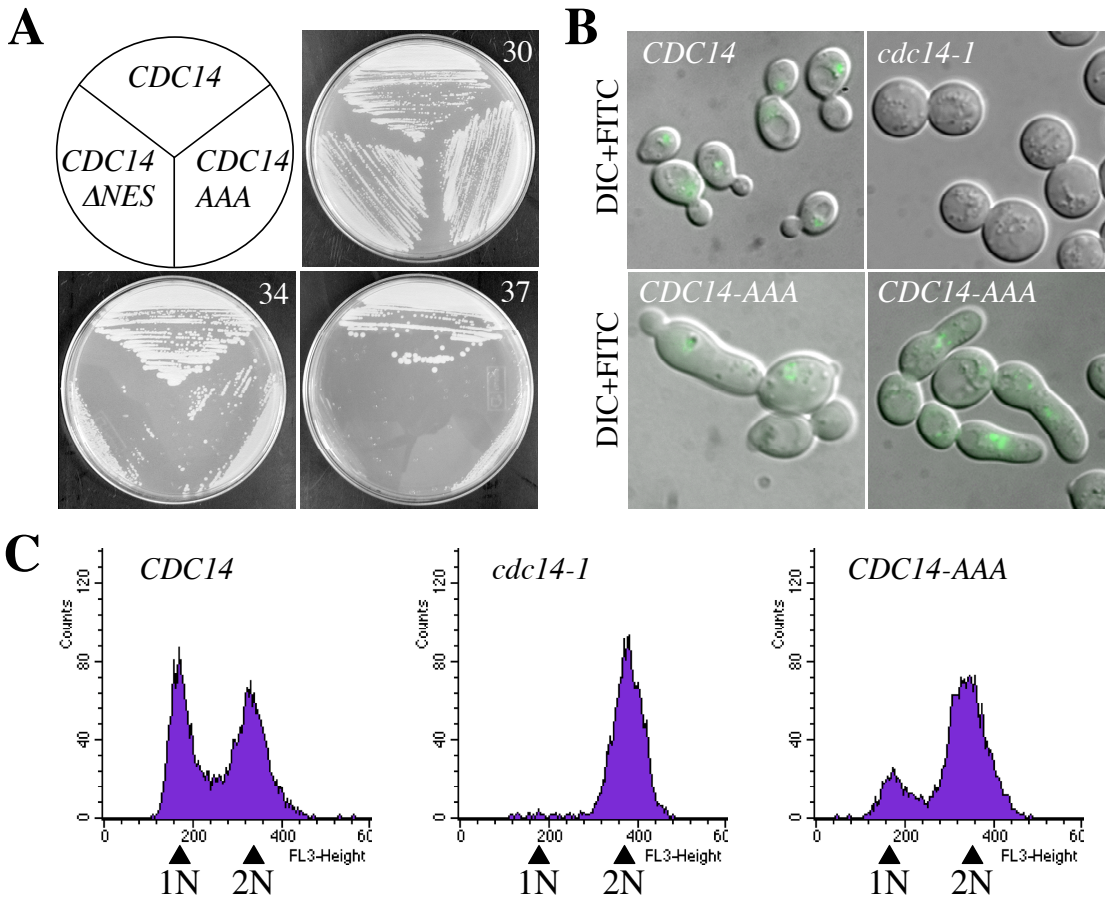
Figure 3-4. Heterokaryon shuttling assay demonstrates that hCdc14B has a NES. GFP fusion proteins were expressed in HeLa cells overnight. Transfected HeLa cells were then fused to untransfected mouse L929 cell in the presence of cycloheximide to prevent further GFP fusion protein expression. The heterokaryons were fixed and costained with DAPI. The hCdc14B protein is able to shuttle from the HeLa nucleus (marked with arrow) into the mouse nucleus, which is prevented with the addition of Leptomycin B or mutations in the NES. Nucleophosmin (NPM) is a resident of the nucleolus that does not shuttle in this assay.

NES Mutant Cdc14 Causes a Cytokinesis Failure in S. cerevisiae

To assess the effect of mutating the NES of Cdc14 on cell cycle progression, I chose to work in the genetically tractable *S. cerevisiae*, which contains only one Cdc14 homologue. To analyze Cdc14p in budding yeast, I generated a low copy yeast expression plasmid with a uracil marker which encodes Cdc14p fused to GFP at the C-terminus under the control of the endogenous *CDC14* promoter. This Cdc14p-GFP

expression plasmid was transformed into a diploid heterozygous *CDC14* deletion strain obtained from Research Genetics, which was then sporulated to obtain viable *cdc14*Δ haploid cells rescued by the Cdc14p-GFP expression plasmid. I used plasmid shuffling with FOA selection to obtain yeast strains with several different *CDC14* NES mutant alleles and analyzed the temperature-dependent growth of these *CDC14* NES mutant strains.

