Net1 Stimulates RNA Polymerase I Transcription and Regulates Nucleolar Structure Independently of Controlling Mitotic Exit

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Summary
The budding yeast RENT complex, consisting of at least three proteins (Net1, Cdc14, Sir2), is anchored to the nucleolus by Net1. RENT controls mitotic exit, nucleolar silencing, and nucleolar localization of Net1. Here, we report two new functions of Net1. First, Net1 directly binds Pol I and stimulates rRNA synthesis both in vitro and in vivo. Second, Net1 modulates nucleolar structure by regulating rDNA morphology and proper localization of multiple nucleolar antigens, including Pol I. Importantly, we show that the nucleolar and previously described cell cycle functions of the RENT complex can be uncoupled by a dominant mutant allele of CDC14. The independent functions of Net1 link a key event in the cell cycle to nucleolar processes that are fundamental to cell growth.

Introduction
Unexpected links between the nucleolus and the cell cycle have been uncovered (reviewed by García and Pillus, 1999; Cockell and Gasser, 1999). In the budding yeast Saccharomyces cerevisiae, the RENT protein complex regulates mitotic exit, nucleolar silencing, and Nop1 localization through its three known components: Cdc14, Sir2, and Net1 (also known as Cfi1; Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999).

The yeast nucleolus, which contains ~150 consecutive repeats of the rDNA, is the center for ribosomal RNA synthesis and ribosome assembly (reviewed in Shaw and Jordan, 1999). The chromatin structure in the nucleolus's silences transcription by RNA polymerase II without interfering with highly active transcription by RNA polymerase I (Pol I; reviewed in Guarante, 2000). This restriction, termed "nucleolar silencing," may be the consequence of a mechanism that evolved to suppress recombination among rDNA repeats and thereby reduce the production of RNA circles that have been shown to cause cellular senescence (reviewed in Guarante, 2000). The RENT complex, tethered to the nucleolus by its core subunit Net1, influences the nucleolus in at least two ways. First, both Net1 and Sir2 are essential for nucleolar silencing, and Net1 influences rDNA chromatin in part by tethering Sir2 to the nucleolus (Straight et al., 1999). Sir2 in turn mediates silencing, suppression of recombination, and extension of longevity through its NAD-dependent histone deacetylase activity (reviewed by Gottschling, 2000; Guarante, 2000) and/or other mechanisms such as NAD breakdown and generation of O-acetyl-ADP-ribose (Tanner et al., 2000; Tanny and Moazed, 2000). Second, Net1 is required to maintain nucleolar localization of Nop1 (Straight et al., 1999), a protein implicated in pre-rRNA processing, methylation, and ribosome assembly (Tollervey et al., 1999). It is not known whether defective localization of Nop1 in net1 cells is specific or whether it results from a general perturbation of nucleolar structure. Despite the key role of the nucleolus in cellular metabolism, the molecular basis for nucleolar assembly/organization and the role of Net1 in this process remain mysterious.

Besides its role in nucleolar processes, the RENT complex also controls mitotic exit. When cells exit mitosis, the mitotic exit network (MEN) triggers accumulation of the Cdk inhibitor Sic1 and degradation of B-type mitotic cyclins (Cibs) and consequently inactivation of mitotic Cdc28 (reviewed by Zachariae and Nasmyth, 1999). Members of the MEN include Tem1 (a GTP binding protein), Lte1 (a putative guanine nucleotide releasing factor), Cdc14 (a protein phosphatase), Cdc15 (a kinase), Db2/Db120 (kinases), Cdc5 (a kinase), Mob1 (a protein that binds to Db2), and Nud1 (reviewed in Morgan, 1999; Gruneberg et al., 2000). When cells harboring conditional-lethal temperature sensitive (ts) mutations in any of the MEN genes are shifted to the restrictive temperature, they uniformly arrest in late anaphase as large budded cells with segregated chromosomes, elongated spindles, and in all tested cases, elevated Cdc28 activity.

To address how the MEN is organized and regulated, we previously sought telophase arrest bypassed (tab) mutants that alleviate the essential requirement for CDC15 and TEM1. One of these mutants, tab2-1 (net1-1), enabled
Clb2 degradation and Sic1 accumulation even in the absence of Tem1 (Shou et al., 1999). Further characterization of Net1 led us and others to propose the “RENT control” hypothesis: Throughout most of the cell cycle, Net1 sequesters Cdc14 in the nucleolus and inhibits its phosphatase activity. As cells exit mitosis, a TEM1/ CDC15-dependent signal leads to disassembly of the RENT complex, causing Cdc14 and Sir2 to vacate the nucleolus (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). The evicted Cdc14 subsequently catalyzes Cdc28 inactivation (Visintin et al., 1998; Jaspersen et al., 1999). However, it is unknown whether release of Cdc14 from the nucleolus is sufficient to bypass cdc15Δ and temΔ.

