Cell, Vol. 97, 233–244, April 16, 1999, Copyright ©1999 by Cell Press

Exit from Mitosis Is Triggered by Tem1-Dependent Release of the Protein Phosphatase Cdc14 from Nucleolar RENT Complex

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Summary

Exit from mitosis in budding yeast requires a group of essential proteins—including the GTPase Tem1 and the protein phosphatase Cdc14—that downregulate cyclin-dependent kinase activity. We identified a mutation, *net1-1*, that bypasses the lethality of *tem1*Δ. *NET1* encodes a novel protein, and mass spectrometric analysis reveals that it is a key component of a multifunctional complex, denoted RENT (for *r*egulator of *n*ucleolar silencing and *t*elophase), that also contains Cdc14 and the silencing regulator Sir2. From G1 through anaphase, RENT localizes to the nucleolus, and Cdc14 activity is inhibited by Net1. In late anaphase, Cdc14 dissociates from RENT, disperses throughout the cell in a Tem1-dependent manner, and ultimately triggers mitotic exit. Nucleolar sequestration may be a general mechanism for the regulation of diverse biological processes.

Introduction

Activation and inactivation of cyclin-dependent kinases (Cdks) governs cell cycle transitions. In the budding yeast *Saccharomyces cerevisiae*, a single Cdk, Cdc28, orchestrates various stages of the cell cycle by taking on different cyclin partners: Cln1–3 for G1 phase, Clb5–6 for S phase, and Clb1–4 for M phase (reviewed by Deshaies, 1997). There are at least three key cell cycle transitions in this organism: G1→S, metaphase→anaphase, and the exit from mitosis (reviewed by King et al., 1996; Deshaies, 1997). Cdc28 inactivation mediated by proteolysis of Clb2 and/or accumulation of the Cdc28/Clb inhibitor Sic1 seems to lie at the heart of the latter transition (Schwab et al., 1997; Visintin et al., 1998).

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Anaphase-promoting complex/cyclosome ubiquitin ligase (APC/C) and its substrate-specific activator Hct1/Cdh1 are required for Clb2 degradation. Clb degradation was originally thought to be required for exit from mitosis (Surana et al., 1993). However, cells devoid of Hct1/Cdh1 fail to degrade Clb2 and are viable (Schwab et al., 1997; Visintin et al., 1997), probably because Sic1 accumulates and turns off Cdc28 activity. Whereas cells devoid of Sic1 can degrade Clb2 and exit mitosis, cells devoid of both Hct1 and Sic1 are inviable, presumably due to their inability to extinguish Cdc28 activity during telophase (Schwab et al., 1997).

In addition to APC/C, another set of genes plays a pivotal role in exit from mitosis. They include *TEM1*, *LTE1*, *CDC15*, *DBF2/DBF20*, *CDC5*, *MOB1*, and *CDC14* (reviewed in Deshaies, 1997; Luca and Winey, 1998). When cells that harbor conditional-lethal temperature-sensitive (ts) mutations in any of these genes are shifted to the restrictive temperature, they uniformly arrest in late anaphase/telophase as large-budded cells with segregated chromosomes, fully elongated microtubule spindles, and elevated Cdc28/Clb2 protein kinase activity. Clb2 proteolysis is not completed, and *SIC1* transcripts and protein fail to accumulate to high levels (Surana et al., 1993; Shirayama et al., 1994b; Toyn and Johnston, 1994; Irniger et al., 1995; Charles et al., 1998; Jaspersen et al., 1998; Visintin et al., 1998). Furthermore, manipulation of Sic1 and Clb2 levels has dramatic effects on these mutants (Donovan et al., 1994; Shirayama et al., 1994b; Toyn et al., 1997; Charles et al., 1998; Jaspersen et al., 1998): overexpression of Clb2 or deletion of Sic1 typically exacerbates their phenotype, whereas overexpression of Sic1 has the opposite effect.

Most of these proteins required for exit from mitosis resemble components of signaling pathways: Tem1 is a GTP-binding protein (Shirayama et al., 1994b); *LTE1* encodes a putative guanine nucleotide exchange factor (Shirayama et al., 1994a); Dbf2/Dbf20, Cdc15, and Cdc5 are protein kinases (Toyn and Johnston, 1994; Hardy and Pautz, 1996; Jaspersen et al., 1998); Mob1 is a novel protein that associates with Dbf2 (Komarnitsky et al., 1998; Luca and Winey, 1998); and Cdc14 is a dual specificity protein phosphatase (Taylor et al., 1997). Consistent with the notion that these proteins constitute elements of a signaling pathway, the corresponding genes display a variety of genetic interactions with each other (Parkes and Johnston, 1992; Kitada et al., 1993; Shirayama et al., 1994b, 1996; Jaspersen et al., 1998; Komarnitsky et al., 1998; Luca and Winey, 1998; Visintin et al., 1998). Thus, for simplicity, we will refer to this group of genes as the mitotic exit network.

Although the functional organization of the mitotic exit network is poorly understood, recent work suggests that the protein phosphatase Cdc14 might act directly on cell cycle regulators to promote mitotic exit. First, overexpression of Cdc14 can activate ectopic degradation of Clb2 and accumulation of Sic1 (Visintin et al., 1998; W. S. and R. J. D., unpublished). Second, mutation of Cdk consensus phosphorylation sites in Swi5 and Hct1 activates their ability to promote *SIC1* transcription and

Clb2 degradation, respectively (Moll et al., 1991; Zachariae et al., 1998), and Cdc14 antagonizes the phosphorylation of both proteins (Visintin et al., 1998; Jaspersen et al., 1999). Although these data suggest that Cdc14 is required to dephosphorylate Swi5 and Hct1 as cells exit mitosis, they fail to address the key question of whether Cdc14 is a regulated component of the biochemical switch that flips the cell from mitosis to G1.

We report here the identification of a protein complex, named RENT, that tethers Cdc14 to the nucleolus throughout most of the cell cycle. As cells progress through mitosis, Cdc14 is released from the nucleolus in a Tem1-dependent manner. We propose that the Tem1-dependent discharge of Cdc14 from the nucleolar RENT complex lies at the heart of the biochemical engine that drives cells from mitosis to G1.

Results

net1-1 Enables *TEM1*-Independent Clb2 Degradation and Sic1 Accumulation

To delineate how *CDC15* promotes exit from telophase, we conducted a genetic screen to isolate *tab* (*telophase arrest bypassed*) mutants that allow *cdc15*Δ cells containing a complementing *[CDC15, URA3]* plasmid to survive without the plasmid (W. S. and R. J. D., in preparation). The gene corresponding to one of these mutants, *TAB2*, was cloned by complementation of the ts growth defect of *tab2-1* cells and identified as *YJL076W*. It encodes a 129 kDa protein with only one obvious homolog in sequence databases: the *S. cerevisiae* topoisomerase-I interacting protein Tof2 encoded by *YKR010C*, which is 22% homologous to Net1 over 828 amino acids. *TAB2* and the *tab2-1* allele were subsequently renamed *NET1* (for *n*ucleolar silencing *e*stablishing factor and *t*elophase regulator) and *net1-1*, respectively. The name *NET1* also reflects its independent identification as *NUS1* (nucleolar specific silencing protein; Straight et al., 1999 [this issue of *Cell*]), *ESC5* (establishes silencing; E. D. Andrulis and R. Sternglanz, personal communication), and *TAB2* (this report; W. S. and R. J. D., in preparation). *NET1* was also identified in a two-hybrid screen for Cdc14-interacting proteins (C. B. and H. C., in preparation). Disruption of *NET1* was not lethal, although *net1*Δ cells grew very slowly (Straight et al., 1999; W. S. and R. J. D., unpublished data).

Double mutant *cdc15*Δ *net1-1* cells were able to form colonies (W. S. and R. J. D., in preparation), as were *tem1*Δ *net1-1* and *cdc15*Δ *net1*Δ cells (Figure 3; data not shown). These genetic data formally suggest that Net1 is an inhibitor of mitotic exit that acts either downstream of, or parallel to, Tem1 and Cdc15. To address whether *net1-1* can efficiently bypass the requirement for Tem1 in Clb2 degradation and Sic1 accumulation, we constructed a *tem1*Δ*::GAL1-UPL-TEM1* strain that allowed for the rapid, conditional depletion of Tem1. UPL, which stands for ubiquitin-proline-LacI, acts as a destabilizing module that permits rapid degradation of appended proteins (Johnson et al., 1992). *tem1*Δ*::GAL1-UPL-TEM1* cells grew at a normal rate in YP-galactose medium (YPG; *TEM1* expressed) but exhibited first cycle arrest in telophase upon transfer to YP-glucose medium (YPD; *TEM1* repressed). We also tried to construct a similar conditional allele for *CDC15*, but first cycle arrest was not achieved.

