Whi5 and Stb1 define redundant pathways through which the G1 cyclin Cln3 promotes cell division.

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Abstract

In the budding yeast *S. cerevisiae*, commitment to cell division, Start, is promoted by a trio of G1 cyclins, Cln1, Cln2, and Cln3, that activate the CDK kinase Cdc28. The active kinases somehow activate two transcription factors, SBF and MBF, leading to induction of about 100 genes for budding, DNA synthesis, and other early cell cycle processes. Activation of the transcription factors is opposed by a repressive protein called Whi5, and also by a second repressive protein called Stb1. Both Whi5 and Stb1 contain many potential sites for phosphorylation by CDK kinase, and is thought that relief of transcriptional repression involves the phosphorylation of Whi5 and Stb1 by CDK. Phosphorylation site mutants have been studied for Whi5, but not for Stb1. Here, we create phosphorylation site mutants of Stb1, and combine them with site mutants of Whi5. We find that the G1 cyclin Cln3 activates cell cycle transcription effectively when at least one of these proteins has its phosphorylation sites. However, when both Whi5 and Stb1 simultaneously lack all consensus phosphorylation sites, Cln3 is unable, or almost unable, to induce any gene expression, or any advancement of Start. Thus the G1 cyclin signaling pathway to Start has a requirement for CDK phosphorylation sites on either Whi5 or Stb1.

Introduction.

In the yeast *S. cerevisiae*, entry into the cell cycle ("Start") is promoted by three G1 cyclins, