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Multiple *telophase arrest bypassed (tab)* mutants alleviate the essential requirement for Cdc15 in exit from mitosis in *S. cerevisiae*

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Abstract

Background: The Mitotic Exit Network (MEN) proteins – including the protein kinase Cdc15 and the protein phosphatase Cdc14 – are essential for exit from mitosis in *Saccharomyces cerevisiae*. To identify downstream targets of the MEN, we sought *telophase arrest bypassed (tab)* mutations that bypassed the essential requirement for *CDC15*. Previous studies identified *net1*$^{tab2\text{-}1}$ and *CDC14*$^{TAB6\text{-}1}$ as mutations in the RENT complex subunits Net1 and Cdc14, respectively, and revealed that the MEN acts by promoting release of Cdc14 from its nucleolar Net1 anchor during anaphase. However, the remaining *tab* mutants were not characterized.

Results: Fourteen out of fifteen *tab* mutants were mapped to three recessive (*tab1-tab3*) and three dominant (*TAB5-TAB7*) linkage groups. We show that *net1*$^{tab2\text{-}1}$ enables growth of *tem1Δ*, *cdc15Δ*, *dbf2Δ dbf20Δ*, and *mob1Δ*, but not *cdc5Δ* or *cdc14Δ*, arguing that whereas the essential task of the first four genes is to promote exit from mitosis, *CDC5* possesses additional essential function(s). *net1*$^{tab2\text{-}1}$ but not *CDC14*$^{TAB6\text{-}1}$ resulted in a high rate of chromosome loss, indicating that Net1 promotes accurate chromosome segregation in addition to its multiple known roles. Finally, *TAB1* was shown to be *MTR10*, a gene encoding nuclear transport receptor/adaptor. In some of the *tab* mutants including *mtr10*$^{tab1\text{-}1}$, defective nuclear export of the ribosomal protein Rpl11b was observed. Furthermore, the transport-defective *-31* allele of the karyopherin *SRP1*, but not the transport competent *-49* allele, exhibited a *tab* phenotype.

Conclusions: Transport-defective mutations in two karyopherins can bypass *cdc15Δ*, suggesting that the function of the MEN is to promote mitotic exit by regulating nuclear transport.

Background

In *S. cerevisiae*, a key event accompanying exit from mitosis is the inactivation of Clb/Cdc28 protein kinase achieved through degradation of Clb and accumulation of the Clb/Cdc28 inhibitor Sic1 (reviewed in [1]). Anaphase-Promoting Complex/Cyclosome (APC/C) ubiquitin ligase and its substrate specific activator Hct1/Cdh1 are required for degradation of Clb2, the major mitotic cyclin in the budding yeast (reviewed in [2]). Whereas *hct1Δ* and *sic1Δ* cells appear relatively normal, *hct1Δ sic1Δ* cells are inviable, presumably due to their inability to extinguish Cdc28 activity in telophase [3].

The complexity in the regulation of mitotic exit is underscored by the existence of a set of genes essential for this process, referred to as the "mitotic exit network (MEN)".

They encode the GTP-binding protein Tem1 and its putative guanine nucleotide releasing factor Lte1, the dual specificity protein phosphatase Cdc14, protein kinases Cdc5, Cdc15, and Dbf2/Dbf20, the Dbf2-binding protein Mob1 (reviewed in [4]) and the spindle-pole-body component Nud1 [5]. Conditional-lethal temperature sensitive (ts) mutations in any of the MEN genes cause cells to arrest in late mitosis with elevated mitotic Cdk activity when shifted to the restrictive temperature. Inactivation of Cdc28 by overexpression of Sic1 suppresses multiple MEN mutants and hyperactivation of Cdc28 by overexpression of Clb2 exacerbates their phenotypes, consistent with the idea that a key function of the MEN proteins is to eradicate Cdc28 activity during the transition from anaphase/telophase to G1 [6–9].

Recently, important advances have been made in our understanding of how the MEN is switched on during anaphase. Tem1 is thought to be kept inactive throughout early mitosis by the two-component GTPase-activating protein complex Bub2/Bfa1 which co-localizes with Tem1 at the spindle pole body [10,11]. When the mitotic spindle penetrates the mother-daughter neck, the Tem1-bound spindle pole body is translocated into the daughter cell where a cortical pool of Lte1 is thought to activate Tem1 [12,13]. In addition to this spatial regulation, the GTPase activity of Bfa1 is thought to be inactivated through its phosphorylation by Cdc5 [14], but it remains unknown how this process is coordinated with the presumed juxtaposition of Lte1 and Tem1 in the daughter cell. Despite this rapid progress in understanding the signals that regulate the MEN, the direct downstream targets of the MEN have yet to be apprehended.

To address how the MEN components downstream of Cdc15 are organized and what their targets are, we sought *tab (telophase arrest bypassed)* mutants that alleviate the essential requirement for *CDC15.* In theory, *tab* mutants could act downstream of or parallel to *CDC15*, and could be either loss-of-function mutations in genes that inhibit exit from mitosis or gain-of-function mutations in genes that promote exit from mitosis. To our surprise, mutations in six linkage groups were uncovered, with three groups being recessive (*tab1-tab3*) and three groups being dominant (*TAB5-7*). We have shown in earlier studies that *tab2-1* is a reduction-of-function mutation in *NET1* (a.k.a. *CFI1*) [15–17] and that *TAB6* is a gain-of-function mutation in *CDC14*[18]. Net1 sequesters Cdc14 in the nucleolus and inactivates it in preanaphase cells. During exit from mitosis, a *TEM1/CDC15*-dependent signal frees Cdc14 from the nucleolus and allows it to promote inactivation of Clb/Cdc28 [15,17,19]. The fact that both Net1 and Cdc14 are directly involved in exit from mitosis validates the effectiveness of the tab screen and heightens the potential value of the uncharacterized *tab* mutants. Here, we present a detailed description of the *tab* mutant screen, and describe a third *TAB* gene, *TAB1/MTR10.*

Results

Cdc15 promotes activation of Clb proteolysis by a post-translational mechanism

Transcription has been postulated to play an important role in triggering exit from mitosis [20]. For example, Dbf2, a protein kinase that promotes exit from mitosis, is associated with the CCR4 transcription regulatory complex [21]. In addition, the Swi5 transcription factor activates *SIC1* expression during late anaphase, and both *sic1Δ* and *swi5Δ* are synthetically lethal with *dbf2Δ*[22,7]. To address if production of new proteins is essential for exit from mitosis, we examined the effects of the protein synthesis inhibitor cycloheximide on Clb2 degradation, a key aspect of this process. Mutant *cdc15-2* cells were uniformly arrested in late anaphase/telophase by incubation at the restrictive temperature 37°C. The cultures were then supplemented with 0, 10, or 100 μg/ml cycloheximide before being returned to 25°C to reverse the *cdc15-2* arrest. In the absence of cycloheximide, Sic1 accumulated and Clb2 disappeared as cells exited mitosis (Figure 1, lanes 1–4). In the presence of cycloheximide, Sic1 accumulation was abolished, but Clb2 degradation still occurred with apparently normal kinetics (Figure 1, lanes 5–8 and 9–12). Although this result does not exclude the likely possibility that synthesis of new proteins (e.g. Sic1) normally facilitates the exit from mitosis, it reveals that a key aspect of mitotic exit – inactivation of Cdc28 protein kinase via degradation of the mitotic cyclin Clb2 – can proceed beyond the Cdc15-dependent step without synthesis of new proteins.

A screen for telophase arrest bypassed (tab) mutants

To understand how the MEN mobilizes post-translational mechanisms to trigger mitotic exit, we conducted a genetic screen for 'telophase arrest bypassed' (*tab*) mutants that bypassed the essential requirement for *CDC15* (Figure 2). Because the essential requirement for *CLNs 1, 2,* and *3* in entry into S phase can be bypassed by deletion of a gene (*SIC1*) that inhibits entry into S phase [23,24], we reasoned by analogy that our screen might reveal key negative regulators of the anaphase/telophase -> G1 transition.

Ninety independent cultures of *cdc15Δ [pMET3-cdc15-2, URA3]* were first grown in inducing medium (- methionine) at permissive temperature (25°C) to allow occurrence of spontaneous mutations. The cultures were subsequently plated on repressing medium (+ methionine) at a temperature semi-restrictive for the *cdc15-2* allele (30°C) to enrich for mutations that supported the growth of *CDC15*- deficient cells. To identify mutations that allowed complete bypass of *cdc15Δ*, representative colonies from each plate were tested for their ability to

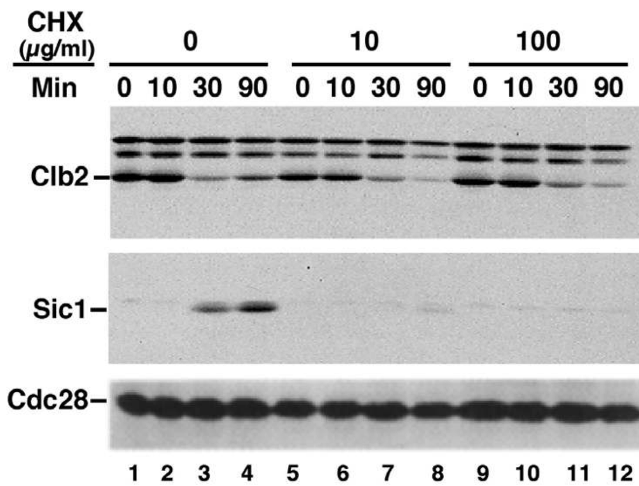


Figure 1
Post-translational control of Clb2 proteolysis by *CDC15*. Exponentially growing *cdc15-2* (RJD619) cells were arrested in late anaphase by shifting the culture to 37°C for three hours. The culture was split in three and either mock-treated (lanes 1–4) or supplemented with the protein synthesis inhibitor cycloheximide (CHX) at 10 μg/ml (lanes 5–8) or100 μ g/ml (lanes 9–12). After five minutes (time = 0), the cultures were released from cell cycle arrest by downshift to 25°C. At indicated time points, samples were withdrawn, and the levels of Clb2, Sic1 and Cdc28 proteins were assayed by SDS-PAGE and immunoblotting.

grow independent cultures of *cdc15Δ [pMET3-cdc15-2, URA3]* in methionine-free medium @ 25 °C (*cdc15-2* induced)

plate cells on methionine -containing medium @ 30 °C, one culture per plate (*cdc15-2* repressed)

test 1-2 representative colonies from each plate for viability on 5-FOA. (only *ura3-* cells survive on 5-FOA)

verify plasmid loss in 5-FOA-resistant colonies by PCR

Figure 2
Scheme for isolating *telophase arrest bypassed* (*tab*) mutants. See text for details.

grow on 5-FOA-containing medium (5-FOA selectively blocks the growth of plasmid-bearing *URA3*+ cells). The parental strain failed to grow due to its inability to proliferate without the [*MET3-cdc15-2, URA3*] plasmid. In contrast, twenty five mutants survived on 5-FOA. Furthermore, all of them had lost the plasmid as determined by PCR analysis (data not shown). Fifteen of these mutants were amenable to genetic manipulation and harbored single mutations. Fourteen mutants were assigned to three recessive and three semi-dominant/dominant linkage groups (Table 2). We referred to these mutants as *tab* mutants for their telophase-arrest-bypass (Tab+) phenotype. In addition, we serendipitously found that *srp1-31* but not *srp1-49* could bypass *cdc15Δ* (Table 2; data not shown).

Earlier studies identified *tab2* and *TAB6* as mutations in *NET1* and *CDC14*, respectively [15,18]. Net1 detains Cdc14 in the nucleolus and inactivates it until a Tem1-dependent signal sets Cdc14 free to promote exit from mitosis [15–17]. The fact that both Net1 and Cdc14 are directly involved in exit from mitosis prompted us to further analyze *tab* mutants.

Does bypass of cdc15Δ require Sic1 accumulation and Clb2 degradation?

Either Sic1 accumulation or Clb2 degradation is proposed to be sufficient to sustain mitotic exit [3]. Although *cdc15-2* mutants are defective in both processes, ectopic activation of either one should restore exit from mitosis and growth. Thus, to gain insight into their molecular targets, we asked if bypass of *cdc15Δ* by *tab* mutations required *SIC1* or *HCT1*. Whereas all *tab* mutants tested were able to bypass *cdc15Δ* in the absence of *HCT1* and two ($net1^{tab2\text{-}1}$ and $CDC14^{TAB6\text{-}1}$) were able to bypass *cdc15Δ* in the absence of either *SIC1* or *HCT1*, four *tab* mutants (*tab1-1*, *tab3-1*, *TAB5-1*, and *TAB7-1*) failed to bypass in the absence of *SIC1* (Table 3).

Efficient bypass of mitotic arrest by both SIC1-dependent and SIC1-independent tab mutants is accompanied by ectopic Sic1 accumulation and Clb2 degradation

We have previously shown that both $net1^{tab2\text{-}1}$ and $CDC14^{TAB6\text{-}1}$ efficiently bypassed the mitotic arrest caused by Tem1-depletion [15,18]. Furthermore, bypass was accompanied by ectopic Sic1 accumulation and Clb2 degradation. Because both $net1^{tab2\text{-}1}$ and $CDC14^{TAB6\text{-}1}$ could bypass *cdc15Δ* in the absence of *SIC1*, we tested if the same bypass efficiency could be achieved by *tab3-1*, a

Table 2: Characterization of *tab* mutants

Name	# alleles	recessive/dominant[b]	identity	growth at 37°C[d]	Nop1 pattern[e]
tab1	5	recessive	*MTR10*[c]	inviable	-
tab2	2	recessive	*NET1*	slow	-
tab3	1	recessive		slow	+
TAB5	2	semi-dominant		normal	+
TAB6	1	dominant	*CDC14*	slow	+
TAB7	3	semi-dominant (-1) dominant (-2)		normal	+
srpl-31[a]		recessive			-

[a]We have not determined if *srp1-31* is allelic to *tab3*, *TAB5*, or *TAB7*.[b]*All* alleles were tested for recessiveness/dominance of Tab+ except for *TAB7-3*.[c]See Methods. [d]*All* alleles of a single linkage group displayed similar ts growth phenotype. [e]All strains were grown to exponential phase at 25°C, and subjected to indirect immunofluorescence using anti-Nop1 antibodies. The wild type staining pattern was scored as "+", and partial delocalization or fragmented staining patterns were scored as "-".