As expected, cells containing the wild-type *CDC14* are viable at all temperatures. Strains carrying *CDC14* mutations with the NES deleted (*CDC14*Δ*NES*) or with three hydrophobic residues of the NES mutated to alanine (*CDC14–AAA*) grow poorly at 34°C and are inviable at 37°C (Figure 3-5A). To understand the nature of the growth defect I compared the growth defect of the strains grown at 37°C. Analysis of the cells by DIC microscopy shows that the wild-type *CDC14* strains grew normally at 37°C, as expected (Figure 3-5B; upper left panel). The previously characterized *cdc14-1* strain arrests with large budded cells as previously characterized (Figure 3-5B; upper right panel). In contrast, cells carrying only NES mutant *CDC14* cannot complete cell division, continue to grow an elongated bud tip beyond the normal mitotic arrest, and in some cases can rebud (Figure 3-5B lower panels). The majority of the population of *cdc14-1* and *CDC14* NES mutant haploid cells grown at 37°C for 2 hours are arrested with 2N DNA content, whereas wild-type cell populations grow asynchronously, as revealed by FACS analysis (Figure 3-5C). I also monitored the budding index after 3 hours of growth at 26°C or 37°C (Figure 3-5D). The wild-type strain grew normally under both conditions, as expected. At restrictive temperature, the *cdc14-1* strain was 94% arrested with large-budded cells and several isolated examples could be found with



D

	♂	♂	♂	♂
CDC14 26˚C	42%	58 %	0 %	0 %
CDC14 37˚C	53%	44 %	2 %	1 %
CDC14-AAA 26˚C	43 %	51 %	5 %	1 %
CDC14-AAA 37˚C	6 %	22 %	16 %	56 %
cdc14-1 26˚C	22 %	73 %	5 %	0 %
cdc14-1 37˚C	0 %	5 %	94 %	1 %

Figure 3-5. Phenotype of Budding Yeast with NES mutations in Cdc14. A, *CDC14* NES mutant strains were grown on YPD at the indicated temperatures, showing temperature sensitive growth. B, DIC microscopy of *CDC14*, *CDC14-AAA*, and *cdc14-1* cells grown at 37˚C for 3 hours. The *cdc14-1* shows the classical large-budded arrest, whereas the NES mutant grows beyond anaphase arrest. C, FACS analysis of the strains grown at 37˚C shows that the *CDC14* NES mutant cells are shifted to the 2N peak similar to the *cdc14-1* strain. D, Budding index given as percentage of cells with the diagramed phenotype of the indicated strains grown at either 26˚C or 37˚C.

cells growing beyond the normal mitotic arrest. In comparison, 56% of the *CDC14* NES mutant cells arrested with either abnormal elgongated growth or multiple buds and another 16% arrested as large mitotic cells. Therefore the novel NES mutant alleles of

Cdc14 confer temperature sensitive growth to haploid budding yeast cells, characterized by a defect in cytokinesis.

The NES Mutants of Cdc14p Fail to Localize to the Bud Neck and Spindle Pole Bodies

To determine the effect of NES mutations on Cdc14 localization, I first analyzed the localization of the wild-type Cdc14p-GFP protein through time-lapse microscopy at 37°C or 26°C (Figure 3-6A, 3-6B respectively). Consistent with previous reports, I found that Cdc14p-GFP localizes to the nucleolus during most of the cell cycle and is released as cells near completion of mitosis. Surprisingly, Cdc14p-GFP not only localizes to the centrosome when released, but also appears at a bifurcated ring at the bud neck (Figure 3-6B). The bud neck localization of Cdc14p appears quickly after nucleolar release in anaphase and persists for a short time after a majority of the Cdc14p has returned to the nucleolus.

We next analyzed the localization patterns of the NES mutant of Cdc14p-GFP, Cdc14p-AAA-GFP, at 37˚C. This NES mutant strain progressed normally into mitosis. Cdc14p-AAA-GFP was released from the nucleolus in late anaphase. However, Cdc14p-AAA-GFP failed to accumulate at the SPB and bud neck (Figure 5). These cells also failed to complete cytokinesis. They continued to grow at the distal tip of the daughter cell in the absence of cytokinesis (Figure 3-7A). This abnormal growth eventually led to cell lysis (data not shown).