In this study, we describe new functions of Net1 in the nucleolus: It directly activates synthesis of rRNA by RNA Pol I and plays a general role in regulating nucleolar structure. The perturbed nucleolar structure in net1-1 cells raises the possibility that mislocalized nucleolar proteins other than Cdc14 could cause or contribute to the cdc15Δ bypass phenotype. Characterization of a second tab locus, TAB6-1, proves this possibility unlikely and provides key support to the RENT control hypothesis. TAB6-1 is a dominant allele of CDC14 that bypasses cdc15Δ and temΔ without perturbing nucleolar structure or Pol I function. Thus, the release of Cdc14 from the nucleolus is sufficient to trigger mitotic exit in the absence of MEN proteins, and the role of RENT in cell cycle control can be uncoupled from its roles in maintenance of nucleolar integrity and Pol I transcription.

Results

RRN3 Suppresses Temperature Sensitivity of net1 Mutants

NET1 has been implicated in three potentially distinct cellular processes: mitotic exit, nucleolar silencing, and proper localization of the nucleolar antigen Nop1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). net1-1 cells grow slowly, and net1Δ cells fail to grow at 37°C, which could result from improper mitosis, an abnormal nucleolus, or defects in some other unknown functions of NET1. To distinguish between these possibilities, we screened a CEN/ARS-based yeast genomic library to identify genes that rescued the ts growth defect of net1-1. In addition to NET1, this effort yielded RRN3 (Figure 1A; see Experimental Procedures), a transcription activator for RNA Pol I (Yamamoto et al., 1996). Strikingly, centromeric plasmids containing NET1 ([NET1]) or RRN3 ([RRN3]) driven by their respective native promoters rescued the temperature sensitivity of net1Δ to similar extents (Figure 1B). The level of Rrn3 protein in net1 mutants was not diminished at 25°C or 37°C (Figure 1C), suggesting that the ts growth defects of net1 mutants did not result from reduced amounts of Rrn3.

It is unlikely that RRN3 suppressed the growth defect of net1 mutants by restoring nucleolar silencing because a lack of silencing in sir2Δ does not affect growth. It is also unlikely that suppression involved mitotic exit, since in the presence of [RRN3], net1-1 still bypassed cdc15Δ (W.S., data not shown). Nop1, a protein involved in pre-rRNA processing and ribosome assembly, is delocalized from the nucleolus in net1Δ cells (Straight et al., 1999). Although it is not known whether delocalization of Nop1 impedes cell growth, we tested whether the RRN3 plasmid affected Nop1 localization in net1 mutants. Whereas Nop1 assumed a characteristic nucleolar localization pattern (a crescent-shaped structure abutting the nucleus) in 98% of wild-type cells, only 17% of net1-1 cells showed a significant or complete Nop1 localization to the nucleolus at 37°C (Figure 1D). However, this fraction was increased to 64% by the RRN3 plasmid (Figure 1D). Similarly, the fraction of cells with significant or complete localization of Nop1 to the nucleolus was increased from 9% in net1Δ to 46% in net1Δ [RRN3] at 25°C (Figure 1D). Thus, RRN3 on a centromeric plasmid partially restored correct Nop1 localization in net1 mutants, suggesting that the growth defect of these mutants arose from a disruption of nucleolar structure and function.

rRNA Synthesis Is Reduced in net1-1

Given that extra Rrn3, a transcription factor for Pol I, suppressed the ts growth defect of net1 mutants, a key function of Net1 might be to sustain Pol I activity. To test this hypothesis, we evaluated rRNA and actin mRNA accumulation in net1-1 mutants by ethidium bromide staining and Northern blotting, respectively. The level of rRNA in net1-1 cells at 37°C, normalized to actin mRNA, was reduced by ∼40% compared with that in wild-type cells (Figure 2A).