Table 3: Does bypass require *SIC1* or *HCT1*?

Strain	*sic1Δ*[a]	*hct1Δ*[b]	+[c]
tab1-1	-	+	+
tab2-1	+/-	+	+
tab3-1	-	+	+
TAB5-1	-	+	+
TAB6-1	+/-	+	+
TAB7-1	-	+	+

[a]To assay if bypass required *SIC1*, *cdc15Δ::TRP1 tab [pMET3-CDC15/URA3]* cells were transformed with an *SICl*-deletion cassette (*S. pombe his5*+ DNA fragment whose termini were engineered to be homologous to *SIC1* 5' and 3' untranslated regions). All transformants were tested for their ability to bypass *cdc15Δ* using the 5-FOA growth assay (sample size n = 4–17). They were also screened for the absence of *SIC1* as the result of homologous integration. In the case of *tab2* and *TAB6*, about half of *sic1Δ* transformants could bypass. This incomplete penetrance is not well understood. There are at least two sources of variability in this experiment. First, *sic1Δ* transformants of *tab cdc15Δ [CDC15, URA3]* were pregrown in rich media to accumulate cells that had lost the [*CDC15*, *URA3*] plasmid. Because plasmid-loss is a low-probability event and because cells without the plasmid are at a growth-disadvantage, the number of plasmid-free cells spotted on a 5-FOA plate probably was small and varied among different transformants. Second, mutant individuals can exhibit more variability than wild-type individuals, a phenomenon frequently observed in worms and flies. Therefore, it is possible that in only a fraction of plasmid-free cells, the amount of Cdc14 activity reached a level high enough to tolerate *sic1Δ*. Thus, one can imagine that for some transformants, the number of plasmid-free cells spotted on 5-FOA was small and that by chance they or their limited numbers of progeny could not exit mitosis, resulting in the absence of bypass colonies. In other transformants, the opposite could occur, giving rise to mixed results in the plate assay. [b]Similar assay was conducted for *HCT1*. Sample size n = 1–7. [c]Transformants from a and b that resulted from non-homologous integration (and hence no deletion of *SIC1* or *HCT1*) were used as a control. Sample size n = 12–86.

SIC1-dependent bypass mutant. We used a *tem1Δ::GAL1-UPL-TEM1* strain that allowed for conditional depletion of Tem1 [15] to impose a conditional late mitotic block, and introduced the *tab3-1* mutation into this background. Wild type or *tab3-1* derivatives of *tem1Δ::GAL1-UPL-TEM1* were grown in galactose medium (*TEM1* induced), arrested in G1 with α factor, and released into glucose-containing medium to repress *TEM1* expression (time 0). As expected, the majority (~80%) of *TAB*+ cells arrested with large buds. In the *tab3-1* strain, however, a significant fraction (~36%) of cells had ≥ 3 cell bodies at 12 hours after release, indicating that they had undergone at least one extra round of budding (Figure 3A). Thirty-six percent represents a minimum estimate of *TEM1* bypass efficiency in *tab3-1* cultures, since cells with 1–2 cell bodies could have arisen from bypass events followed by successful cell separation.

Having established that *tab3-1* indeed bypassed Tem1 deficiency at a cellular level, we tested whether Sic1 accumulation and Clb2 degradation, two hallmark events that accompany exit from mitosis, also occurred. *tem1Δ::GAL1-UPL-TEM1* cells carrying *TAB3* or *tab3-1* alleles were arrested in G1 with α factor, and released into glucose medium (*TEM1* repressed, time 0). After cells had exited G1, α factor was added back to stop cells from cycling beyond the next G1 phase. As Tem1-depleted *TAB3* cells exited G1, Clb2 accumulated and remained at high levels, whereas Sic1 was degraded and remained at low levels (Figure 3B, left panels). In contrast, as Tem1-depleted *tab3-1* cells progressed through mitosis, Clb2 was almost completely degraded and Sic1 accumulated to high levels (Figure 3B, right panels). These data suggest that like *tab2-1* and *TAB6-1*, *tab3-1* efficiently bypassed *tem1Δ* by enabling Tem1-independent degradation of Clb2 and accumulation of Sic1. We conclude that the apparent *SIC1*-dependence of bypass by *tab3-1* and other *tab* mu-

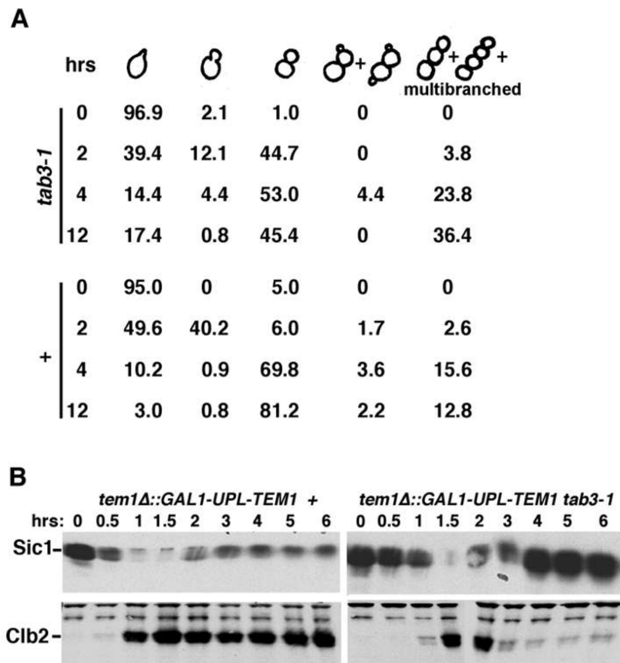


Figure 3
tab3-1 enables TEM1-independent degradation ofClb2 and accumulation of Sic1. *tem1Δ::GAL1-UPL-TEM1 tab3-1* (WY97) and *tem1Δ::GAL1-UPL-TEM1* (WY46) cells grown in galactose medium (*TEM1* expressed) at 25°C were arrested in G1 with α factor, and released into glucose medium (*TEM1* repressed) at time = 0. (A) At indicated time points after release, budding index was monitored. (B) The experiment in (A) was repeated, except that at either 2 h (TAB3) or 3 h (*tab3-1*) following release from α factor arrest, α factor was added back to prevent cells from proceeding through a second cell cycle. At indicated time points, samples were withdrawn to measure Sic1 and Clb2 levels by SDS-PAGE and immunoblotting.

tants relates more to the strength of the particular mutation, rather than the mechanism of bypass. In support of this hypothesis, we recently observed that the *SIC1*-independent bypass mutant 15D2 is allelic to the *SIC1*-dependent *tab1-1* mutant (R. Azzam, unpublished data).

tab2-1 bypasses multiple deletion mutants of MEN

All MEN genes are required for exit from mitosis, but it has not yet been tested whether this is the sole essential function of all MEN genes, or if some MEN genes play critical roles in other processes. This question is amplified by the observations that Cdc5 participates in both chromosome segregation and cytokinesis ([25,26]; Park et al., submitted), Mob1 binds an essential protein kinase involved in spindle pole body duplication [27], and Tem1, Cdc15, Dbf2, and Mob1 have all been implicated in cytokinesis ([28–31]; reviewed in [1]. The *tab* mutants provide a unique opportunity to address this issue. Accordingly,

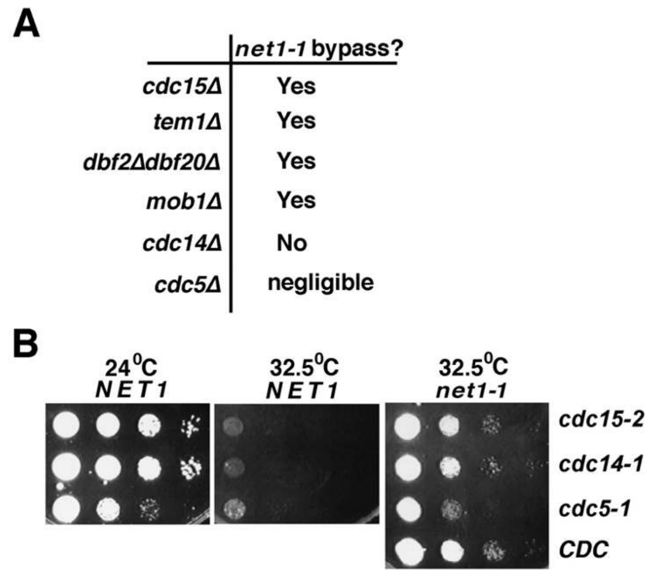


Figure 4
$net1^{tab2-1}$ efficiently bypasses multiple deletion mutants of MEN and extends the permissive temperature range for *cdc15-2*, *cdc14-1*, and *cdc5-1*. (A) The bypass specificity of $net1^{tab2-1}$ (*net1-1*). (B) Cells with specified combinations of *NET1* alleles (indicated at the top) and *CDC* alleles (indicated on the side) were spotted onto YPD plates in 5-fold serial dilutions. Plates were incubated at 24° (left panel) or 32.5° (center and right panels) for 2–4 days before being photographed. $net1^{tab2-1}$ was observed to suppress the ts phenotype of *dbf2-1* at 35.5°C (not shown).

we tested *if* $net1^{tab2-1}$ could bypass deletion mutants of other MEN genes. $net1^{tab2-1}$ bypassed *cdc15Δ, tem1Δ, dbf2Δ dbf20Δ, mob1Δ,* but not *cdc14Δ* (Figure 4A; [15]). In addition, it bypassed *cdc5Δ* with negligible (1000–10,000 fold lower) efficiency (Fig. 4A), suggesting that although the sole essential function of *CDC15, TEM1, DBF2,* and *MOB1* is to drive cells out of mitosis, *CDC5* has other critical cellular functions.

Some but not all tab mutants exhibit additional phenotypes including growth defects, perturbations in the nucleolus, and elevated rates of chromosome loss

To further characterize the *tab* mutants, we tested whether they display other phenotypes in an otherwise wild type background. *tab* mutants grown to exponential phase at 25°C and shifted to 37°C for 3 or 6 hours did not arrest at a particular stage of the cell cycle (data not shown). When *tab* mutants were plated on YPD plates and grown at 37°C, *TAB5* and *TAB7* grew normally (Table 2), suggesting that bypass of *CDC15* does not necessarily cause defective growth. However, *tab1* failed to form colonies, and $net1^{tab2-1}$, *tab3*, and $CDC14^{TAB6-1}$ grew slowly at 37°C, suggesting that at least some *tab* mutations reside in genes

that have multiple functions. Indeed, *NET1* additionally modulates nucleolar structure and transcription [16,18].

The release of Cdc14 from nucleolar Net1 promotes mitotic exit [15,17,18]. *net1*$^{tab2\text{-}1}$ cells display a panel of nucleolar defects including reduction of nucleolar silencing, mislocalization of nucleolar antigens, alteration of rDNA morphology, and reduction of rRNA synthesis [16,18]. To test whether perturbation of nucleolar structure is consistently associated with bypass of *cdc15Δ*, we immunolocalized the nucleolar antigen Nop1 in all *tab* mutants. Nop1 localization was also perturbed in *tab1-1* and *srp1-31*, but was normal in *tab3*, *TAB5*, *TAB6*, and *TAB7* cells (Table 2; [18]), suggesting that a gross change in nucleolar structure is not required for bypass.

Because the putative Tem1 antagonists and mitotic checkpoint proteins Bub2 and Bfa1 negatively regulate exit from mitosis and cytokinesis in response to both DNA damage and spindle misorientation ([32]; reviewed in [33]), we asked whether bypass of *TEM1*-dependent regulation can cause chromosome instability. All *tab* mutants are in the W303 background in which the *ade2-1* mutation makes colonies red after adenine in the growth medium has been depleted. A *URA3-marked* tester mini-chromosome carrying the *SUP11* suppressor transfer RNA gene [34]) was introduced into *net1*$^{tab2\text{-}1}$ and *CDC14*$^{TAB6\text{-}1}$, the two *tab* mutants with well-characterized bypass mechanisms. Cells that retain the mini-chromosome should form white colonies due to suppression of *ade2-1* by *SUP11*, whereas loss of this chromosome should result in a red sector or sectors. The extent of chromosome loss (expressed as % colonies that were red or had red sectors) was low in wild-type and *CDC14*$^{TAB6\text{-}1}$ cells (< 5%), and high (94%) in *net1*$^{tab2\text{-}1}$ cells (Figure 5). In comparison, in a strain lacking the anaphase-inhibitor Pds1, ~18% of the colonies suffered chromosome loss events (Figure 5). Since *net1*$^{tab2\text{-}1}$but not *CDC14*$^{TAB6\text{-}1}$ also perturbs nucleolar functions [18], *CDC14*$^{TAB6\text{-}1}$ is a more specific and representative bypass mutant. Thus, *TEM1* regulation can be short-circuited without a major effect on chromosome loss. Consistent with this notion, cells lacking Bub2 (an inhibitor of Tem1) showed a similarly moderate extent of chromosome loss (5%) (Figure 5). We propose that additional functions of Net1 ensure faithful transmission of chromosomes.

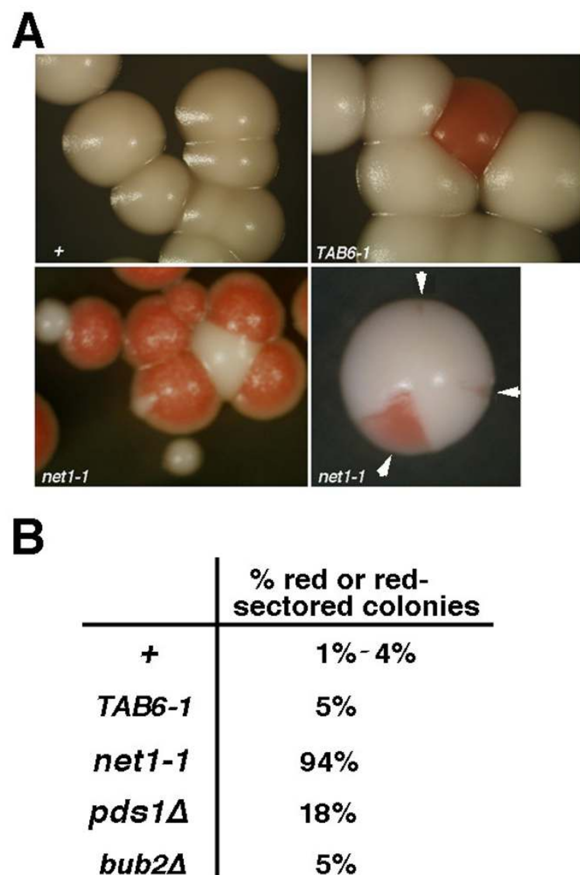
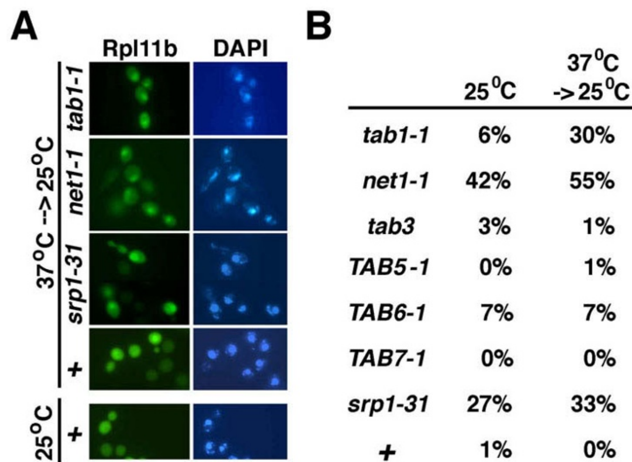


Figure 5
net1$^{tab2\text{-}1}$ cells display strong chromosomal segregation defect. A *URA3*-marked tester mini-chromosome carrying the *SUP11* suppressor transfer RNA gene [34] was introduced into the indicated genetic background also harboring an *ade2* mutation. Cells that retained the mini-chromosome would form white colonies due to suppression of *ade2* by *SUP11;* the loss of this chromosome resulted in a red sector or sectors. When the loss event occurred at a very high frequency, a red colony could form. Cells were pre-grown in the uracil-lacking SD agar medium, and plated on YPD plates at room temperature. (A) After the initial signs of colony coloration appeared, the plates were kept at 4°C to enhance color development. White arrowheads indicate three sectoring events in a *net1*$^{tab2\text{-}1}$(*net1-1*) colony. (B) Percentages of colonies that were red or had red sectors were calculated.

Molecular cloning of TAB genes and implication of nuclear transport in the regulation of mitotic exit

We attempted to clone by complementation genes corresponding to the recessive *tab* mutations by exploiting the fact that all of the recessive *tab* mutants are ts for growth (see below). By this approach, *TAB1* was revealed to be *MTR10,* a gene previously implicated in nuclear transport [35–37]. We were unable to isolate plasmids that complemented *tab3* in all libraries tested.

Both *MTR10* (*TAB1*) and *SRP1* belong to the nuclear transport receptor/adaptor family (reviewed by [38]). Mutant *srp1-31* and *mtr10*$^{tab1\text{-}1}$ cells accumulate the ribosomal protein Rpb11b in the nucleus [39]. Thus, to evaluate a potential link between nuclear transport and the *tab* phenotype, we evaluated the localization of Rpl11b-GFP [39]



	25°C	37°C -> 25°C
tab1-1	6%	30%
net1-1	42%	55%
tab3	3%	1%
TAB5-1	0%	1%
TAB6-1	7%	7%
TAB7-1	0%	0%
srp1-31	27%	33%
+	1%	0%

Figure 6
Some of the *tab* mutants show nuclear transport defects. Strains of the indicated genetic background were transformed with a CEN/ARS plasmid expressing Rpl11b-GFP, and grown at 25°C. A portion of the cultures were then shifted to 37°C for 1 hr and shifted back to 25°C for 35 min before the 37°C -> 25°C samples were taken. (A) Rpl11 b-GFP was visualized as described previously [39]. (B) The percentage of cells (+/- 1%) with preferential nuclear Rpl11b-GFP staining in each sample was counted (n ≥ 100).

in all *tab* mutants. Mutants expressing Rpl11b-GFP were grown to early-log phase at 25°C, shifted to 37°C to induce the ts mutant phenotype (which also reduces synthesis of ribosomal proteins including Rpl11b), and shifted back to 25°C to induce new ribosomal protein synthesis. If the rate of mutant phenotype reversal is slower than restoration of ribosome synthesis, then Rpl11b should accumulate in the nucleus. Whereas Rpl11b-GFP was distributed relatively evenly in wild-type cells, it accumulated in the nucleus in a significant fraction of *srp1-31* and $net1^{tab2\text{-}1}$ cells at 25°C and after 25°C ->37°C -> 25°C shift (Figure 6; [39]). In addition, although $mtr10^{tab1\text{-}1}$ cells were relatively normal at 25°C, 30% of them accumulated nuclear Rpl11b after 25°C ->37°C -> 25°C shift (Figure 6). Thus, multiple *tab* mutants accumulate Rpl11b in the nucleus, implying that nucleocytoplasmic trafficking is involved in the regulation of mitotic exit. Other *tab* mutants (such as *tab3*, *TAB5*, $CDC14^{TAB6}$ and *TAB7*) showed no defect in this assay, suggesting that bypass *of cdc15Δ* can be achieved without perturbing nucleocytoplasmic transport.