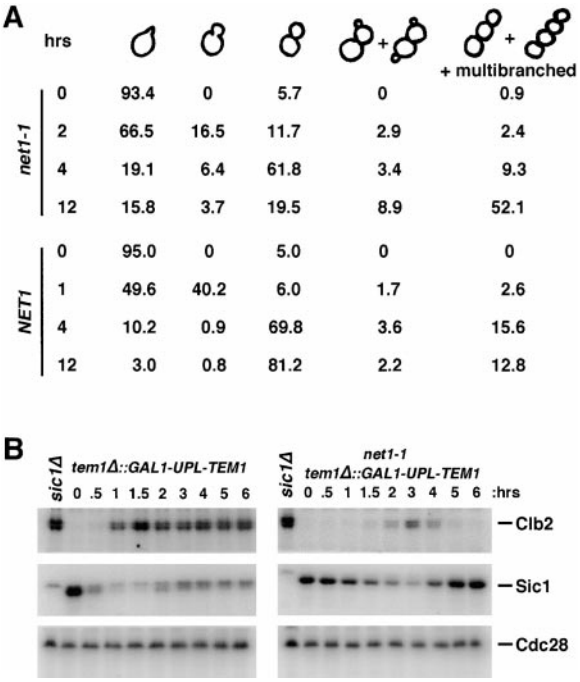


Figure 1. *net1-1* Bypasses *tem1*Δ by Inducing Ectopic Clb2 Degradation and Sic1 Accumulation

*tem1*Δ*::GAL1-UPL-TEM1 net1-1*and *tem1*Δ*::GAL1-UPL-TEM1* cells grown in YPG (*TEM1* expressed) at 25°C were arrested in G1 phase with α factor and released into YPD (*TEM1* repressed) at time = 0.
(A) At 0, 1, 2, 4, and 12 hr, samples were taken to measure budding index.
(B) Same as (A), except that at either 2 hr (*NET1*) or 3 hr (*net1-1*) following release from α factor arrest, α factor was added back to prevent cells from proceeding through a second cell cycle. At the indicated time points (hr), samples were taken to measure Clb2, Sic1, and Cdc28 protein levels by immunoblotting.

*tem1*Δ*::GAL1-UPL-TEM1* cells in the wild-type or *net1-1* background were arrested in G1 phase with the mating pheromone α factor and synchronously released into YPD to extinguish expression of *UPL-TEM1*. As expected, the majority (~80%) of *NET1 tem1*Δ*::GAL1-UPL-TEM* cells arrested with large buds. At 12 hr after release, however, ~60% of *net1-1 tem1*Δ*::GAL1-UPL-TEM1* cells exhibited ≥3 cell bodies (Figure 1A), indicating a further round of division without cell separation. Those cells with one to two cell bodies could have resulted from bypass events followed by successful cell separation. Extensive chains of cells with multiple nuclei were commonly observed in the *net1-1* culture but rarely observed with *NET1* cells (data not shown). These data suggest that *net1-1* efficiently bypasses the cell division arrest caused by depletion of Tem1, although *net1-1 tem1*Δ*::GAL1-UPL-TEM1* cells still appear to exhibit a cytokinesis or cell separation defect.

Since Clb2 degradation and Sic1 accumulation normally accompany exit from mitosis, we tested if *net1-1* influenced the levels of these two proteins in Tem1-deficient cells. *tem1*Δ*::GAL1-UPL-TEM1* cells in the wild-type or *net1-1* background were arrested in G1

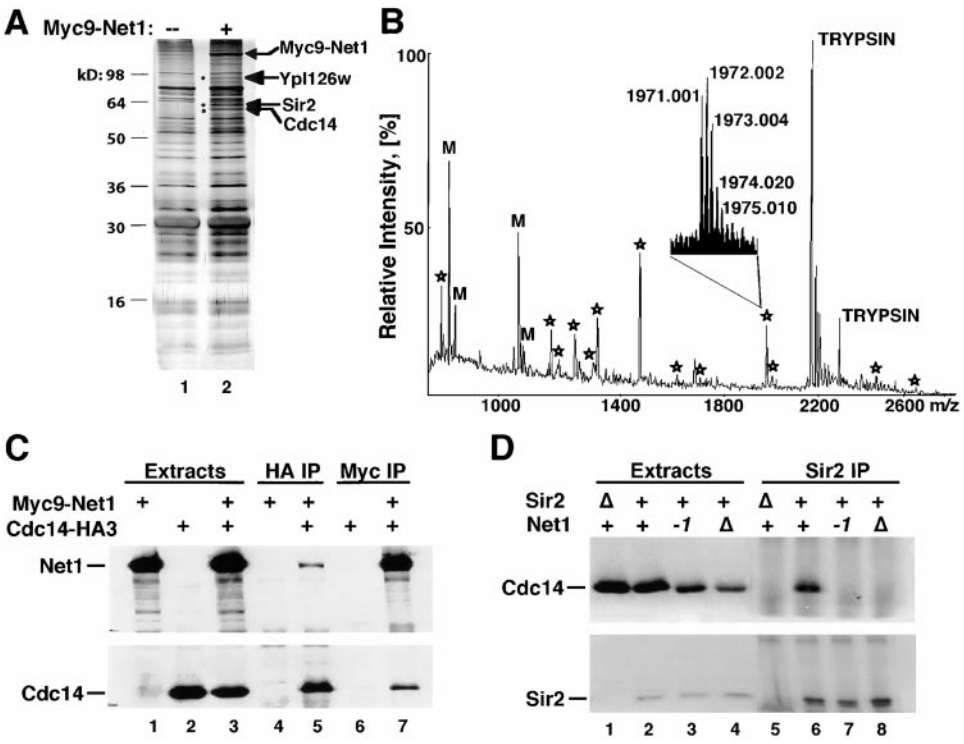


Figure 2. Identification of the RENT Complex

(A) Purification of Net1. Extracts (35 mg) of *myc9-NET1* (lane 2) and untagged control (lane 1) strains were fractionated on beads containing covalently linked anti-Myc monoclonal antibody 9E10. Eluted proteins were separated on a 10%–15% SDS-polyacrylamide gradient gel and visualized by silver staining. Protein bands specifically detected in the immunoprecipitates from *myc9-NET1* but not the untagged control strain were excised and identified by mass spectrometry.

(B) Protein identification by high mass accuracy MALDI peptide mass mapping. Mass spectrum acquired from a 0.5 μL aliquot of in-gel digest of the 62 kDa band revealed 13 peptide ions that matched the calculated masses of protonated tryptic peptides from Cdc14 with accuracy better than 50 ppm (designated with asterisks). These peptides covered more than 26% of the protein sequence. Ions originating from the matrix are designated by "M." Peptide ions of known trypsin autolysis products are also marked. The inset shows an isotopically resolved peptide ion having the monoisotopic weight 1971.001. Mass resolution was better than 7000 FWHM (full width at half maximum) despite the very low amount of protein present in the gel.

(C) Net1 associates with Cdc14. Extracts from strains with the indicated genotypes were immunoprecipitated with either 9E10 antibodies or anti-haemagglutinin (HA) monoclonal antibodies 12CA5. The immunoprecipitates (IP) and the input extracts were immunoblotted with 9E10 (top panel) and 12CA5 (bottom panel) to detect Net1 and Cdc14 proteins, respectively.

(D) Net1-dependent association between Sir2 and Cdc14. Extracts of *CDC14-HA3* cells with the indicated genotypes were immunoprecipitated with anti-Sir2 antibodies. Δ, strains deleted for *SIR2* or *NET1*; +, wild type; −*1*, the *net1-1* allele. The immunoprecipitates (IP) and the input extracts were immunoblotted with 12CA5 (top panel) and anti-Sir2 antibodies (bottom panel) to detect the Cdc14 and Sir2 proteins, respectively.

phase with α factor and released into YPD (Tem1 synthesis repressed; time 0). After cells had exited G1, α factor was added back to trap any cycling cells in the next G1 phase. Cells were harvested at various time points after α factor release and assayed for Clb2, Sic1, and Cdc28 by immunoblotting. In Tem1-deficient *NET1* cells, Clb2 accumulated and remained at high levels, whereas Sic1 was degraded as cells exited G1 and remained at low levels (Figure 1B, left panels). In Tem1-deficient *net1-1* cells, Clb2 accumulation and Sic1 degradation were delayed, presumably due to the reduced growth rate of *net1-1* (data not shown). Nevertheless, eventually Clb2 was completely degraded and Sic1 accumulated to high levels (Figure 1B, right panels). These data suggest that *net1-1* bypassed *tem1*Δ by enabling both Clb2 degradation and Sic1 accumulation.