The reduced level of rRNA in net 1-1 could result from a reduced rate of transcription or defective processing of pre-rRNA. To distinguish between these possibilities, we performed pulse-chase experiments using [3H]-methionine, which has the advantage of being rapidly incorporated into rRNA through methylation and even more rapidly chased (Warner, 1991). net1 mutants did not exhibit a major defect in 35S, 27S, and 20S pre-rRNA processing (Figure 2B, lower panel). However, the net1 mutants incorporated much less [3H]-methionine (Figure 2B, lower panel; note the 7-fold longer exposure time for net1Δ), which could result from reduced rRNA methylation or transcription. We therefore repeated the pulse-chase experiment using [3H]-uracil and adjusted exposure time such that the labeling of tRNA (synthesized by Pol III) was approximately equivalent. In net1 mutants, mature 25S and 18S rRNA transcripts were produced at a slower rate (Figures 2C, lower panel, and 2D). We suggest that the large difference in rate of appearance of mature rRNA in wild-type versus net1 cells is due to changes in the actual rate of transcription by Pol I and not due simply to changes in nucleotide pool sizes. Curiously, the net1Δ cells analyzed in this experiment displayed a less severe defect in rRNA synthesis than the net1-1 cells. In our experience, the slow-growth phenotype of net1Δ cells was neither uniform nor stable: The two net1Δ spores from a single tetrad frequently displayed unequal growth rates, and upon subculturing, net1Δ cells always began to grow faster (W.S., data not shown). Thus, it is possible that net1Δ cells accumulated second site mutations more efficiently than net1-1 or acquired more rRNA repeats through mitotic recombination to compensate for the severe reduction in rRNA synthesis.
Figure 1. A CEN/ARS Plasmid Containing RRN3 ([RRN3]) Suppresses the Temperature-Sensitive (ts) Growth Defect and Nop1 Mislocalization Phenotype of net1 Mutants

(A and B) [RRN3] suppresses net1 ts growth defect. (A) net1-1 cells transformed with a CEN/ARS/LEU2 plasmid harboring either no insert (vector) or RRN3 under its natural promoter were plated onto synthetic dextrose medium lacking leucine. After 25°C for one day, both plates were shifted to 37°C for two more days, and then photographed. (B) NET1 and net1Δ cells transformed with a CEN/ARS/LEU2 plasmid harboring either no insert (vector). RRN3 or NET1 (driven by their natural promoters) were streaked onto synthetic dextrose medium lacking leucine. Two independent colonies were analyzed in each case. The plates were photographed after four days at 25°C or five days at 37°C.

(C) Wild-type and net1 mutants were pregrown at 25°C to exponential phase and shifted to 25°C or 37°C for three more hours. Extracts were fractionated by SDS-PAGE and immunoblotted with 9E10 antibodies (against the Myc epitope) to measure the level of Rrn3-Myc9. Cdc28 served as a loading control.

(D) [RRN3] partially restores Nop1 nucleolar localization in net1 mutants. Wild-type and net1 mutants with or without [RRN3] plasmid were grown at 25°C to exponential phase. Some cultures were further incubated at 37°C for 3 hr as indicated. Cells were subjected to indirect immunofluorescence with anti-Nop1, and the extent of Nop1 delocalization in net1 mutants was categorized into four classes: none, complete delocalization such that Nop1 staining is uniform across the entire nucleus; slight, strong nuclear staining with stronger nucleolar staining; significant, intense nucleolar staining and subdued nuclear staining; and complete, nucleolar localization with no detectable nuclear staining. Fractions of cells that fell in the four different classes were quantitated.
puriﬁed Pol I complexes also efﬁciently captured His6-Myc9-Net1 (Figure 4C, lanes 3 and 4). This observation indicates that Pol I bound directly to the Net1 subunit of RENT.

Net1 Stimulates Pol I Activity In Vitro

To test whether Net1 could directly activate Pol I, lysates from insect cells either mock infected or infected with a baculovirus that expressed His6-Myc9-Net1 were adsorbed to nickel-NTA resin, and speciﬁcally bound proteins were eluted and added to a standard transcription reaction that contained linearized rDNA template, Pol I, Rm3, TATA binding protein (TBP), upstream activating factor (UAF), and core factor (CF; Keener et al., 1998). His6-Myc9-Net1 stimulated rRNA synthesis 2.2-fold relative to the mock sample (Figure 5A). To conﬁrm Net1’s stimulatory effect, we repeated this experiment with His6-Myc9-Net1 that was further enriched on SP- and Q-sepharose columns, resulting in a preparation with >90% purity (Figure 5B). The production of [32P]-labeled runoff transcripts (arrow in Figure 5C) increased as a function of input Net1 (Figures 5C and 5D), with the maximum stimulation being slightly more than 4-fold. Furthermore, maximum stimulation occurred at a concentration of Net1 that was approximately equimolar with Pol I, suggesting that Net1 stimulates Pol I transcription by helping to recruit Pol I to the rDNA templates present in our in vitro reactions. Taken together, our data indicate that Net1 is required for optimal activity of Pol I, both in vivo and in vitro.

Nucleolar Structure Is Perturbed in net1 Mutants

The failure of Cdc14, Sir2, and Pol I to localize properly to the nucleolus in net1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999; Figure 3A) could be explained by the fact that they all directly bound Net1 (Traverso et al., 2001; Figures 4B and 4C). In contrast, delocalization of Nop1 in net1 mutants (Figure 3B) is harder to explain given that Nop1 did not stably associate with Net1 (Figure 4A). The simplest model predicts that Net1 directly or indirectly inﬂuenced nucleolar structure, which in turn dictated the localization pattern of Nop1 and possibly other nucleolar proteins.