Discussion

MEN: A signalling network that can post-translationally trigger exit from mitosis

Both transcriptional and post-translational controls have been implicated in the operation of the Mitotic Exit Network and the regulation of the anaphase/telophase -> G1 transition [40,7,19,41]. To address whether the portion of the MEN that acts downstream *of CDC15* can trigger a key aspect of the anaphase/telophase -> G1 transition (i.e. inactivation of Clb/Cdc28 via proteolysis of Clb2) in the absence of new gene expression, we evaluated the turnover of Clb2 in cells released from a *cdc15-2* arrest in the presence of cycloheximide. Interestingly, Clb2 was rapidly and efficiently degraded upon reversal of the *cdc15-2* block regardless of whether cycloheximide was present (Figure 1) although both cytokinesis and cell separation require protein synthesis [42]. This observation suggests that the exit from mitosis in budding yeast may be controlled by mechanisms fundamentally similar to those employed by animal cells, which proceed through mitosis with highly condensed, transcriptionally silent chromosomes. Interestingly, all of the MEN genes have putative homologues in the *C. elegans* genome [http://genome-www.stanford.edu/Saccharomyces/worm/] .

Isolation of tab mutants

A deliberate search for bypass mutants was a key aspect of the screen reported here, since numerous genetic interactions have been observed among components of the MEN (consult [9] for a summary). Formally speaking, it is not possible to order gene functions based on suppressive interactions involving reduction-of-function (e.g. ts) alleles. For example, the *net1-1* allele suppressed ts mutations in both its downstream target *CDC14* and its upstream regulators *CDC5*, *CDC15*, and *DBF2* (Figure 4B). Since we sought mutants that bypassed *cdc15Δ*, the *tab* genes are predicted to function either downstream of or parallel to *CDC15*, but not upstream of *CDC15*.

The tab screen yielded both dominant and recessive mutants which mapped to six linkage groups. The pattern of alleles (Table 2) does not satisfy a Poisson distribution, suggesting that the screen has not reached saturation. The recessive *tab* mutants are likely to represent reduction-of-function mutations in genes that inhibit exit from mitosis, and the dominant *TAB* mutants are likely to result from gain-of-function mutations in genes that promote exit from mitosis. This prediction is supported by our prior characterization of the *tab2-1* and *TAB6-1* alleles of *NET1* and *CDC14*, respectively (see next section). The fact that we recovered recessive, semi-dominant, and dominant mutants in additional *tab* genes suggests that there are likely to be multiple dosage-sensitive positive and negative regulatory controls that act downstream of or parallel to *CDC15*.

tab mutants can be classified into two groups: those that bypass *cdc15Δ* only in the presence of *SIC1* (e.g. *tab3-1*), and those that can bypass in the absence of *SIC1* (e.g. $net1^{tab2\text{-}1}$ and $CDC14^{TAB6\text{-}1}$). It remains unclear whether mutants distribute into these classes based on bypass

mechanism or allelic potency, but we favor the latter possibility, because some *tab1* alleles required *SIC1* to bypass *cdc15Δ*, whereas others did not (R. Azzam, unpublished data). Importantly, *tab* mutants from both classes appeared to bypass mitotic arrest in Tem1-depleted cells by enabling degradation of Clb2 and accumulation of Sic1 – two hall-marks of mitotic exit (Figure 3; [15,18]).

A genetic pathway for anaphase -> G1 transition

Biochemical and cell biological experiments indicate that Net1 acts as part of a complex named RENT that tethers Cdc14 to the nucleolus and inhibits Cdc14 phosphatase activity, and that *CDC15* and *TEM1* are required for the release of Cdc14 from Net1 at the end of mitosis [15–17,43]. Consistent with this notion, both *net1*$^{tab2\text{-}1}$ (which encodes a mutant version of Net1 with presumably reduced affinity for Cdc14) and *CDC14*$^{TAB6\text{-}1}$ (which encodes a mutant version of Cdc14 with reduced affinity for Tab2/Net1) bypass *cdc15Δ*[15,18]. Both *net1*$^{tab2\text{-}1}$ and *CDC14*$^{TAB6\text{-}1}$ impinge directly on mitotic exit, suggesting that other *TAB* genes may encode physiological regulators and effectors of the Mitotic Exit Network.

The *net1*$^{tab2\text{-}1}$ mutation efficiently bypassed *tem1Δ*, *cdc15Δ*, *dbf2Δdbf20Δ*, and *mob1Δ*, consistent with the notion that the sole essential function of all these genes is to inhibit Net1. *net1*$^{tab2\text{-}1}$ failed to bypass *cdc14Δ*, because Cdc14 is the downstream target of Net1 [15,17]. Cdc5 also regulates Net1 (Shou et al., manuscript in preparation), but *net1*$^{tab2\text{-}1}$ bypassed *cdc5Δ* with very low efficiency, presumably because *CDC5* has other essential functions [44,25,26,45].

The most parsimonious model that unifies all of these observations is as follows: Tem1 functions upstream of Cdc15, which in turn activates the Dbf2-Mob1 protein kinase complex [46–49]. Together with Cdc5, these proteins dislodge Cdc14 from nucleolar Net1. Cdc14 subsequently dephosphorylates (and thereby activates) proteins involved in Sic1 expression (Swi5) and C1b degradation (Hct1/Cdh1) [19,41,50], thereby eliminating mitotic Cdk activity and promoting exit from mitosis. The mechanisms by which Cdc5 and Dbf2/Mob1 promote the disassembly of RENT remain unknown, raising the possibility that other Tab proteins serve as intermediaries in this process. One attractive hypothesis based on our identification of *MTR10* as a *tab* gene is that MEN proteins regulate the nucleocytoplasmic distribution of a key regulator of the release process.

Chromosome instability in tab mutants

The Bub2/Bfa1 GTPase-activating protein (GAP) complex inhibits the MEN and cytokinesis in response to DNA damage and spindle misorientation ([32]; reviewed in [33]). *bub2Δ* and *CDC14*$^{TAB6\text{-}1}$ cells show similar chromosome instability as wild-type cells. In contrast, *net1*$^{tab2\text{-}1}$ cells have a dramatic chromosome instability phenotype, with some colonies showing multiple red sectors indicative of multiple chromosome-loss events, and many colonies completely red on the surface (Figure 5A). Net1 is unlikely to directly regulate transmission of the tester chromosome, because it is only localized to rDNA in the nucleolus [15–17]. Thus, the difference between *net1*$^{tab2\text{-}1}$ and *CDC14*$^{TAB6\text{-}1}$ presumably results from molecular defects unique to the former mutant, including defective nucleolar structure and function, or perhaps defective nuclear transport ([18]; Figure 6). Further study is required to reveal how the multifunctional Net1 protein helps to ensure chromosome transmission fidelity.

Multiple tab mutations influence nucleocytoplasmic transport

Nuclear transport-defective mutations in *MTR10* and *SRP1* (*mtr10*$^{tab1\text{-}1}$ and *srp1-31*, respectively) bypassed *cdc15Δ*. Mtr10 assembles with and functions as a nuclear import receptor for the mRNA-binding protein Npl3 [36,37]. Interestingly, *srp1-31* also inhibits nuclear import of multiple proteins including Npl3 [51,52]. Remarkably, reduction-of-function alleles of the karyopherin *KAP104* were also recovered in a screen for mutations that suppressed *cdc15-2*^{ts} (A. Toh-e, personal communication). *KAP104* is required for proper localization of mRNA binding proteins Nab2 and Hrp1 to the nucleus [53].