Net1 Physically Associates with Cdc14 and Sir2

To delineate the mechanism by which *NET1* influences mitotic exit, we sought to identify proteins that interact with Net1. The chromosomal copy of *NET1* was modified to encode a protein with nine copies of the Myc epitope at its N terminus, and Myc9-Net1 was affinity purified from cell extracts on a 9E10 monoclonal antibody matrix. Besides Myc9-Net1, three proteins were specifically detected in silver-stained SDS-polyacrylamide gels of Myc9-Net1 immunoprecipitates but not in control immunoprecipitates (Figure 2A). Protein bands were identified by high mass accuracy matrix-assisted laser desorption/ionization (MALDI) peptide mapping and nanoelectrospray tandem mass spectrometric sequencing combined in a layered approach (Shevchenko et al., 1996a). One Net1-interacting protein (Cdc14) was identified by MALDI (Figure 2B). The identity of the other two proteins (Sir2 and Ypl126w) was revealed by subsequent tandem mass spectrometric sequencing.

To verify the interactions between Net1, Sir2, and Cdc14, we carried out coimmunoprecipitation experiments. Myc9-Net1 was specifically detected in anti-HA immunoprecipitates prepared from a strain containing

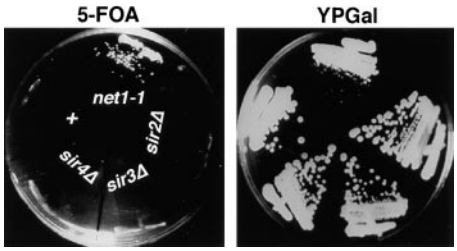


Figure 3. Loss of Silencing Fails to Bypass the Essential Requirement for *TEM1*

Wild-type (+), *net1-1*, *sir2*Δ, *sir3*Δ, or *sir4*Δ cells that carried *tem1*Δ and were sustained by a *[GAL1-TEM1, URA3]* plasmid were grown on YPG (*TEM1* expressed) and then plated onto either YPG (right panel) or synthetic glucose medium supplemented with 5-FOA (left panel) to select for colonies that were able to grow in the absence of the *[GAL1-TEM1, URA3]* plasmid. The plates were incubated at 25°C and photographed after 1 (YPGal plate) or 2 weeks (5-FOA plate).

chromosomal *CDC14* modified to encode a protein with three copies of the haemagglutinin epitope at its C terminus (Cdc14-HA3), but not from a strain that expressed untagged Cdc14 (Figure 2C, compare lanes 4 and 5). Conversely, Cdc14-HA3 was selectively recovered in anti-Myc immunoprecipitates prepared from strains that express Myc9-Net1 (Figure 2C, compare lanes 6 and 7). By a similar analysis, it was confirmed that Myc9-Net1 bound specifically to Sir2 (Straight et al., 1999). Cdc14-HA3 was also detected in anti-Sir2 immunoprecipitates prepared from wild-type cells (Figure 2D, lane 6) but not those prepared from *sir2*Δ (lane 5), *net1-1* (lane 7), or *net1*Δ (lane 8) mutants, suggesting that Net1 bridges the interaction between Cdc14 and Sir2. We refer to the Cdc14-Net1-Sir2 complex as "RENT," for *re*gulator of *n*ucleolar silencing and *t*elophase (see Discussion and Straight et al., 1999).

We have only performed a cursory analysis of the third Net1-associated protein, Ypl126w. *YPL126W* was determined to be an essential gene, and Myc9-tagged Ypl126w was localized to the nucleolus by indirect immunofluorescence, consistent with it being an authentic Net1-binding partner (W. S., unpublished data). To reflect its known properties, we suggest *YPL126W* be renamed *NAN1* (*N*et1-*a*ssociated *n*ucleolar protein).

Although *net1-1* Is Defective in rDNA Silencing, Loss of Silencing Does Not Bypass *tem1*Δ

Sir2 regulates transcriptional silencing at telomeres and the silent mating type loci (reviewed by Lustig, 1998). Sir2 also mediates silencing of some RNA polymerase II–transcribed genes integrated within the rDNA, a phenomenon referred to as "rDNA silencing" (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997). Net1 was independently discovered as a Sir2-binding protein (Straight et al., 1999), and both *net1*Δ and *net1-1* were shown to be defective in rDNA silencing. This raised the possibility that loss of silencing accounted for the *tem1*Δ bypass activity of *net1-1*. To test this hypothesis, we compared the bypass activity of *net1-1*, *sir2*Δ, *sir3*Δ, and *sir4*Δ in *tem1*Δ cells kept alive by a *URA3* plasmid harboring *GAL1-TEM1*. The parental *tem1*Δ *[GAL1-TEM1, URA3]* strain failed to grow on 5-fluoroorotic acid (5-FOA)-containing medium (5-FOA selectively prevents the growth of Ura^+ cells) due to its inability to survive without the *[GAL1-TEM1, URA3]* plasmid. The *net1-1* allele, but not *sir2*Δ, *sir3*Δ, or *sir4*Δ, allowed *tem1*Δ *[GAL1-TEM1, URA3]* cells to survive on 5-FOA (Figure 3). PCR analysis confirmed that the viable colonies indeed lacked the *TEM1*-containing plasmid (data not shown). Thus, the effects of *net1-1* on *tem1*Δ bypass and rDNA silencing can be uncoupled.

Net1 Is an Inhibitor of and a Candidate Substrate for Cdc14

Cdc14 is a dual specificity protein phosphatase essential for mitotic exit (Culotti and Hartwell, 1971; Taylor et al., 1997). Net1 bound to Cdc14 and behaved genetically as a negative regulator of mitotic exit (Figure 2; W. S. and R. J. D., in preparation). Therefore, we postulated that Net1 might either be an inhibitor of Cdc14 or a mitotic exit inhibitor whose activity was counteracted by Cdc14. To test if Net1 is an inhibitor of Cdc14, we measured Cdc14 activity in wild-type, *net1-1*, and *net1*Δ cell extracts. Cdc14-HA3 was immunoprecipitated from asynchronous cultures of *net1-1*, *net1*Δ, and wild-type strains and incubated with ^{32}P-labeled Sic1. It is not clear if phospho-Sic1 is a physiological substrate for Cdc14, but it provides a convenient assay to measure the phosphatase activity of Cdc14. The specific activity of Cdc14-HA3 isolated from *net1-1* and *net1*Δ cells was on average 3.6 and 3.8 times as much as that from wild-type cells, respectively (Figure 4A). This elevation in Cdc14-HA3-specific activity was not due to altered cell cycle kinetics, since the specific activity of Cdc14-HA3 purified from G1-synchronized *net1-1* cells was still 3.8- to 4.5-fold as high as that obtained from G1-synchronized wild-type cells (Figure 4B).

To address whether the effect of *net1* mutations on Cdc14 protein phosphatase activity was direct, we tested if immunopurified Myc9-Net1 could inhibit recombinant GST-Cdc14 purified from *E. coli*. Wild-type or catalytically inactive GST-Cdc14 (Taylor et al., 1997) was recruited to matrices that contained either immobilized Myc9-Net1 or immobilized anti-GST antibodies. To prepare Net1 beads lacking endogenous Cdc14 activity, Myc9-Net1 was immunopurified from extracts treated with phosphatase inhibitor sodium orthovanadate, which reduced the amount of endogenous Cdc14 protein bound to Net1 beads (data not shown); to prepare anti-GST beads, rabbit α-GST antibodies were absorbed to protein A matrix. Following the recruitment step, bead-bound GST-Cdc14 was assayed for its phosphatase activity toward ^{32}P-Sic1. GST-Cdc14 recovered on the Myc9-Net1 matrix had 50-fold lower specific activity than GST-Cdc14 bound to the anti-GST matrix (Figure 4C). Taken together, these experiments suggest that Net1 directly inhibits the protein phosphatase activity of Cdc14.