Nuclear Export of Cdc14p Is Not Required for Clb2p Degredation

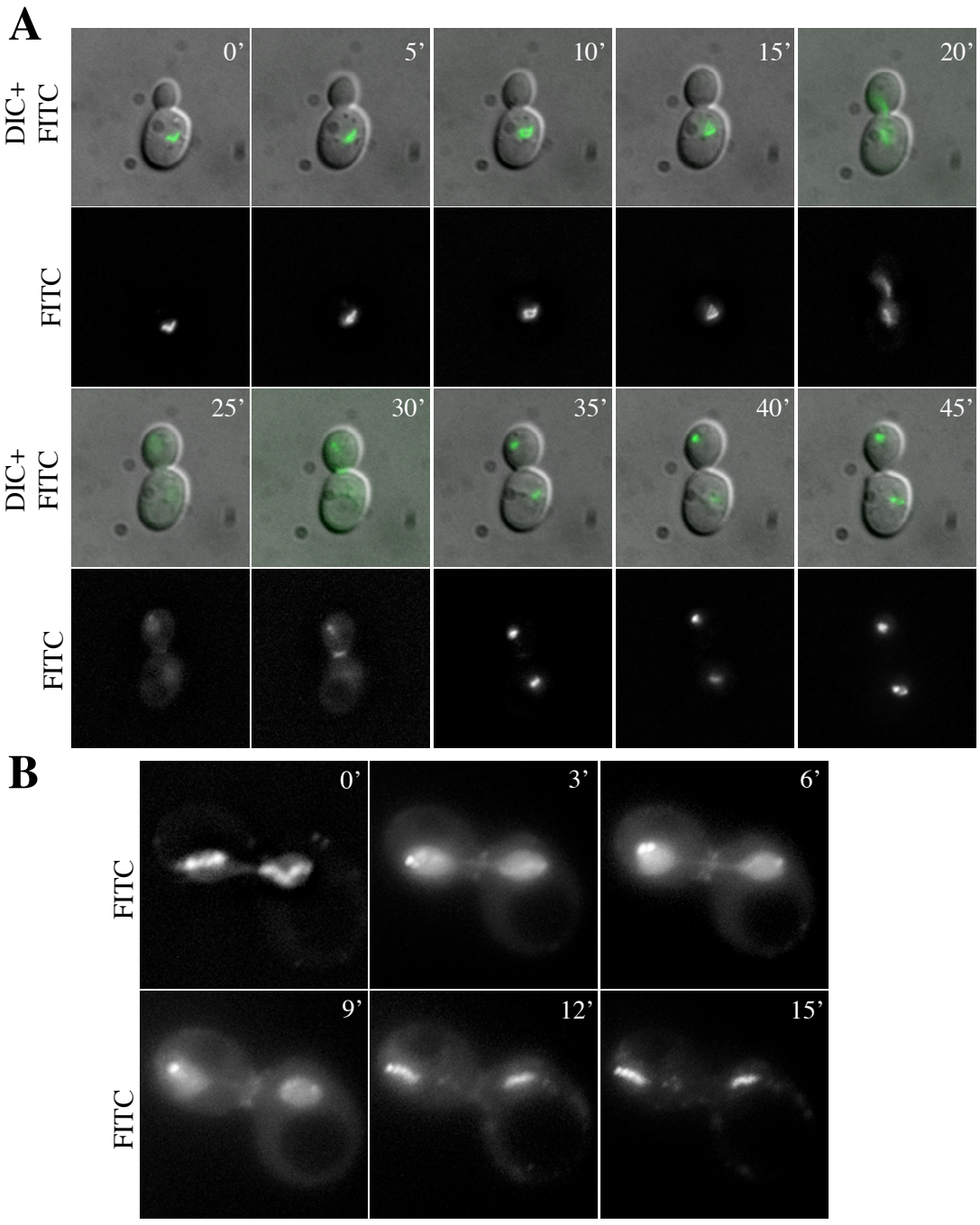


Figure 3-6. Time-lapse Microscopy of Cdc14p-GFP or -YFP in Budding Yeast. A, The Cdc14p-YFP strain was grown on cover slips at 37˚C and images were captured at the indicated time points. B, The Cdc14p-GFP strain was grown at 26˚C and images were taken every two minutes to clearly observe the bifurcated ring at the bud neck. Several z-planes were captured for each time point, deconvolved and stacked.

Cdc14p activates APC/C^{Cdh1} through dephosphorylation of Cdh1p. It is thus required for the ubiquitin-mediated degradation of Clb2p. However, it is known that high levels of

Clb2p-Cdc28p kinase activity prevent re-budding and abnormal bud growth of yeast cells. Because the *CDC14-AAA* strain was able to re-bud in the absence of cytokinesis, it is possible that Clb2p is degraded normally in the *CDC14-AAA* strain even at the restrictive temperature. We therefore examined the levels of Clb2p in the wild-type *CDC14* and *CDC14-AAA* strains after the release from an α-factor-mediated G1 arrest at 37˚C. Both strains accumulated and degraded Clb2p with similar kinetics (Figure 3-7B). Thus, the nuclear export of Cdc14p is not required for Clb2p degradation.