To test this model, we examined the localization patterns of two additional nucleolar proteins in various mutants. Nop2 (de Beus et al., 1994) exhibited a nucleolar staining pattern in wild-type and net1-1 but not net1 mutants (Figure 3C, top three rows). Mislocalization of Nop2 in net1 mutants (Figure 3B) is harder to explain given that Nop2 did not stably associate with Net1 (Figure 4A). The simplest model predicts that Net1 directly or indirectly inﬂuenced nucleolar structure, which in turn dictated the localization pattern of Nop1 and possibly other nucleolar proteins.

To test this model, we examined the localization patterns of two additional nucleolar proteins in various mutants. Nop2 (de Beus et al., 1994) exhibited a nucleolar staining pattern in wild-type and net1-1 but not net1 mutants (Figure 3C, top three rows). Mislocalization of Nop2 in net1-1 was not due to increased levels of this protein (Figure 3E, bottom panel). In contrast, the localization pattern of Fpr3 (Benton et al., 1994) was altered in a different way in both net1 mutants: Despite near equiva

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Figure 2. net1 Mutants Have a Reduced Rate of rRNA Synthesis

(A) The steady-state level of rRNA is reduced in net1-1. Total RNA was extracted from cells grown at 23°C and shifted to 37°C for three hours. Equal amounts of total RNA were separated by agarose gel electrophoresis, ﬁrst stained with ethidium bromide to show the levels of 25S and 18S rRNA (upper panel), and then probed for actin mRNA in a Northern blot (lower panel). The ratio of 18S rRNA to actin mRNA is shown below each lane.

(B–D) net1 mutants have almost normal rRNA processing but reduced rRNA transcription. Cells were grown at 25°C, shifted to 37°C for 3 hr, and kept at 37°C thereafter. They were pulse-labeled with [3H]-methionine (B) or [3H]-uracil (C) for 5 min and chased with unlabeled methionine (B) or uracil (C) for 0, 3, 6, 15, or 30 min, as indicated. Total RNA was separated by agarose gel electrophoresis, stained with ethidium bromide (upper panel), and subjected to autoradiography for the indicated amounts of time (Texp) (lower panel). The amount of the [3H]-uracil-labeled 25S rRNA from equivalent OD600 cells was quantitated and plotted in (D).
Figure 3. Net1 Is Required for the Proper Localization of Multiple Nucleolar Proteins

(A–D) Cells with indicated genotypes were grown at 25°C and subjected to indirect immunofluorescence with anti-A190 (A), anti-Nop1 (B), anti-Nop2 (C), or anti-Fpr3 (D) antibodies (column 1). They were also stained with DAPI to show the position of nuclei (column 2). The images in columns 1 and 2 were merged in column 3. In 40%–50% of sir2Δ and 20% of wild-type cells, Nop2 and Fpr3 assumed diffused staining patterns that covered the majority of the nucleus or even exceeded the boundary of the nucleus (white arrows in panels C and D).

(E) Protein levels of the four nucleolar antigens in wild-type and mutant cells were compared in Western blots, with Cdc28 serving as the loading control.

(F) rDNA morphology is altered in net1Δ and sir2Δ but not TAB6-1 mutants. Cells were arrested in nocodazole and subjected to fluorescence in situ hybridization (FISH) using DIG-labeled probes against rDNA followed by rhodamine-anti-DIG (column 1). DNA was visualized by DAPI staining (column 2). The merged image is shown in column 3.
Figure 5. Net1 Stimulates Pol I Activity

(A) His6-Myc9-Net1 (lane 2) or a control sample from mock-infected cells (lane 1) purified in parallel was added to a standard transcription reaction that employed a linearized plasmid template that contained rDNA linked to its full promoter (extending to 210) as the template and 10–15 nM Pol I (Keener et al., 1998). An arrow indicates the position of [32P]-labeled runoff transcripts.

(B) Net1 enriched to 90% purity (Experimental Procedures) was fractionated by SDS-PAGE and stained with Coomassie Blue. M refers to molecular weight markers.

(C) Net1 stimulates Pol I activity. Dilution buffer (lanes 1 and 2) or increasing amounts of Net1 (lanes 3 to 5) were added to the transcription reaction. Quantitation of the amount of transcripts (arrow in C) as a function of Net1 concentration is plotted in (D).

Figure 4. Pol I but Not Nop1 Binds Stably to Net1

(A) A190 but not Nop1 binds stably to Net1. Extracts from strains with the indicated genotypes were immunoprecipitated with 9E10 antibodies (−−−− refers to a strain with a natural [that is, untagged] NET1 allele). The immunoprecipitates (Myc IP) and the input extracts were fractionated by SDS-PAGE and immunoblotted with antibodies against A190 (first row), Nop1 (second row), and Myc9-Net1 (third row).