The only phenotype known to be shared by *srp1*, *mtr10*, and *kap104* mutants is accumulation of the 60S ribosomal protein Rpl11b in the nucleus [39]. *SRP1*, *MTR10*, and *KAP104* may also collaborate to maintain the proper nucleocytoplasmic partitioning of α factor (or factors) that regulates mitotic exit. An alternative possibility is that they sequester an inactive stockpile of α factor that, upon mutation or signalling by the MEN, is released from these transportins and enables exit from mitosis [54,55]. Finally, it is possible that the transportin mutants enable bypass through an indirect perturbation of nucleolar architecture (Table 2). Given that some of the MEN proteins reside in the cytoplasm whereas their target – the RENT complex – resides in the nucleolus, it is tempting to speculate that nuclear transport is a target of MEN action. Our failure to observe nuclear accumulation of Rpl11b in *tab3* and *TAB5-TAB7* mutants suggests that these *tab* genes bypassed *cdc15Δ* by a distinct mechanism. Regardless of the exact mechanisms of bypass, it is clear from these analyses that the release of Cdc14 from Net1 is more intricately connected to cell regulation than was previously imagined.

Conclusions

Three main conclusions on how exit from mitosis is regulated in the budding yeast can be derived. First, the sole es-

sential function of the Tem1, Cdc15, Dbf2/Dbf20, and Mob1 (but not Cdc5) proteins are to promote the release of Cdc14 from the nucleolus, as cells lacking these proteins can grow upon simultaneous reduction *of NET1* activity. Second, high chromosome loss rate was observed in *net1*$^{tab2\text{-}1}$ but not *CDC14*$^{TAB6\text{-}1}$, suggesting that although *CDC15*-independent mitotic exit does not dramatically affect chromosome transmission, additional functions of Net1 do. Third, both *mtr10*$^{tab1\text{-}1}$ and *srp1-31* affect nuclear transport, suggesting that nuclear transport regulates mitotic exit.

Materials and Methods

Strains and plasmids

All yeast strains used in this study (listed in Table 1) are isogenic to W303. Standard methods were employed for the culturing and manipulation of yeast [56]. All plasmid constructions were based on the pRS vector series [57]. To replace *CDC15* with *TRP1*, polymerase chain reaction (PCR) products containing *TRP1* flanked by 200-base pair (bp) homology to the 5' and 3' untranslated regions of the targeted gene were used to transform diploid strain RJD381. Correct integrants were verified by PCR using primers that amplified a DNA fragment that spanned the recombination junction. Deletions of *CDC5* and *TEM1* were carried out similarly. To construct [*pMET3-CDC15, URA3*] (pWS100) and [*pMET3-cdc15-2, URA3*] (pWS109), the corresponding genes (from start codon to 300 bps downstream of the stop codon) were amplified by PCR from genomic DNA of wild type (RJD381) or *cdc15-2* (RJD619) cells, and cloned into the SpeI and SacII sites of RDB620 (a derivative of pRS316 containing the *MET3* promoter inserted between the HindIII and EcoRI sites).

Table 1: *S. cerevisiae* strains

Strain	Genotype[a]
RJD381	*MAT**a**/MATα*
RJD619	*cdc15-2 pep4Δ::TRP1 MAT**a***
WY4	*TAB7-2 MAT**a***
WY9	*cdc15Δ::TRP1 TAB5-1 [pMET3-CDC15, URA3] [pRS315, LEU2] MATα*
WY10	*TAB5-1 [pRS315, LEU2] MATα*
WY11	*cdc15Δ::TRP1 tab2-1(net1-1) [pMET3-CDC15, URA3] MATα*
WY14	*tab3-1 MAT**a***
WY17	*cdc15Δ::TRP1 TAB6-1 [pMET3-CDC15, URA3] [pRS315, LEU2] MAT**a***
WY18	*TAB6-1MATα*
WY21	*cdc15Δ::TRP1 tab1-1 [pMET3-CDC15, URA3] [pRS315, LEU2] MATα*
WY34	*cdc15Δ::TRP1 TAB7-1 [pMET3-CDC15, URA3] [pRS315, LEU2] MAT**a***
WY38	*tab1-1 MAT**a***
WY39	*tab2-1(net1-1) MAT**a***
WY41	*cdc15Δ::TRP1 tab3-1 [pMET3-CDC15, URA3] MAT**a***
WY46	*tem1::GAL1-UPL-TEM1/TRP1 bar1::hisG MAT**a***
WY97	*tab3-1 tem1::GAL1-UPL-TEM1/TRP1 bar1:: LEU2 MAT**a***
WY214	*cdc5Δ::TRP1 [pMET3-CDC5, URA3] MATα*
WY217	*cdc15Δ::TRP1 [pMET3-CDC15, URA3] MATα*
WY218	*tem1Δ::TRP1 [pGAL1-TEM1, URA3] MATα*
WY221	*cdc15Δ::TRP1 [pMET3-cdc15-2, URA3] MATα*
WY243	*cdc14Δ::his5^{+} [HA3-CDC14, URA3] MATα*
WY280	*dbf2Δ::LEU2 dbf20Δ::TRP1 [DBF2, URA3] MATα*
WY288	*mob1Δ::HIS3 [MOB1, URA3] MATα*

[a]All strains are in the W303 background (*ade2-1 can1-100 his3-11-15 leu2-3-112 trp1-1 ura3-1*), which was provided by B. Fuller. *[ ]* indicates *CEN/ARS* plasmid. WY280 and WY288 were derived after backcrossing the original strains into W303 three times.

Isolation of tab mutants

The scheme is outlined in Figure 2. Ninety independent cultures of *cdc15Δ [pMET3-cdc15-2, URA3*] (WY221) were grown at 25°C in synthetic minimal medium + 2% glucose (SD) in the absence of methionine. For each culture, ~8 × 10^{6} cells were plated on SD+ methionine (2 mM) at 30°C (to simultaneously repress expression and partially inactivate the *cdc15-2* allele). One to two colonies were picked from each plate and tested for viability on SD+ 5-fluoroorotic acid (5-FOA) plates [58]. When more than one colony was picked from a single plate, care was taken to pick colonies of different size or morphology. Out of ~90 colonies tested, 25 survived on 5-FOA medium (Tab^{+}). PCR analysis confirmed that all twenty five colonies had lost the [*pMET3-cdc15-2, URA3*] plasmid, indicating that they were true bypassers of *cdc15Δ*.