Because Cdc14p dephosphorlyates Sic1p and its transcription factor Swi5p and stabilizes Sic1p, we also examined the levels of Sic1p in these strains. Consistent with earlier findings, Sic1p levels increased as cells underwent mitotic exit in the wild-type strain. Interestingly, Sic1p failed to accumulate in late anaphase in the *CDC14-AAA* strain (Figure 3-7B). Our data suggest that the nuclear export of Cdc14p is required for the stabilization of Sic1p in late anaphase.

DISCUSSION

The Role of the MEN During Mitotic Exit

The data presented herein demonstrates that nucleocytoplasmic shuttling is a central component of the regulation of Cdc14 in both mammalian cells and budding yeast. The localization of both full-length homologues at the midbody and centrosome, as well as various defects observed during cytokinesis upon overexpression of different human Cdc14 constructs provides further evidence that the human Cdc14 homologues regulate cytokinesis. Similarly, a role for Cdc14 in cytokinesis in budding yeast as

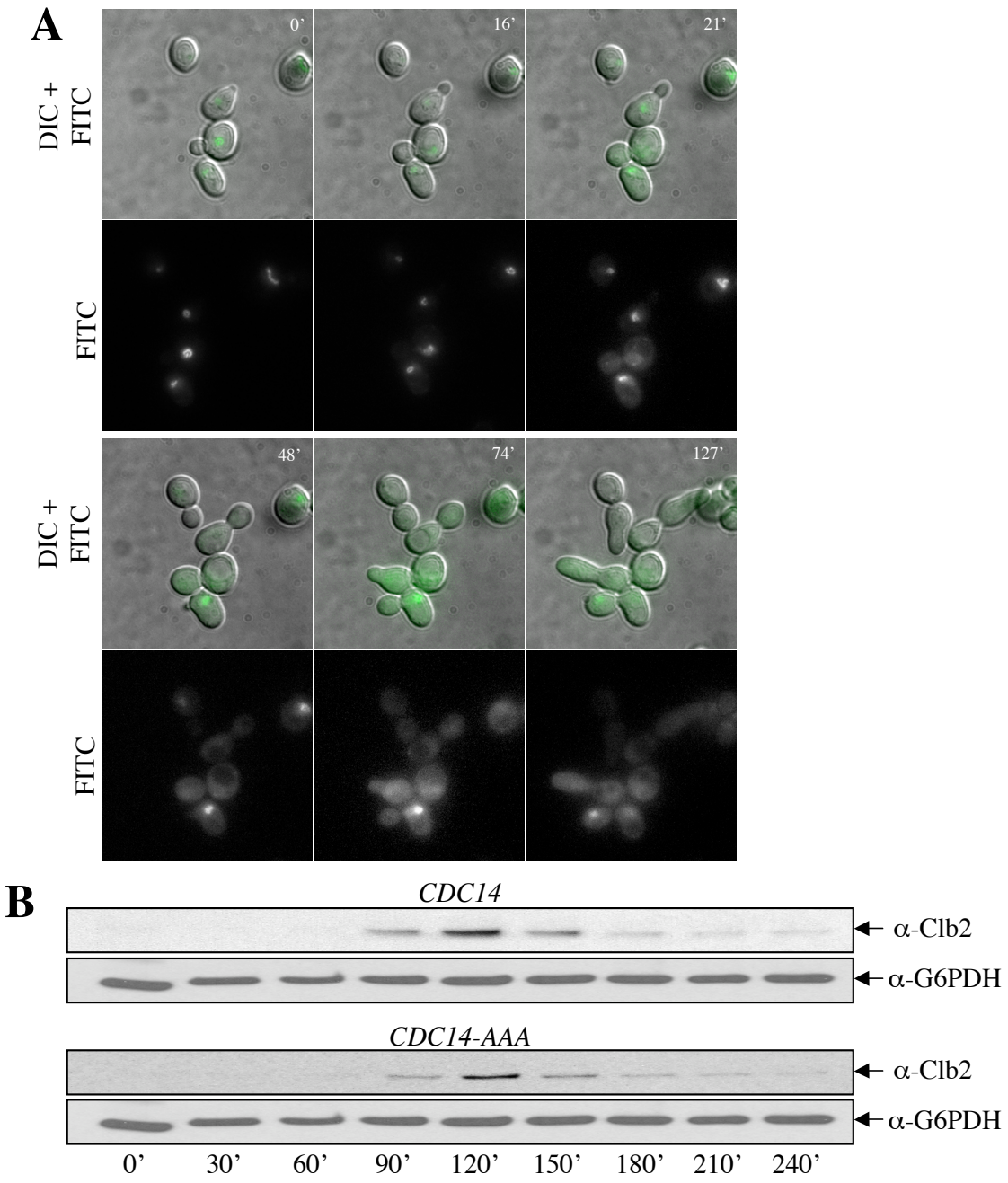


Figure 3-7. Time-lapse Microscopy of budding yeast with Cdc14-AAA-YFP. A, The strain carrying Cdc14p-AAA-YFP was grown on a cover slip at 37°C for 2 hours and images were taken at the indicated time points. Several z-planes were captured for each time point, deconvolved and stacked. B, Samples from cultures release from alpha factor arrest at the indicated time points were blotted with a-Clb2p and a-G6PDH antibodies.

revealed by the NES mutant *CDC14* alleles provides important insight into the conserved function of Cdc14 homologues. It also begins to resolve the disparate models for the function of the MEN and SIN during the final stages of mitosis. While the precise

mechanistic details needed to explain the phenotypic variance between MEN and SIN mutations are currently unknown, my data indicate that these networks may ultimately perform similar tasks at the end of mitosis. Despite the emphasis on cyclin regulation, genetic studies have previously implicated a role for MEN components during cytokinesis, similarly to the SIN, for a review see (38). For example, Bub2 was originally identified as a regulator of the spindle checkpoint. The spindle checkpoint prevents APC/C-mediated protein degradation and the onset of anaphase prior to bioriented attachment of sister chromatids to opposite poles of the mitotic spindle. Unlike most spindle checkpoint components that target the APC/C^{Cdc20} complex, Bub2 acts to stabilize cyclins by restraining MEN activity until anaphase has been initiated (145). In the absence of Bub2, cells cannot remain arrested in metaphase and actomyosin ring formation occurs prematurely (146). Thus unrestrained MEN activity leads to the premature initiation of structures involved in the final stages of cytokinesis.