Having established that Net1 is associated with potent Cdc14 inhibitory activity, we tested if Net1 might also be a substrate for Cdc14. First, Net1 was a phosphoprotein in vivo, since a radioactive band with the expected molecular weight of Myc9-Net1 was apparent in immunoprecipitates prepared from ^{32}P-labeled *myc9-NET1* cells but not from untagged cells (Figure 4D). Second,

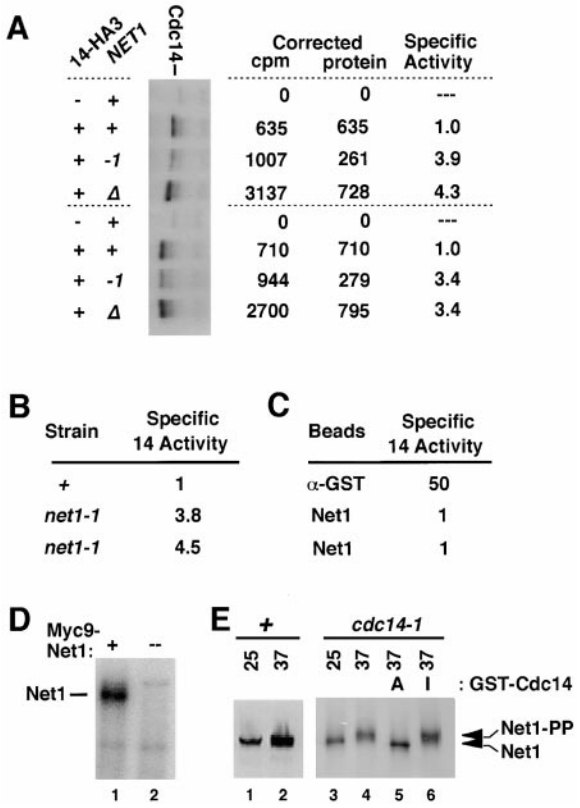


Figure 4. Net1 Is an Inhibitor of and a Candidate Substrate for Cdc14

(A and B) Cdc14 activity is elevated in *net1* mutants. Cdc14-HA3 was retrieved from lysates of the indicated strains (in the "14-HA3" column: −, untagged; +, *CDC14-HA3*; in the "*NET1*" column: +, *NET1*; −*1*, *net1-1*; Δ, *net1*Δ) by immunoprecipitation with 12CA5 antibodies and was incubated with ^{32}P-Sic1 for 20 min at 22°C. The specific activity of Cdc14-HA3 in each reaction was determined by measuring trichloroacetic acid–soluble counts released during the incubation and dividing by the relative amount of Cdc14-HA3 as determined by immunoblotting. All values were corrected by subtracting out background signals obtained with untagged control sample; relative amounts of proteins were normalized such that the specific activity of Cdc14 was 1.0 in wild-type cells. The background signals were ~350 and ~27 in the ^{32}P release and immunoblot assays, respectively. The results of duplicate reactions are presented. In (A), asynchronous cells were used, whereas in (B), cells were synchronized in G1 phase with α factor prior to analysis.

(C) Net1 inhibits Cdc14 phosphatase activity in vitro. Extracts of *myc9-NET1* cells were immunoprecipitated with 9E10 antibodies, and protein A beads were coated with anti-GST antibodies to prepare Net1 and α-GST beads, respectively. Both matrices were washed extensively and incubated with wild-type or mutant GST-Cdc14 purified from *E. coli* (20 ng for GST beads and 60 ng for Net1 beads). The specific activity of bead-bound GST-Cdc14 was evaluated as described above using ^{32}P-Sic1 as the substrate. Levels of GST-Cdc14 recruited to the Net1 and α-GST beads were assessed by immunoblotting with α-GST antibodies. The activity of mutant Cdc14 bound to the α-GST beads was used for background correction. For simplicity, in (B) and (C) only the final values (^{32}P-Sic1 dephosphorylation divided by relative amount of Cdc14 antigen) are shown.

(D and E) Net1 is a candidate substrate for Cdc14. (D) Net1 is a phosphoprotein in vivo. *myc9-NET1* (lane 1) and untagged (lane 2) strains were labeled with ^{32}P-inorganic phosphate. Extracts of labeled cells were immunoprecipitated with 9E10 antibodies, and recovered proteins were fractionated by SDS-PAGE and detected by autoradiography. (E) Net1 can be dephosphorylated by Cdc14 in vitro. *CDC14 myc9-NET1* (+, lanes 1 and 2) and *cdc14-1 myc9-NET1* (*cdc14-1*, lanes 3–6) cells were grown at 25°C or shifted to 37°C for 3 hr, as indicated. Myc9-Net1 was immunoprecipitated from cell extracts with 9E10 antibodies, and portions of the Net1 immunoprecipitate from the 37°C *cdc14-1* culture were either left untreated (lane 4) or incubated with wild-type (A; lane 5) or C283S mutant (I; lane 6) GST-Cdc14 for 60 min at 30°C. All immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with 9E10 to detect Myc9-Net1.

immunoblotting of anti-*myc* immunoprecipitates revealed that Myc9-Net1 isolated from a 37°C *cdc14-1* culture (nonpermissive temperature) migrated slower in SDS-PAGE than that from a 25°C culture (permissive temperature; Figure 4E, lane 3 vs. lane 4). In contrast, no effect of temperature was observed for Myc9-Net1 isolated from wild-type cells (Figure 4E, lanes 1 and 2). The slower migrating form of Myc9-Net1 from the 37°C *cdc14-1* culture was collapsed down to a faster migrating form upon treatment with wild type (A) but not mutant (I) GST-Cdc14 purified from *E. coli* (Figure 4E, lanes 5 and 6). Since Cdc14 modulates the phosphorylation state of Net1 in vivo and in vitro, we conclude that Net1 is most likely one of its physiological substrates. In the Discussion, we consider the possibility that Net1 and Cdc14 reciprocally control each others' activities via a negative feedback loop.

Net1 Resides in the Nucleolus and Is Required for Cdc14 Localization to the Nucleolus

Both Net1 (Straight et al., 1999) and a portion of Sir2 (Gotta et al., 1997) localize to the nucleolus. These data suggest that the entire RENT complex, including Cdc14, might be tethered to the nucleolus. To test this possibility, we compared the localization of Myc9-Net1 and Cdc14-HA3 by indirect immunofluorescence. Myc9-Net1 displayed a crescent-shaped staining pattern characteristic of nucleolar localization (Figure 5A, middle row). A similar pattern was observed for Cdc14-HA3 (Figure 5A, bottom row). To confirm that both proteins do in fact localize to the nucleolus, we probed cells with antibodies directed against the relevant epitope tag and antibodies directed against the RNA polymerase 1 A190 subunit (RPA190), a nucleolar marker (Oakes et al., 1998). A striking overlap in the antibody staining patterns was observed (Figure 5A), indicating that both Net1 and Cdc14 localize to the nucleolus.

Net1 was required for localization of Sir2 to the nucleolus (Straight et al., 1999), which prompted us to examine if Net1 was also required for the nucleolar localization of Cdc14. Cdc14 localization was visualized in wild-type, *net1-1*, and *net1*Δ cells. In wild-type and *net1-1* cells, Cdc14 assumed a sharp nucleolar staining pattern (Figure 5B, center rows). However, in *net1*Δ cells, Cdc14 was diffused throughout the entire cell (Figures 5B and 5C, bottom rows). Although there was 4-fold less Cdc14 protein in *net1*Δ cells (Figure 5D), the level of delocalized Cdc14 staining was clearly greater than that seen in *CDC14-HA3 NET1* cells or in cells that lacked tagged *CDC14*. These data suggest that Net1 anchors the RENT complex to the nucleolus. The significance of the nucleolar localization of Cdc14 in *net1-1* is considered in the Discussion.

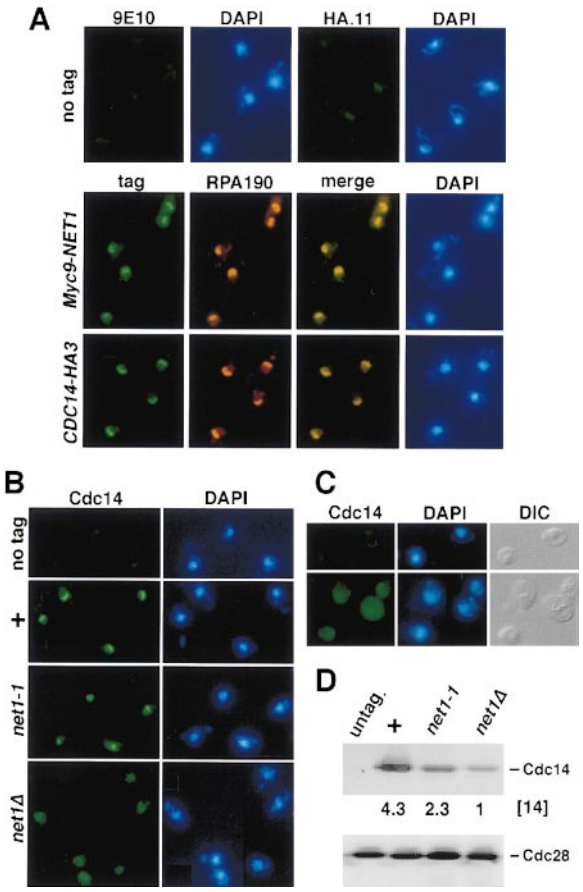


Figure 5. Net1-Dependent Localization of Cdc14 to the Nucleolus

(A) Net1 and Cdc14 both localize to the nucleolus. Top row: untagged wild-type cells were probed with 9E10 antibodies or anti-hemagglutinin monoclonal antibody HA.11 plus the DNA-binding dye DAPI. Center and bottom rows: cells with the indicated genotype were probed with monoclonal antibodies (9E10 or HA.11, column 1) and rabbit anti-RPA 190 antibodies (column 2). The third column shows a merge of the two antibody staining patterns in columns 1 and 2. The fourth column shows the position of nuclei, as revealed by DAPI staining.