(B) His6-Myc9-Net1 and GST-Sir2 interact in vitro. His6-Myc9-Net1 and GST-Sir2 were expressed and purified from insect cells and bacteria, respectively. 9E10 antibody beads were incubated with GST-Sir2 in the presence (+) or absence (−) of Myc9-Net1. Proteins captured by the beads were immunoblotted with 9E10 and α-GST antibodies.

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

The abnormal localization patterns of most nucleolar proteins examined so far in net1 mutants led us to examine whether the organization of rDNA itself was perturbed in these cells. Cells were arrested in mitosis with nocodazole and subjected to fluorescence in situ hybridization (FISH; Guacci et al., 1994). Whereas wild-type cells displayed line-shaped rDNA that appeared to be spooled away from the focus of 4′,6-diamidino-2-phenyindole dihydrochloride (DAPI) staining, the majority (93%) of net1-1 cells possessed more condensed rDNA semicircles that abutted directly against the DAPI-stained material (Figure 3F, compare rows 1 and 3). net1Δ cells did not arrest well in nocodazole, but they showed similar rDNA morphology as net1-1 (data not shown), suggesting that Net1 is not an essential determinant of the unusual chromatin structure observed for rDNA in FISH experiments. Thus, in net1 mutants, localization patterns of multiple nucleolar antigens as well as rDNA morphology are altered, although rDNA still congregates into a structure distinct from the bulk DNA.

net1 and sir2 Mutations Have Distinct Effects on the Nucleolus

Sir2 plays an important role in the nucleolus by suppressing intrachromosomal recombination within the rDNA repeats and silencing transcription by Pol II (reviewed in Guarente, 2000). Because Net1 tethers Sir2 to rDNA (Straight et al., 1999), all of the phenotypes observed for net1 could, in theory, have been caused by a loss of Sir2 function within the nucleolus. However, this is clearly not the case because net1 cells had either distinct or more severe phenotypes than sir2Δ in all
assays that were conducted. First, unlike in net1 mutants, accumulation of rRNA was normal in sir2Δ (Figure 2A). Second, A190, Nop1, Nop2, and Fpr3 displayed normal (or nearly normal) nucleolar localization patterns in sir2Δ (Figures 3A–3D). Third, rDNA in sir2Δ cells, as revealed by FISH, displayed considerable heterogeneity and seemed to be less compact than that in wild-type or net1-1 cells (Figure 3F). Thus, Net1 and Sir2 regulated nucleolar structure and function in distinct ways.

The TAB6-1 Allele of CDC14 Bypasses tem1Δ and cdc15Δ

The pleiotropic defects of the nucleolus in net1 mutants add a confounding twist to the RENT control hypothesis. We originally proposed that Tem1-dependent disassembly of the RENT complex and the subsequent release of Cdc14 from the nucleolus drive cells from mitosis to interphase. tem1Δ and cdc15Δ cells arrest in late mitosis, but the net1-1 mutation (also known as tab2-1 for telophase arrest bypass) enables these cells to exit mitosis, presumably by allowing Cdc14 to escape the nucleolus (Shou et al., 1999). However, given the profound disruption of nucleolar structure in net1-1 cells, it is also possible that displaced nucleolar proteins other than Cdc14 caused or contributed to the tem1Δ and cdc15Δ bypass phenotype.

To test the RENT control hypothesis rigorously, we sought to identify a mutant form of Cdc14 with reduced binding affinity for Net1. If our hypothesis is correct, two predictions can be made for this mutant: (1) Like net1-1, it should be a tab mutant, because both mutations should allow ectopic release of Cdc14 from the nucleolus. (2) The tem1Δ and cdc15Δ bypass phenotype should be dominant. Based on this reasoning, we tested dominant mutants recovered in our earlier tab screen and found that the dominant TAB6-1 mutation demonstrated tight linkage to CDC14 (zero recombinants in thirty wild-type levels of phosphatase activity toward p-nitrophenyl phosphate and tyrosine-phosphorylated myelin basic protein (Figure 7A), it was bound less efficiently (Figure 7B) by a purified N-terminal fragment (amino acids 1–600) of Net1, which has been shown to bind and im-

Cdc14TAB6 Has Reduced Affinity for Net1, which Renders Cib5 Essential for Spore Viability