Several additional lines of evidence implicate a role for MEN components in cytokinesis. The mitotic exit defect of Tem1 mutants can be bypassed in the presence of the *net1-1* allele which allows a partial release of Cdc14p without MEN activity (60). The *tem1*Δ*net1-1* double mutant cells exit mitosis, however are incapable of contracting the actomyosin ring during cytokinesis and form chains of cells (74). Cells carrying the *cdc15lyt-1* mutant allele are also capable of bypassing the normal late mitotic arrest found in *CDC15*Δ mutants, and continue to grow without cytokinesis until eventual lysis (77), a phenotype nearly identical to my *CDC14* NES mutants. Similarly, overexpression of Cdc15p truncations that do not localize to the SPB in a *cdc15*Δ background leads to cytokinesis defects (54). The Mob1 protein interacts with the Dbf2 kinase and is required

for Dbf2 function. Mob1 mutations cause cells to arrest in late mitosis, which can be bypassed by overexpression of the mitotic Cdk inhibitor, Sic1p. This bypass of Mob1-induced mitotic arrest mutations prevents bud neck constriction and leads to the continued growth of cells into chains (76). The Cdc5 polo-like kinase, which plays multiple roles during progression through the cell cycle including participation in FEAR and MEN signaling, is required for cytokinesis (78). This evidence supports the notion that the role of the MEN and Cdc14p during cytokinesis is masked by the inability of the mutant cells to properly degrade cyclins and remove the inhibition of cytokinesis by mitotic Cdks.

The players of the MEN act in a positive feedback loop with Cdc14p. Several upstream MEN components are required to maintain the release of Cdc14p. The released Cdc14p then binds Bfa1p, which is thought to relieve Bfa1p GAP activity towards Tem1p, contributing to Tem1p activation (69). Cdc14p also contributes to the full activation of the MEN pathway by dephosphorylating Lte1p and Cdc15p. Cdc5p, Cdc15p, Dbf2p, Dbf20p, and Mob1p have all been previously shown to appear at the bud neck during cytokinesis. Interestingly, a recent study has demonstrated that the translocation of several MEN kinases from the SPB to the bud neck requires the inactivation of mitotic Cdks (132). Thus Cdc14p promotes cytokinesis indirectly through direct activation of several MEN components, and by triggering the inactivation of mitotic Cdks, which allows translocation of MEN components to the bud neck. I cannot currently determine the role of Cdc14p at the bud neck and whether the cytokinesis defect I observe is a direct result of mislocalized Cdc14p or insufficient activity of the other MEN components. My data suggests that Cdc14p plays a direct role in cytokinesis by

appearing at the bud neck with other MEN components and presumably dephosphorylating important targets at the bud neck. I support a model whereby Cdc14p coordinates the inactivation of mitotic Cdks with the onset of cytokinesis by directly playing a role in both processes.