(B) Untagged control (top row) and *CDC14-HA3* cells carrying *NET1*, *net1-1*, or *net1*Δ alleles (rows 2–4, respectively) were stained with HA.11 antibodies (column 1) and DAPI (column 2).

(C) Untagged control (top row) and *net1*Δ *CDC14-HA3* (bottom row) cells were stained with HA.11 antibodies (column 1) and DAPI (column 2). DIC images are shown in column 3 to reveal the outline of cells.

(D) The levels of Cdc14-HA3 and Cdc28 proteins in the indicated strains were evaluated by immunoblotting with 12CA5 antibodies. The relative levels of Cdc14-HA3 ([14]) are indicated below the top panel.

Cdc14 Is Released from the Nucleolus during Progression through Mitosis

If Net1 sequesters the bulk of Cdc14 in the nucleolus in a presumably inactive form, how does Cdc14 fulfill its essential role in promoting mitotic exit? To resolve this puzzle, we looked more carefully at the localization of Net1 and Cdc14 during the cell cycle. Asynchronous populations of cells expressing Myc9-Net1 and Cdc14-HA3 were probed with antibodies directed against the relevant epitope tags and with anti-tubulin antibodies to visualize the mitotic spindle. Cells in late anaphase and telophase contain elongated spindles, whereas cells in other stages exhibit either focal tubulin staining (G1 and early S phase cells) or a short spindle (S phase and preanaphase cells). Like the well-characterized nucleolar protein Nop1 (Aris and Blobel, 1988), Net1 exhibited a characteristic nucleolar staining pattern throughout the cell cycle (Figure 6A, upper and middle rows; Straight et al., 1999). Intriguingly, the localization of Cdc14 varied as a function of the cell cycle (Figures 6A, bottom row, and 6B): in cells with short spindles, Cdc14 was localized to the nucleolus (Figure 6B, bottom row). However, in late anaphase/telophase cells with long spindles, Cdc14 was diffused throughout the nucleus (Figure 6B, middle row) or the entire cell (Figure 6B, top row; note the diffuse cytoplasmic staining that extends beyond the nucleus).

To evaluate more rigorously the relationship between long spindles and delocalized Cdc14, we carried out a cell cycle block-release experiment. *myc9-NET1 CDC14-HA3* cells were synchronized in G1 with α factor, and at various time points after release, samples were processed for indirect immunofluorescence. The percentage of cells with long mitotic spindles, delocalized Cdc14-HA3, and delocalized Myc9-Net1 were plotted as a function of time. Although Myc9-Net1 remained localized to the nucleolus throughout the cell cycle, release of Cdc14-HA3 from the nucleolus mirrored the appearance of long spindles (Figure 6C).

The Release of Cdc14 from Nucleolus Requires Tem1

An intriguing possibility is that *tem1* cells fail to exit mitosis due to a failure to dislodge Cdc14 from its Net1 tether in the nucleolus. To test this hypothesis, we examined whether the release of Cdc14 from the nucleolus requires Tem1. *CDC14-HA3 tem1Δ::GAL1-UPL-TEM1* cells were either maintained in YPG (*TEM1* expressed) as an exponentially growing culture or were released from α factor–induced G1 arrest into YPD (*TEM1* repressed) to yield a synchronous population of cells arrested in late anaphase/telophase due to a deficit of Tem1 activity. In cells grown in YPG, Cdc14 was largely delocalized in 92% of cells containing a long mitotic spindle (Figure 7B), whereas the nucleolar antigen RPA190 retained a distinctive nucleolar staining pattern (Figure 7A, top row). In contrast, in cells that were arrested in late anaphase/telophase by depletion of Tem1, Cdc14 (Figure 7A, bottom row) and the nucleolar marker RPA190 (data not shown) displayed overlapping focal staining patterns in the vast majority (93%) of cells with long spindles (Figure 7B).

A simple view would argue that *net1-1* bypasses *tem1*Δ by causing ectopic release of Cdc14 from the nucleolus. Surprisingly, Cdc14-HA3 appears correctly localized in *net1-1* cells (Figure 5B). However, it is important to note that the bypass activity of *net1-1* would not necessarily require constitutive delocalization of Cdc14. To test whether Tem1-independent release of Cdc14 from the nucleolus occurred in mitotic cells, we compared Cdc14-HA3 localization in Tem1-depleted *net1-1* and *NET1* cells. Whereas Cdc14-HA3 was localized to the nucleolus in $>$90% of Tem1-deficient *NET1* cells that contained a long spindle, it was delocalized in $\sim$40% of the equivalently staged Tem1-depleted *net1-1* cells

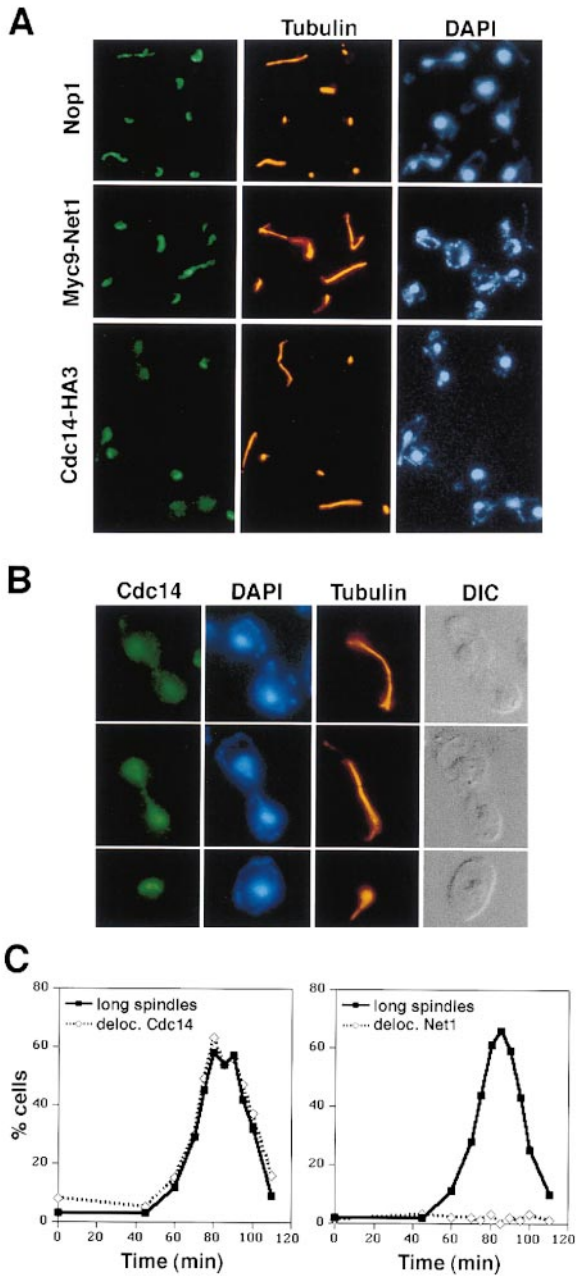
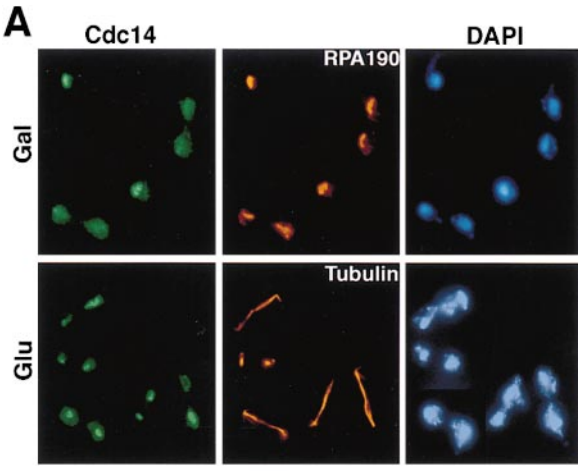


Figure 6. Cdc14 Is Released from the Nucleolus during Anaphase/Telophase

(A) Asynchronous *myc9-NET1 CDC14-HA3* cells were double labeled with rabbit anti-tubulin antibodies (column 2) and one of the following mouse antibodies (column 1): anti-Nop1 (top panel), 9E10 (center panel), or HA.11 (bottom panel). The positions of nuclei, as revealed by DAPI staining, are shown in Column 3.

(B) *Cdc14-myc9/CDC14* diploid cells were probed with 9E10, DAPI, and anti-tubulin antibodies (columns 1–3, respectively). DIC images are shown in column 4. Cdc14 exhibits three different types of staining patterns: delocalized over the entire nucleus (center row), the entire cell (top row) during anaphase/telophase, or restricted to the nucleolus (bottom row) during interphase. Note the absence of extranucleolar Cdc14 in the G1 cell shown in the bottom row.

(C) *myc9-NET1 CDC14-HA3* cells were synchronized with α factor and then released into YPD at time = 0. Samples withdrawn and fixed at the indicated time points were double labeled with anti-tubulin and either 9E10 (to detect Myc9-Net1) or HA.11 (to detect Cdc14-HA3) antibodies. For each sample, more than 200 cells were counted to calculate the percentage of cells with delocalized Cdc14 or Net1 and the percentage of cells with long spindles.



B

Strain	Growth Condition	% long spindles w/ focal Cdc14 1 focus	2 foci	Total
tem1Δ::GAL1-UPL-TEM1 +	Gal	1.9	5.8	7.7
tem1Δ::GAL1-UPL-TEM1 +	Glu	15.1	77.4	92.5
net1-1	Glu	5.6	55.5	61.1

Figure 7. Release of Cdc14 from the Nucleolus Requires Tem1

(A) *tem1Δ::GAL1-UPL-TEM1 CDC14-HA3* cells grown in YPG (*TEM1* expressed) were either sampled directly (top panel) or arrested in G1 phase with α factor and subsequently released into YPD (*TEM1* repressed) for 3 hr (bottom panel). Cells were double labeled with HA.11 to visualize Cdc14-HA3 (column 1) and either anti-RPA190 or anti-tubulin antibodies to visualize nucleoli or mitotic spindles, respectively (column 2).

(B) A similar block/release protocol was conducted with a *tem1Δ::GAL1-UPL-TEM1 CDC14-HA3 net1-1* strain, except that samples were processed for indirect immunofluorescence 4–6 hr after release from α factor arrest. A longer release time was required for *net1-1* strains to progress from G1 to anaphase/telophase due to the ~2-fold longer doubling time of this mutant (W. S., data not shown). The percentages of cells with long spindles and focal Cdc14-HA3 staining were quantitated for this sample as well as the samples from (A). More than 50 cells were counted for each sample.

(Figure 7B). These results argue that Tem1 is normally required for release of Cdc14 from the nucleolus during anaphase/telophase and that *net1-1* allows ectopic release of Cdc14 (from at least a fraction of cells) during late mitosis, even in the absence of *TEM1* function.

Discussion

RENT Control: A Model for the Control of Mitotic Exit in Budding Yeast

Tem1, Cdc15, and Cdc14 are required for mitotic exit. These proteins have been implicated in ensuring a rapid and irreversible drop in mitotic cyclin/Cdk activity at the end of mitosis by promoting the proteolysis of the mitotic cyclin Clb2 and the accumulation of the cyclin/Cdk inhibitor Sic1. Despite the appeal of this scenario, it has remained unclear how the activities of these proteins are mobilized at the end of mitosis.

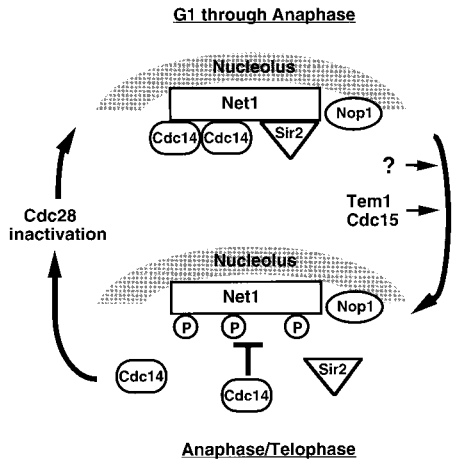


Figure 8. The "RENT Control" Hypothesis
See text for details.

Based on the data presented here, we propose the following model (Figure 8). Throughout G1, S, and early M phase, a pool of Cdc14 is confined to the nucleolus as a subunit of the RENT complex (which also contains Net1 and Sir2). As cells proceed through anaphase, Tem1-dependent signaling impinges on the RENT complex, enabling the release of Cdc14 (and a portion of Sir2; Straight et al., 1999) from Net1. Unfettered Cdc14 diffuses into the nucleus and cytoplasm, where it modulates the phosphorylation state of proteins involved in the proteolysis of Clb2 (Hct1) and transcription of *SIC1* (Swi5) (Visintin et al., 1998; Jaspersen et al., 1999). The RENT complex then reassembles once Clb/Cdk activity is quenched and Tem1-dependent signaling has been terminated, possibly via a negative feedback loop that involves Cdc14-dependent dephosphorylation of Net1.

We present five major lines of evidence in support of this model. First, Net1 and Cdc14 assemble into a complex, and Net1 is required to anchor Cdc14 to the nucleolus throughout interphase. Second, binding of Net1 to Cdc14 inhibits the protein phosphatase activity of Cdc14 in vitro, and *net1* mutants display elevated Cdc14 protein phosphatase activity. Third, Cdc14 is released from its inhibitory nucleolar tether as wild-type cells proceed through mitosis. Fourth, Cdc14 is not released from the nucleolus in cells arrested in late mitosis due to deficiency of *TEM1* function. Fifth, recessive mutations in *NET1* bypass the essential requirement of Tem1 for (1) release of Cdc14 from the nucleolus during late mitosis, (2) destruction of Clb2 and accumulation of Sic1 during late mitosis, and (3) growth.

The "RENT control" model opens up several key questions regarding the function and regulation of RENT. What is the nature of the stimulus that initiates Tem1-dependent signaling? What are the proximal biochemical events that trigger the release of Cdc14 from RENT? How is the RENT complex reestablished as cells return to G1 phase? What functions does the RENT complex perform in the regulation of nucleolar processes? Identification of the dynamic RENT complex provides a focus for exploring the molecular events that culminate in the release of active Cdc14 during anaphase/telophase.

Net1 Is a Multifunctional Protein

Affinity purification of Net1 revealed at least two binding partners—Sir2 and Cdc14—that possess disparate cellular functions. Net1 also appears to associate with the essential nucleolar protein Ypl126w (Nan1), but we do not know whether Nan1 is a component of the RENT complex or part of a distinct Net1-containing complex. Sir2 mediates the silencing of RNA polymerase II–dependent transcription in specific regions of the genome (the rDNA locus, the mating type loci, and telomeres) (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997; reviewed by Lustig, 1998). Consistent with the company it keeps, Net1 also participates in the silencing of RNA polymerase II–dependent transcription in the nucleolus (but not at the telomeres or silent mating loci) by tethering Sir2 to rDNA (Straight et al., 1999). In addition, *net1* mutations cause partial delocalization of the nucleolar protein Nop1 and alter the structure of the nucleolus (Straight et al., 1999; W. S. and R. J. D., unpublished data). The nucleolar and cell cycle functions of Net1 are at least partially separable: unlike *net1-1*, *sir2*Δ was not able to bypass *cdc15*Δ, does not cause growth defects, and has no obvious effect on the ultrastructure of the nucleolus (Ivy et al., 1986). Taken together, these observations suggest that the nucleolar functions of Net1 extend beyond its ability to recruit Sir2. Point mutations that specifically disrupt one function at a time will be of great value in dissecting the activities of this multifaceted protein.

On the Relationship between Net1 and Cdc14

Net1 inhibits Cdc14 by two independent mechanisms. First, it physically assembles with Cdc14 and inhibits its protein phosphatase activity, as evidenced by the fact that the specific activity of Cdc14 is elevated in *net1* mutants and Cdc14 recruited to Net1-containing beads is inactive. Multiple protein kinase inhibitors (e.g., p16, p21, p27; reviewed in Xiong, 1996) and serine/threonine protein phosphatase inhibitors (reviewed in Shenolikar, 1995) have been reported. To our knowledge, Net1 is the first known protein inhibitor of a member of the protein tyrosine phosphatase family that does not work by a dominant-negative mechanism. It will be interesting to determine whether human Cdc14 homologs (Li et al., 1997; H. C., unpublished data) and other members of the dual-specificity protein phosphatase family are regulated by Net1-like molecules.

As described in the previous section, Net1 also tethers Cdc14 to the nucleolus from G1 until late mitosis. Whereas Cdc14 is completely delocalized in *net1*Δ cells at all stages of the cell cycle, a nucleolar pool of Cdc14 persists in *net1-1* mutants during interphase. However, Cdc14 is released from the nucleolus in ~40% of Tem1-deficient *net1-1* cells in late anaphase/telophase but is released in only 8% of the equivalent Tem1-deficient *NET1* cells. These observations are consistent with the notion that release of Cdc14 from its nucleolar bonds requires the action of two independent signals: an unknown signal and the Tem1-dependent signal, both of which are active only during mitosis (Figure 8). We suggest that *net1-1* relieves the requirement for the latter but not the former, such that Cdc14 is still localized to the nucleolus in Tem1-depleted *net1-1* cells during

interphase but is delocalized once they proceed through mitosis.

The specific activity of Cdc14 is elevated in *net1-1* to about the same extent as in *net1*Δ, although the majority of Cdc14 is associated with the nucleolus in *net1-1* but not *net1*Δ cells. One explanation for this discrepancy is that the enfeebled Net1-1/Cdc14 protein complex is not stable under our immunoprecipitation conditions such that *net1-1* behaves like a null allele in this assay. Alternatively, the Net1-1 protein could have lost its inhibitory activity, but not its binding affinity, toward Cdc14. It is interesting to note that the level of Cdc14 is reduced in *net1-1* and further reduced in *net1*Δ, suggesting that Cdc14 bound to Net1 is more stable than the released protein. Degradation of free Cdc14 may contribute to the extinction of Cdc14 activity once the exit from mitosis is completed.

Intriguingly, we found that Net1 is a phosphoprotein and can be dephosphorylated by Cdc14. This raises the possibility that there is a negative feedback loop connecting Net1 and Cdc14. We suggest the following scenario. From G1 to anaphase, hypophosphorylated Net1 binds to and sequesters Cdc14 in the nucleolus. In anaphase/telophase, Tem1-dependent signaling activates a Net1 kinase, which in turn hyperphosphorylates Net1, thereby stimulating Cdc14 release. As cells enter G1, Tem1 signaling ceases, the Net1 kinase becomes inactive, and Cdc14 dephosphorylates Net1. The dephosphorylated Net1 subsequently binds Cdc14, thereby resetting Cdc14 activity to the ground state (Figure 8).

Although Net1 influences cell cycle progression by sequestering Cdc14 in the nucleolus, it is not yet clear whether Cdc14 contributes to the assembly or function of this organelle. *cdc14ts* but not *cdc15ts* mutants fail to completely segregate the nucleolar antigen Nop1 (Granot and Snyder, 1991) and the rDNA-bound proteins Net1 and Sir2 (Straight et al., 1999), suggesting that Cdc14 might contribute to the organization or segregation of nucleolar chromatin.

Net1 Provides a Missing Link between Tem1/Cdc15 and Cdc14

Exit from mitosis in *S. cerevisiae* involves an elaborate signaling network consisting of at least seven essential genes: *CDC5*, *CDC14*, *CDC15*, *DBF2/DBF20*, *LTE1*, *MOB1*, and *TEM1*. Since overexpression of the GTP-binding protein Tem1 bypasses the essential requirement (at low temperature) for the nucleotide exchange factor Lte1, and overexpression of the protein kinase Cdc15 bypasses the essential requirement for Tem1 (Shirayama et al., 1994b), it is likely that Lte1 activates Tem1, which in turn activates Cdc15. In support of this model, the *S. pombe* Cdc15 homolog Cdc7 binds to and is genetically downstream of *S. pombe* Tem1 homolog Spg1 (Schmidt et al., 1997). Since the recessive *net1-1* mutation bypasses *cdc15*Δ and *tem1*Δ, *NET1* formally behaves as a mitotic exit inhibitor acting downstream of or parallel to *TEM1* and *CDC15* (W. S. and R. J. D., in preparation). Based on our observation that Net1 binds to and inhibits Cdc14, the most parsimonious model is as follows. During progression through mitosis, an unknown signal activates Lte1, which in turn activates Tem1. Tem1 then activates Cdc15, which in turn relieves the inhibition of Cdc14 by Net1. Although we have yet to test this directly, we suggest that Cdc5, Dbf2/Dbf20, and Mob1 are also required for the release of Cdc14 from RENT. Active Cdc14 then promotes Clb2 degradation and Sic1 accumulation (Visintin et al., 1998; Jaspersen et al., 1999; W. Reynolds, W. S., and R. J. D., unpublished data), which shuts off Cdc28 activity.

Nucleolus as a Sequestration Center?

The nucleolus is a discrete subnuclear area for ribosomal RNA synthesis and preribosome assembly (reviewed in Shaw and Jordan, 1995). Some nucleolar proteins—including nucleolin, B23, and Nopp140—were initially shown to localize to the nucleolus by immunofluorescence and later shown to shuttle between the nucleolus and the cytoplasm (reviewed in Shaw and Jordan, 1995). Therefore, it is possible that Net1 also shuttles between the nucleolus and the cytoplasm to ferry cytoplasmic and nuclear Cdc14 to the nucleolus.

Cdc14 is localized to the nucleolus at times during the cell cycle when it is not needed and is released from the nucleolus when its activity is required for cell cycle progression. Might the nucleolus serve an unappreciated role as a "sequestration center" for proteins that are to be kept inactive? Interestingly, a $p34^{cdc2}$ homolog has been shown to localize to the nucleoli of neurons and glia in the mitotically quiescent murine central and peripheral nervous systems (Ino et al., 1993). This notion raises the possibility that some of the many proteins that have been localized to the nucleolus may not act solely (or even primarily) in the nucleolus but may be stockpiled there in anticipation of their eventual release. It is not clear how many other proteins might be regulated by nucleolar sequestration; only future research can reveal the generality of this mode of regulation.

Experimental Procedures

Yeast Strain Constructions

All yeast strains used in this study are in the W303 background (*can1-100*, *leu2-3*, *-112*, *his3-11*, *-15*, *trp1-1*, *ura3-1*, *ade2-1*). The pRS vector series was described previously (Sikorski and Hieter, 1989). The details of plasmid and strain construction are available upon request. To obtain *tem1*Δ*::TRP1 [GAL1-TEM1, URA3]* (RJD1017), the diploid strain RJD1005 (*tem1*Δ*::TRP1/TEM1, MATa/*α) was transformed with pWS102 *[pRS316: GAL1-TEM1, URA3]* and dissected. To construct *GAL1-UPL-TEM1*, the UPL degron of the UFD (ubiquitin fusion degradation) pathway (Johnson et al., 1992) was amplified by PCR and inserted upstream of *TEM1* in pWS102. The *GAL1-UPL-TEM1* cassette was then excised and cloned into pRS304 (TRP1), which was subsequently digested with both BglII and KpnI, blunt ended with Klenow, and self-ligated to generate pWS103 (*[GAL1-UPL-tem1*Δ*C/TRP1]*). pWS103 was linearized with EcoRI to direct integration at the *TEM1* locus, yielding WY46 (*tem1*Δ*::GAL1-UPL-TEM1, bar1::hisG, MATa*). To construct *net1::myc9-NET1/LEU2* (WY53), pWS104 (containing nucleotides [NT] −293→+3 and +4→+844 of *NET1* flanking Myc9) was linearized with Bsg1 and integrated into a wild-type strain. To construct *cdc14::CDC14-Flag-His6-HA3/HIS3* strain (RJD 1191), pGJR (pRS315 containing NT 1336–1653 of the *CDC14* ORF, followed by Flag-His6-HA3, followed by NT 1654–2095 of *CDC14* 3′ untranslated region) was linearized with EcoRV and transformed into a wild-type strain. To delete *SIR* genes, strains were transformed with *sir*Δ*::LEU2* plasmids (Ivy et al., 1986), and correct transformants were identified based on their sterility. The *NET1* locus was replaced by *S. pombe his5*$^{+}$ using the PCR amplification/transformation method described

previously (Wach et al., 1997). Correct integrants were verified by PCR, sporulated, and dissected (WY 69 and 70).