To interpret the phenotype of TAB6-1 cells properly, it is important to understand the molecular mechanism by which TAB6-1 bypassed tem1Δ and cdc15Δ. In net1-1 mutants, bypass of the anaphase arrest that normally occurs upon depletion of Tem1 is accompanied by ectopic Cib2 degradation and Sic1 accumulation (Shou et al., 1999). TAB6-1 cells behaved similarly to net1-1 cells upon depletion of Tem1 (Figure 6C, compare lanes 8–14 [TAB6-1] with lanes 1–7 [-]). Moreover, like net1-1, TAB6-1 bypassed anaphase arrest in almost all cells, since Cib2 degradation proceeded to near completion in Tem1-depleted cells (Figure 6C). We conclude that net1-1 and TAB6-1 most likely bypassed cdc15Δ by related mechanisms. To address whether TAB6-1, like net1-1, compromised the stability of RENT, we evaluated the Net1 binding and enzymatic activity of Cdc14 and Cdc14TAB6. Whereas purified Cdc14TAB6 had nearly wild-type levels of phosphatase activity toward p-nitrophenyl phosphate and tyrosine-phosphorylated myelin basic protein (Figure 7A), it was bound less efficiently (Figure 7B) by a purified N-terminal fragment (amino acids 1–600) of Net1, which has been shown to bind and inhibit Cdc14 (Traverso et al., 2001). Similarly, Cdc14TAB6, but not Sir2, was less efficiently recovered in association with Myc9-Net1 upon immunoprecipitation of the RENT complex from yeast cell extracts (Figure 7D). These data imply that the P116L substitution allowed bypass of cdc15Δ and tem1Δ by reducing the affinity of Cdc14TAB6 for Net1.

Unexpectedly, TAB6-1 cells grew robustly at 25°C (Figure 7E), even though loss of temporal control over Cdc14 activity would be expected to perturb cell division and growth. Interestingly, whereas TAB6-1 cib2Δ cells were viable, TAB6-1 cib5Δ spores failed to form colonies (Figure 7F), suggesting that in the absence of the opposing activity of Cib5/Cdc28 (Shirayama et al., 1999), proper control of Cdc14 becomes essential for spore viability.

Cell Cycle Control and Nucleolar Functions of RENT Complex Can Be Uncoupled by the TAB6-1 Allele of CDC14

Whereas both the net1-1 and TAB6-1 mutations destabi-

Figure 6. TAB6-1 Is an Allele of CDC14 that Bypasses tem1Δ

(A) TAB6-1 is an allele of CDC14. tem1Δ::TRP1 [GAL-TEM1/URA3] TAB6-1 cells (center), or tem1Δ::TRP1 [GAL-TEM1/URA3] cells transformed with a HIS3-marked plasmid harboring CDC14 derived from either wild type cells (right), or TAB6-1 cells (left) were grown on YP galactose (YPG, TEM1 expressed) and then plated on synthetic glucose medium containing 5-FOA to select for colonies that had lost the [GAL-TEM1/URA3] plasmid. After ten days at room temperature, the plate was photographed.

(B) TAB6-1 has a single point mutation P116 → L. Cdc14 sequences flanking this amino acid from S. cerevisiae, S. pombe, and Drosophila are aligned.

(C) TAB6-1 bypass of tem1Δ is accompanied by Cib2 degradation and Sic1 accumulation. Cells of the indicated genotypes grown in YPG (TEM1 expressed) at 25°C were arrested in G1 phase with α factor, and released intoYP glucose (TEM1 repressed) at time = 0. One and a half hours later, α factor was added back to prevent cells from proceeding through a second cell cycle. At the indicated times, samples were taken to measure Cib2, Sic1, and Cdc28 protein levels by immunoblotting.

(D) Cdc14TAB6, but not Sir2, was less efficiently recovered in association with Myc9-Net1 upon immunoprecipitation of the RENT complex from yeast cell extracts (Figure 7D). These data imply that the P116L substitution allowed bypass of cdc15Δ and tem1Δ by reducing the affinity of Cdc14TAB6 for Net1.
**Discussion**

The Net1 Subunit of RENT Complex Directly Stimulates Transcription by Pol I

The RENT complex, consisting of at least three proteins (Net1, Cdc14, and Sir2), controls mitotic exit, mediates nucleolar silencing, and sustains proper localization of Nop1 (Shou et al., 1999; Straight et al., 1999; Visintin et al., 1999). The first two functions of RENT are well documented and derive from the ability of Net1 to tether both Cdc14 and Sir2 to the nucleolus, respectively. Besides these phenotypes, we show here that net1 mutants also exhibit de-localization of the A190 subunit of Pol I, a decreased rate of rRNA synthesis, and a reduced accumulation (Figure 2A) were all indistinguishable in net1 mutants (lanes 4–6) and wild-type lanes (lanes 1–3) were immunoblotted with 9E10 antibodies. The input extracts (lanes 1–3) and immunoprecipitates (lanes 4–6) were immunoblotted with 9E10, α-Cdc14, and α-Sir2 antibodies as indicated.