The appearance of Cdc14 homologues at the site of cell cleavage has now been observed in all organisms analyzed to date, suggesting that Cdc14 may perform a conserved function during cytokinesis. Careful analysis of the spatial and temporal regulation of proteins that regulate and carry out cytokinesis will be essential to obtain a deeper understanding of this process. A recent study in budding yeast has generated the first detailed analysis of the dynamics of several MEN components in live cells (147). It was shown that the SPB that migrates through the bud neck generates an active MEN signal during spindle elongation. In fission yeast, a catalogue of factors involved during cytokinesis has been carefully studied in live cells revealing a mechanistic framework for understanding how cells assemble the site of cell scission and accomplish cytokinesis (148). The SIN pathway was shown to act after the formation of a mature division site during the terminal phase of cytokinesis when actomyosin ring contraction occurs. By analogy to the SIN, it is likely that the MEN and Cdc14p also activate the contraction of the bud neck during the final phase of cytokinesis. Determining the downstream targets of Cdc14p and the kinetics of other MEN and SIN components at the site of cell cleavage will be an exciting focus of future studies.

Nucleocytoplasmic Shuttling Regulates Cdc14 and Cytokinesis

Unlike the yeast model organisms that do not undergo nuclear envelope breakdown, mammalian cells disperse both the nucleolus and the nuclear envelope for an open mitosis. This important difference between open and closed mitosis is an unexplored phenomena that has been largely unaddressed and will need to be studied more closely in order better extrapolate the mechanistic models for progression through mitosis from yeast to the mammalian system. The fact that budding yeast undergo cell division with a closed mitosis makes the export from the nucleolus into the cytoplasm required for any role Cdc14p has at the bud neck and centrosome, as my data indicates. However, the open mitosis in mammalian cells raises interesting questions regarding the mechanism of Cdc14 regulation. Since interphase structures are dispersed and the nuclear envelope is broken down during prophase in an open mitosis when cyclins are not destroyed and cytokinesis is prohibited, additional mechanisms besides nucleolar sequestration are expected to regulate the activity of Cdc14. It will be interesting to determine how the Ran-dependent export machinery regulates the activity of human Cdc14 toward mitotic exit and cytokinesis during an open mitosis.

My observations that hCdc14B can shuttle during interphase suggests that nucleolar sequestration is a dynamic equilibrium controlled in part by the nucleocytoplasmic shuttling pathway. This dynamic equilibrium may allow Cdc14 to transiently escape the nucleolus and possibly function during other stages of the cell cycle. Recent work showing that the fission yeast Cdc14 homologue regulates the entry into mitosis through Cdc25 is consistent with this hypothesis (91). More work is needed to verify that hCdc14B shuttles during interphase since I have used an overexpression system and cannot rule out the possibility that the mechanism for tethering hCdc14B in

the mammalian nucleolus is saturated under these conditions, allowing unbound hCdc14B to undergo nucleocytoplasmic shuttling. Despite this caveat I have observed that the relative strength of nucleolar sequestration of hCdc14B in the nucleolus is greater in the NES mutant protein as compared to the WT protein, and the N-terminal truncation mutant (data not shown). My analysis of GFP fusion proteins demonstrates that both a NLS within the N-terminus and a NES in the C-terminus are responsible for localizing the hCdc14A and B homologues to either the centrosome or nucleolus. Consistent with this, addition of 54 N-terminal residues of hCdc14B to the full-length hCdc14A protein tips the shuttling balance toward nuclear import, causing this chimeric protein to localize to the nucleolus (113). These data indicate that what ultimately determines the localization of these two homologues appears to be an equilibrium between the activities of nuclear import relative to nuclear export. It will be important to determine how nucleocytoplasmic shuttling controls the localization of human Cdc14 during an open mitosis.

Intriguingly, components of the nucleocytoplasmic shuttling pathway have previously been identified in budding yeast as genetically interacting with the Cdc15 kinase of the MEN, which is required to maintain Cdc14 release during mitosis. Two independent studies identified the karyopherins MTR10 and Kap104 as suppressor mutations of Cdc15 temperature sensitive alleles. Genetic analysis showed that Spo12, a component of the FEAR network that promotes the initial release of Cdc14p, is required for viability in the KAP104 suppressor mutant. Additionally, Spo12 is required for the partial delocalization of Cdc14p from the nucleolus in cells harboring the Kap104 mutant. The suppressor mutations within these karyophyerins may thus reduce the

efficiency of nuclear import of Cdc14p, overcoming the loss of MEN-induced Cdc14p export during mitosis, although this was not directly shown.