Cell Growth, Synchronization Procedures, and In Vivo Labeling

Cells were grown on 1% yeast extract/2% peptone (YP) + 2% glucose (YPD) or YP + 2% galactose (YPG) medium. 5-FOA plates were prepared as described (Sikorski and Boeke, 1991). To perform *GAL1-UPL-TEM1* shut-off experiments, exponential phase cells grown in YPG were arrested with α factor (10 μg/ml for *BAR1* and 0.1 μg/ml for *bar1*Δ strains) and released into YPD. In some cases, α factor was added back a few hours after release to trap cells in the subsequent G1 phase. To label cells with [^{32}P]phosphate, 5×10^8 exponential phase cells grown in phosphate-free YPD (Warner, 1991) were resuspended in 2.5 ml of phosphate-free YPD and labeled with 0.3 mCi [^{32}P]phosphate (ICN) for 45 min at room temperature. Cells were washed with ice-cold water and harvested. To arrest cells at *cdc* blocks, cells were shifted to 37°C for 2.5–3.5 hr until $\geq$90% cells were arrested.

Cell Extract Preparation, Immunoprecipitation, and Western Blot

To detect proteins by immunoblotting, 200 μl of SDS sample buffer was added to 8×10^7 cells, vortexed for 2 s, and boiled for 3 min. One hundred microliters of acid-washed glass beads (0.5 mm) was added and the mixture was vortexed for 2 min. The samples were boiled again for 2 min, and 8 μl of samples was loaded on SDS-polyacrylamide gels. To prepare extracts for immunoprecipitation (IP), $4–6 \times 10^8$ cells were pelleted, washed once with HBS (20 mM HEPES [pH 7.2], 150 mM NaCl), and resuspended in 200 μl of lysis buffer (HBS + 2 mM dithiothreitol [DTT], 0.2% Triton, and protease inhibitors) (Shou and Dunphy, 1996). Glass beads (200 μl) were added, and cells were lysed by 12 cycles of vortexing for 30 s followed by a 1 min incubation on ice. Extracts were clarified by two consecutive centrifugations (14,000 g for 5 min). Protein concentration of extracts was typically 5–10 mg/ml. Extracts (80–150 μl) were incubated with 1 μl of 12CA5 (for HA-tagged protein) or 9E10 (for Myc-tagged protein) monoclonal antibodies on ice (1 hr), supplemented with 15–20 μl of either protein A (12CA5 antibodies) or anti-mouse IgG beads (9E10 antibodies), and incubated on a rotator at 4°C for 1 hr. Beads were then washed extensively with lysis buffer lacking protease inhibitors. One-third to one-fourth of the washed immunoprecipitates were subjected to SDS-PAGE and immunoblotting as described (Harlow and Lane, 1988). In the experiment described in Figure 4C, 2 mM Na_3VO_4 was included in the Myc9-Net1 IP reaction (but not the wash buffers) to inhibit the endogenous Cdc14 from binding to Net1 in the extracts. To prepare anti-GST beads, 20 μl of protein A beads was incubated with 1 μl of anti-GST antibodies and washed after 1 hr incubation. Western blot detection relied upon either ^{125}I-labeled secondary antibodies (ICN) or HRP-conjugated secondary antibodies followed by ECL+ (Amersham). The blots were scanned and quantitated using Molecular Dynamics STORM system.

Protein Identification by Mass Spectrometry

Protein bands were excised from a single silver-stained SDS-polyacrylamide gel, reduced with dithiothreitol, alkylated with iodoacetamide, and in gel digested with trypsin (37°C, overnight) as described previously (Shevchenko et al., 1996b). An aliquot (0.3–0.5 μL) of the digest was withdrawn and analyzed by high mass accuracy MALDI peptide mapping on a modified REFLEX mass spectrometer (Bruker Daltonics, Bremen, Germany) essentially as described previously (Shevchenko et al., 1996b). If MALDI peptide mass mapping could not provide a conclusive identification of the protein, the gel pieces were further extracted with 5% formic acid and acetonitrile. The extracts were pooled and lyophilized, and the unseparated mixture of tryptic peptides was sequenced by nanoelectrospray tandem mass spectrometry on an API III triple quadruple instrument (PE Sciex, Concord, ON, Canada) as described previously (Wilm et al., 1996). Database searches were performed against a comprehensive nonredundant protein sequence database using PeptideSearch V. 3.0 software developed in the European Molecular Biology Laboratory. No limitations of protein molecular weight and species of origin were imposed.

Detection of Phospho-Net1 and Treatment with Cdc14

To retrieve ^{32}P-Net1, *myc9-NET1* cells were labeled with [^{32}P]phosphate and disrupted with glass beads in lysis buffer supplemented with phosphatase inhibitors as described (Verma et al., 1997), and clarified extracts were immunoprecipitated with 9E10 antibodies. To assay if phospho-Net1 might be a substrate for GST-Cdc14, GST-Cdc14 and catalytically inactive GST-Cdc14 (C283S) were purified from *E. coli* as described (Taylor et al., 1997). Myc9-Net1 was immunoprecipitated in the presence of phosphatase inhibitors (excluding Na_3VO_4) and washed extensively with the final wash being Cdc14 buffer (50 mM imidazole [pH 6.6], 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 25 μg/ml aprotinin, 10 μg/ml PLC). Beads were incubated with 1.2 μg of GST-Cdc14 (wild type or mutant) in 50 μl of Cdc14 buffer at 30°C for 1 hr.

Cdc14 Activity Assay

MBP-Sic1-MycHis6 (10 μg) was phosphorylated by GST-Cdc28/Cln2/Cks1/Cak1 kinase in the presence of [γ-^{32}P]ATP (Skowyra et al., 1997), retrieved from the kinase reaction on Ni-NTA resin (Qiagen), and eluted in 120 μl of 200–250 mM imidazole (pH 7.5). To assay bead-bound Cdc14 activity, the beads were incubated with 30 μl of Cdc14 buffer containing 1 μl of ^{32}P-Sic1 and rotated at 22°C for 18–20 min (or 18°C for 15 min; Figure 4C). Control experiments indicated that, at most, 40%–50% of the input substrate was dephosphorylated under these conditions. The supernatant was removed, and the beads were washed two times with 30 μl HBS + 0.1% Triton X-100. The washes were pooled with the supernatant, adjusted to 10% trichloroacetic acid, incubated for 30 min on ice, and centrifuged for 10 min at 14,000 g. Soluble radioactivity was measured by scintillation counting.

Immunofluorescence

Immunofluorescence was carried out essentially as described previously (Pringle et al., 1991). Cells were fixed with 4.5% formaldehyde at room temperature for 0.5 hr for anti-RPA190 and 1 hr for all other antibodies. Primary antibodies included 1:1,000 mouse monoclonal antibodies HA.11 and 9E10 (Babco), 1:1,000 rabbit anti-RPA190, 1:2,000 rabbit-anti-tubulin antibodies, and 1:12,000 mouse-anti-Nop1. Secondary antibodies included 1:1,000 rhodamine-conjugated-donkey-anti-rabbit antibodies (Jackson Lab) and 1:1,000 fluorescein-conjugated-goat-anti-mouse antibodies (Cappel). Images were captured on a Zeiss Axioskop microscope using Fujichrome Provia 400 slide film.

Acknowledgments

W. S. dedicates this paper to her parents, who have supported her unconditionally. We thank D. Kellogg, M. Nomura, F. Solomon, and J. Aris for anti-Clb2, anti-RPA190, anti-tubulin, and anti-Nop1 antibodies, respectively; A. Varshavsky, K. Nasmyth, P. Philippsen, and J. Hicks for the UPL, Myc9, *S. pombe his5*$^+$, and *sir*Δ*::LEU2* plasmids, respectively; and R. Feldman and R. Verma for the MBP-Sic1 and Sic1 kinase. We thank G. Reynard for constructing CDC14-HA3; J. Archer for sharing immunofluorescence expertise; and R. Verma, R. Feldman, W. Dunphy, and H. Rao for critically reading the manuscript. W. S. thanks B. Hay for insight and encouragement and D. Harrington for assistance with graphics. W. S. is a Howard Hughes Medical Institute predoctoral fellow. H. C. was supported by NIH grant CA59935, and R. J. D. was supported by Junior Investigator Awards from the Searle/Chicago Community Trust and the Beckman Foundation.

Received February 9, 1999; revised March 25, 1999.

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