(F) TAB6-1 cells grow much better than net1-1 cells at 25°C. Wild-type, TAB6-1, and net1-1 cells were struck out on a YPD plate and allowed to grow at 25°C for 2.5 days before the photograph was taken.

(G) TAB6-1 clb5Δ (but not TAB6-1 clb2Δ) spores fail to survive. clb2Δ;LEU2 CDC14/HIS3 and clb5Δ;URA3 CDC14/HIS3 strains were mated with TAB6-1, and the diploid strains were dissected. The number of viable spores of the indicated genotypes were tabulated. Although clb5Δ (Chromosome VI) and TAB6-1 (chromosome VI) are unlinked, clb5Δ TAB6-1 recombinants were never recovered.

**Role of Net1 in Global Nucleolar Structure**

Two proteins (Sir2 and Cdc14) and one protein complex (Pol I) that bind directly to Net1 are delocalized in net1...
Figure 8. TAB6-1 Uncouples the Nucleolar and Cell Cycle Functions of RENT
See Discussion for details.

Wild type

Net1

Nop1

Cdc14

Pol I

Sir2

Fpr3

net1-1

TAB6-1

Phenotypes:

- bypass of tem1Δ and cdc15Δ
- defective nucleolar silencing
- defective rDNA morphology
- delocalized rRNA processing factors
- diminished Pol I function

mutants. However, Nop1, which does not bind stably to Net1, is also delocalized in net1 mutant cells. One explanation comes from the observation that the nucleolar localization of rRNA processing factors Nop1 and Gar1 is reduced upon inhibition of transcription of rDNA (Trumtel et al., 2000). Thus, Nop1 and Nop2, both involved in rRNA processing (Tollervey et al., 1993; Hong et al., 1997), may become delocalized in net1Δ mutants in part due to decreased synthesis of rRNA. Supporting this proposal, RRN3 suppression of net1Δ, presumably involving stimulation of Pol I transcription (Yamamoto et al., 1996; Keener et al., 1998), correlates with partial restoration of Nop1 nucleolar localization (Figure 1D). An alternative possibility is suggested by the recent report that Pol I holoenzyme, some of its transcription activators, and rRNA processing factors assemble together into a functional supercomplex whose integrity does not depend on rRNA transcription by Pol I (Fath et al., 2000). Our observation that net1Δ mutations lead to simultaneous loss of Pol I and rRNA processing components (Nop1, Nop2) from the nucleolus raises the possibility that Net1 contributes to either the integrity or the nucleolar anchorage of the supercomplex.

Intriguingly, in net1Δ cells, the nucleolar protein Fpr3 retains a focal, albeit more compact, localization resembling the morphology of net1-1 rDNA. A similar compaction has been reported for the “nucleolar remnant” visualized by electron microscopy upon thermal inactivation of RNA Pol I (Trumtel et al., 2000). Thus, we suggest that congregation of rDNA repeats occurs even in the absence of Net1 and that Fpr3 is recruited to the nucleolus by a Net1- and transcription-independent pathway.

Although the nucleolar defects of net1Δ cells are likely to arise in part due to diminished Pol I activity and dispersion of Sir2, it seems probable that Net1 serves additional functions within the nucleolus. This prediction is based on four observations. First, the overall growth defect and thermosensitive phenotypes of net1Δ mutants are partially corrected by RRN3 but are not alleviated by ectopic expression of rDNA by Pol II at a level sufficient to support growth of cells lacking a subunit of Pol I (J. Claypool and W.S., data not shown). Second, chromatin IP experiments indicate that Net1 decorates the entire rDNA sequence and is not restricted to the promoter region (Straight et al., 1999). Indeed, Net1 still localized to the altered nucleolus in a rrn5Δ PSW (polymerase switched) strain in which Pol II instead of Pol I transcribed rDNA (I. Siddiqi, M. Oakes, and M.N., unpublished data; for PSW strains, see Oakes et al., 1999). These observations suggest that Net1 “coats” rDNA and regulates its structure in the presence or absence of the Pol I transcriptional machinery. Third, rrn5Δ PSW cells are viable when Pol I is inactivated but are inviable in combination with net1Δ (I. Siddiqi and M.N., unpublished results). Fourth, the morphologies of rDNA in net1-1 and sir2Δ, as judged by FISH analysis, are clearly distinct, suggesting that Sir2 alone cannot account for NET1 functions. On the other hand, a loss of Sir2 function may account for the perturbation of rDNA copy number control that we observed in net1Δ mutants. Chromosome XII ran as a smear in net1Δ cells (W.S., J.D., and M. Oakes, unpublished results), possibly because of increased recombination among rDNA repeats upon delocalization of Sir2 (Gottlieb and Esposito, 1989).