These observations are consistent with my data showing that inefficient nuclear export of Cdc14p creates cytokinesis defects in *S. cerevisiae* due to inefficient Cdc14p localization to the bud neck and centrosome. A model predicted from the available data that would explain Cdc14p regulation through mitosis is diagramed in Figure 3-8. Once the spindle assembly checkpoint is satisfied, securin is degraded leading to the activation of separase and the FEAR network. The FEAR network, through an unknown mechanism that partially involves Net1 and Cdc14p phosphorylation by Cdc5, causes the initial release of Cdc14p from the nucleolus. The Cdc14p that is freed from the nucleolus is then capable of undergoing Crm1-dependent export into the cytoplasm where it activates the MEN. The active MEN signal is required to prevent precocious import of Cdc14p back into the nucleus until the completion of cytokinesis, perhaps by regulating specific nucleocytoplasmic shuttling factors. Concurrent with MEN activation, Cdc14p also activates mitotic Cdk inhibitors, allowing the translocation of several MEN components from the centrosome to the bud neck. Finally, the active MEN together with Cdc14p directly target unknown factors at the bud neck to regulate the final stages of cytokinesis. Once cytokinesis is complete, the MEN is inactivated and Cdc14p is imported and sequestered into the nucleolus until the subsequent mitosis.

Elucidating how the nucleocytoplasmic shuttling of Cdc14 is regulated during the cell cycle should provide better understanding of how signaling pathways coordinate cytokinesis with mitotic exit. In budding yeast, Cdc14p was recently shown to localize to kinetochores where it dephosphorylates the passenger protein Sli15, stimulating the

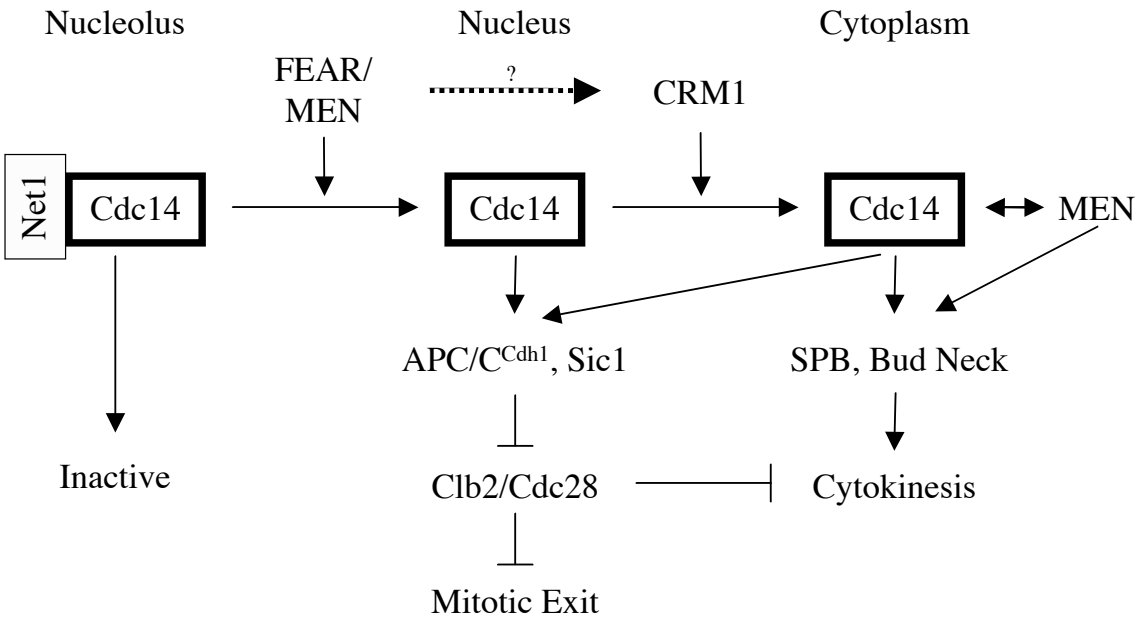


Figure 3-8. Model for Cdc14 Regulation in Budding Yeast

movement of the Ipl1/Sli15 complex to the spindle (149). I have also seen Cdc14p staining patterns during mitosis consistent with the recently reported kinetochore localization (data not shown). The finding that Cdc14p localizes at the kinetochore together with my discovery that Cdc14p localizes to the bud neck later in mitosis officially places it as novel member of the passenger protein family. These passenger proteins appear to regulate the transitions through mitosis and regulate different aspects of cytoskeletal organization. The finding that nucleocytoplasmic shuttling plays an important role in Cdc14p localization leads us to the tantalizing speculation that the behavior of other passenger proteins may be regulated in a similar fashion. Nucleocytoplasmic shuttling has been clearly shown to have multiple functions during several stages of mitosis, and its role during cytokinesis will be an interesting subject of future discoveries.