Mutant characterization

To determine if Tab^{+} phenotypes were due to single mutations, *cdc15Δ tab* strains were crossed to *cdc15Δ [pMET3-cdc15-2, URA3]* and the resulting diploids were sporulated and dissected. Approximately 50% of the viable spores should bypass *cdc15Δ* (as indicated by 5-FOA resistance) if the Tab^{+} phenotype was due to a single mutation. Out of twenty five mutants, fifteen satisfied this criterion, and the other ten were discarded because they either failed to sporulate, or yielded few viable spores after dissection, or did not segregate as single mutations. The same crosses were also used to determine if Tab^{+} was recessive or dominant: for every *tab* mutant, haploid *cdc15Δ tab [pMET3-cdcl5-2, URA3]* and diploid *cdc15Δ/cdc15Δ tab/+ [pMET3-cdc15-2, URA3*] cells were pre-grown on YPD (1% yeast extract/2% peptone + 2% glucose) at 25°C for 2–3 days, spotted in 5-fold serial dilutions onto SD+5-FOA plates, and the number of 5-FOA resistant colonies from the diploid was divided by that from the haploid to obtain ratio P. The mutant was defined to be dominant if P= 0.1-1, semi-dominant if P= 0.01-0.1, and recessive if P < 0.01.

To introduce *tab* mutations into the wild type background, *cdc15Δ tab* strains were crossed to *CDC15 TAB*,

and *tab* spores were verified by crossing to *cdc15Δ [pMET3-CDC15, URA3]*, and demonstrating that half of the resulting *cdc15Δ* segregants were 5-FOA resistant. All *cdc15Δ tab* mutants were also crossed to strains harboring either *[pMET3-CDC15, URA3] [pRS313, HIS3]* or *[pMET3-CDC15, URA3] [pRS315, LEU2]* to introduce these plasmids. The resulting strains grew better than the original *cdc15Δ tab* strains, and harbored selectable markers that facilitated subsequent crosses.

To test whether $net1^{tab2\text{-}1}$ can bypass *tem1Δ*, *dbf2Δ dbf20Δ*, *mob1Δ*, *cdc14Δ*, and *cdc5Δ*, we crossed $net1^{tab2\text{-}1}$ (WY39) to WY218, WY280, WY288, WY243, and WY214, respectively. The diploids were sporulated, and if ~50% of the MEN-deleted spores were resistant to 5-FOA, then *net1-1* could bypass the MEN null mutation.

To test temperature sensitivity (ts) of *tab* mutants, cells were spotted or streaked onto YPD plates, and incubated at 37°C. Their growth was scored after one to two days.

Nop1 immunolocalization was performed as described earlier [15].

The chromosome loss assay was carried out using a strain [34] backcrossed into the W303 background by F. Uhlmann. When cells were pre-grown in YPD liquid (instead of SD-URA solid) media before being tested on YPD plates, the percentage of colonies that were red or had red sectors remained similar for wild-type, $CDC14^{TAB6\text{-}1}$, and *bub2Δ* strains, but was ~25% for $net1^{tab2\text{-}1}$. This number was lower than the 94% value observed in Figure 5, but still significantly higher than the wild-type value (< 5%).

Linkage analysis

To assign *tab* mutants to linkage groups, *cdc15Δ tab [pMET3-CDC15, URA3*] strains were crossed against each other, and the diploids were sporulated and dissected. If two *tab* mutations belonged to the same linkage group, then all spores were expected to survive on 5-FOA. Otherwise, approximately one quarter of the spores would fail to bypass *cdc15Δ*. The alleles of *TAB2*, *TAB3*, *TAB5*, *TAB6*, and *TAB7* (see Table 2) were assigned this way. The recessive *tab* mutants exhibited a ts growth phenotype that co-segregated with the Tab⁺ phenotype. Complementation tests were used to assign two of the ts mutations as alleles of *tab 1*. After *TAB1* was shown to be *MTR10*, other *tab1* alleles were ascertained by their linkage to the *MTR10* locus.

Molecular cloning of TAB1 and TAB2

To clone *TAB1* and *TAB2*, *tab1-1* and *tab2-1* mutant strains were transformed with plasmid libraries harboring yeast genomic DNA fragments, and incubated at 25°C for one day before being shifted to 37°C [59]. Libraries constructed by the laboratories of P. Heiter (ATCC#77164) and R. Young [60] rescued *tab1-1*, and a 2μ library (ATCC#37323, [61]) rescued *tab2-1*. Transformants (enough to cover multiple genome equivalents) were screened, and plasmids retrieved from colonies that grew at 37°C were re-transformed into the original mutant strain to verify their complementation activity. Candidate plasmids were sequenced, and the genomic regions containing the complementing activity were identified. *TAB1* resided on Chromosome XV, 631,500–637,950, and *TAB2*, on Chromosome X, 291,900–299,466. To confirm that these genomic fragments indeed carried the *TAB* genes, complementing fragments were cloned into the integrating vector [pRS305, LEU2], and linearized to target integration into the *tab* locus in a *cdc15Δ::TRP1 tab [pMET3-CDC15, URA3*] strain. The transformants lost their Tab⁺ phenotype, and when crossed to *cdc15Δ::TRP1 [pMET3-CDC15, URA3]*, the diploid yielded no spores that could bypass *cdc15Δ* (n = 40–50). Since both genomic fragments contained multiple open reading frames (ORFs), each ORF was amplified by PCR from yeast genomic DNA and cloned into the integrating plasmid pRS305. The resulting plasmids were transformed into *cdc15Δ::TRP1 tab [pMET3-CDC15, URA3*] to identify the ORF that reversed the Tab⁺ phenotype. This analysis revealed *TAB1* to be *MTR10*, and *TAB2* to be *YJL076W (NET1)*.

Gene replacement

For gene replacement, the *Schizosaccharomyces. pombe his5⁺* PCR amplification/ transformation method was used [62]. Correct integrants were verified by PCR using primers that amplified a DNA fragment that spanned the recombination junction. To assay if *SIC1* or *HCT1* was required for the Tab⁺ phenotype of *tab* mutants, *SIC1* or *HCT1* was replaced by *his5⁺* in *cdc15Δ::TRP1 tab [pMET3-CDC15, URA3*] strains. Each transformant was assayed for the occurrence of correct integration and its ability to survive on 5-FOA.

Cell cycle synchronization

Cells were grown in 1% yeast extract/ 2% peptone (YP) with 2% glucose (YPD), 2% galactose (YPG), or 2% raffinose (YPR). For the experiment described in Figure 1, *cdc15-2* (RJD 619) cells were grown to exponential phase in YPD at 25°C, and shifted to 37°C for three hours so that >95% cells were arrested as large-budded cells. Cycloheximide (CHX) was supplemented to a final concentration of 0, 10, or 100 μg/ml to inhibit translation, and α factor (10 μg/ml) was added to trap cells in the subsequent Gl phase. After five minutes, the cultures were released to 25°C at time 0. Samples were taken at various time points after release. The experiment in Figure 3 was carried out as previously described [15].

Cell extract preparation and protein detection

Detection of proteins from crude yeast extracts was as described [15]. The following primary antibodies were used in immunoblotting: anti-Sic1 (1:8000), anti-Clb2 (1:2500), anti-Cdc28 (1:3000), and 12CA5 (directed against the haemagglutinin (HA) epitope; 1:1000, with 0.5 M NaCl). The first three antibodies were raised in rabbits and affinity purified, and the last one was from mouse.

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