**TAB6-1 Uncouples Nucleolar and Cell Cycle Functions of RENT Complex**

net1-1 was originally isolated as a bypass suppressor of cdc15Δ and tem1Δ, presumably because it allows Cdc14 to be released from the nucleolus during ana-phase in a Tem1/Cdc15-independent manner (Shou et al., 1999). However, the exact mechanism of bypass is
observed by the pleiotropic nucleolar defects of the net1-1 mutant. The possibility that the release of some nucleolar protein other than Cdc14 is responsible for tem1Δ bypass was instigated further by the observation that two other recessive tab mutants (tab1, 15D2) exhibited defects in nucleolar integrity (K.S. and W.S., unpublished data). Furthermore, it is unclear whether the severe growth defect of net1Δ and net1-1 (Shou et al., 1999; Straight et al., 1999) arises from loss of the cell cycle or nucleolar functions of Net1.

The identification of the TAB6-1 allele of CDC14 allowed us to begin to address how the cell cycle and nucleolar functions of RENT are related (Figure 8). Both TAB6-1 and net1-1 efficiently bypass cell cycle arrest in Tem1-depleted cells, presumably because free Cdc14 enables ectopic degradation of Cib2 and accumulation of Sic1. Unlike net1-1 mutants, there are no obvious general defects in nucleolar organization or function in TAB6-1 mutants, as judged by the localization patterns of Nop1, Nop2, Fpr3, and A190, the morphology of rDNA, the number of rDNA repeats (W.S. and J.D., data not shown), and the level of rRNA accumulation. Thus, the more severe growth phenotype observed in net1 mutants is likely due to defective nucleolar functions rather than cell cycle functions. Furthermore, by affecting cell cycle functions without perturbing nucleolar functions of the RENT complex, TAB6-1 confirms that release of Cdc14 from RENT is sufficient to trigger mitotic exit in cdc15Δ cells.

Experimental Procedures

Isolation of RRNA as a Low-Copy Suppressor of net1-1

The net1-1 mutant strain was transformed with yeast genomic plasmid libraries and was incubated at 25°C for one day before being shifted to 37°C. Low-copy CEN/ARS-based libraries from the laboratories of P. Heiter (ATCC#77164) and R. Young (Thompson et al., 1993) yielded a net1-1 complementing activity from chromosome XI, 206,300 210,665. Each open reading frame in this genomic region was amplified by polymerase chain reaction and tested for its ability to complement the temperature sensitivity of net1-1. The complementing activity resided in RRNA3.

RNA Isolation and Northern Blot

Cells grown in 12 ml YPD at 23°C to an OD600 of ~0.8 were shifted to 37°C for 3 hr. Cell pellets were resuspended in 1 ml buffer (50 mM sodium acetate, 10 mM EDTA, 0.1% sodium dodecyl sulfate (SDS), and RNA was extracted with 1 ml of phenol, followed by 1 ml of phenol/chloroform (1:1) by incubation at 65°C for 4 min and on ice for 4 min. RNA was precipitated with ethanol, 5 μg of which was fractionated on a 1.5% agarose gel containing formaldehyde, stained with ethidium bromide, and quantitated using the Alpha 5400 software.

Pulse-Chase Labeling of rRNA

Pulse-chase labeling and analysis of rRNA was carried out essentially as described (Udem and Warner, 1972; Warner, 1991; Powers et al., 2001). In vitro transcription was carried out essentially as described previously (Keener et al., 1998). Protein was harvested on His-bind resin (Novagen, Madison, WI; Traverso et al., 2001). Purification and Analysis of Net1(1-600), Cdc14, and Cdc14(7408)

GST-Cdc14 and GST-Cdc14(7408) were purified from E. coli using glutathione-Sepharose beads, and Net1(1-600)-His6 was purified from E. coli on His-bind resin (Novagen, Madison, WI; Traverso et al., 2001). Phosphatase assays were performed in buffer P (50 mM imidazole [pH 6.6], 1 mM EDTA, 1 mM DTT, 0.5 mg/ml bovine serum albumin) at 30°C using 20 mM p-nitrophenyl phosphate or 4 μM myelin basic protein phosphorylated on tyrosines (Tyr-P-MBP; Tay et al., 1997). The ability of purified Net1(1-600)-His6 to inhibit GST-Cdc14 and GST-Cdc14(7408) was assessed in phosphatase assays performed at 30°C with 10 nM enzyme and 4 μM Tyr-P-MBP in buffer P containing 120 mM KCl.

RNA samples were added at 37°C for 3 hr. The samples were fractionated on a 6.7% formaldehyde-1.5% agarose gel. The gel was stained with ethidium bromide, and quantitated using the Alpha 5400 software.
mouse α-Nop1, 1:1K 9E10 (Babco/CRP, Richmond, CA), and 1:200 rabbit α-Cdc14. FISH analysis was carried out as previously described (Guacci et al., 1994).

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References