CHAPTER FOUR

Perspective and Future Directions

Lessons from cell cycle research in the past have led us to believe that the general principles for setting up the program of mitotic exit and cytokinesis are very likely to be conserved among species. A careful examination of the available data in the literature has already provided hints about how the apparent differences among various organisms might be eventually resolved. For example, an important unresolved question is why APC/C^{Cdc20} is insufficient or prevented from completely degrading mitotic cyclins when the MEN pathway is compromised in budding yeast. In MEN mutants, cells arrest in telophase with high mitotic Cdk activity despite the completion of spindle assembly, sister-chromatid separation, and the loss of spindle checkpoint-mediated inhibition of APC/C^{Cdc20}. In contrast, inactivation of the SIN in fission yeast does not appear to affect the ability of APC/C^{slp1} (slp1 is the fission yeast homologue of Cdc20) to degrade mitotic cyclins. One possible mechanism may be that activation of the FEAR pathway that leads to early anaphase release of Cdc14 following inactivation of the spindle checkpoint might also somehow inactivates APC/C^{Cdc20}. In this vein, it is interesting to note that the APC/C isolated from the Cdc5 mutants is less responsive to Cdh1 activation in vitro (29). Therefore, it is possible that certain MEN genes are required to maintain the integrity of the APC/C core. Alternatively, the MEN genes, such as Cdc5, actively promote the conversion from APC/C^{Cdc20} to APC/C^{Cdh1}. The loss of certain MEN genes would then trap APC/C in an inactive state that cannot fully function with either Cdc20 or Cdh1, thus preventing the degradation of mitotic cyclins.

Why is this feature unique to the budding yeast? The answer might lie in the fact that the cell cycle defects caused by MEN mutations activate the Bub2 spindle-positioning checkpoint. By coupling mitotic cyclin degradation and the status of spindle positioning, this checkpoint would ensure that the activity of the mitotic Cdks persist until proper spindle positioning. In *S. pombe*, the spindle position checkpoint may have already been satisfied because both SPBs have to achieve the correct orientation to allow proper chromosome segregation. If the mechanism that senses spindle position is satisfied earlier in *S. pombe*, the APC/C^{Slp1} complex would then be sufficient to drive mitotic cyclin degradation, allowing exit from mitosis and establishment of G1, even in the absence of flp1 function.

It is apparent that both MEN and SIN regulate cytokinesis. However, the precise mechanism by which these networks regulate cytokinesis remains to be established. Recent reports indicate that the SIN is required for the formation of the actomyosin ring and the eMTOC during late mitosis in *S. pombe* (87,88). Studies in *C. elegans* strongly suggest that Cdc14 might also be required for these processes, as the Zen-4 kinesin cannot localize to the contractile ring in the absence of Cdc14 (150). It will be interesting to determine whether this function of Cdc14 is conserved in other organisms including mammals. The exact role of Cdc14 homologues in regulation of cytokinesis will undoubtedly be an exiting topic of future studies. Determining what downstream targets of Cdc14 and the MEN and SIN at the site of cell division will be an extremely important subject of future studies. Several proteins that localize to the bud neck, such as septins and myosin, have been previously shown to be phosphoproteins. The progression through cytokinesis until the final completion of cell cleavage requires the coordinated

effort of the microtubule and actin cytoskeleton, septins, and the targeted delivery of membrane components. This complex and difficult task may be orchestrated by the active signaling module of the MEN and SIN proteins assembled at the spindle pole to ensure that cytokinesis occurs only after chromosome segregation has been completed.

In conclusion, there are important unresolved differences with respect to the function of Cdc14 in various organisms. These differences might be due to the incomplete understanding of the signaling networks that control mitotic exit and cytokinesis and how they monitor the different mechanisms for cell scission (i.e. budding verses fission). Future studies are needed to resolve the important differences between the MEN and SIN pathways in budding and fission yeast and to delineate similar mitotic exit and cytokinesis signaling networks in mammals and other higher eukaryotes. Based upon my thesis work, it is now clear that Cdc14 homologues from every organism localize to the site of cell cleavage and play an important role during cytokinesis. Cdc14 was originally discovered as being essential for cyclin degradation in budding yeast, and the collective data implies that Cdc14 coordinates the exit from mitosis through Cdk inactivation with the onset of cytokinesis. Determining what role these signaling networks and Cdc14 homologues have in controlling the action of cytoskeletal and membrane-sorting machinery to accomplish cell cleavage will be an exciting challenge for cell biologists in the future.

CHAPTER FIVE

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VITA

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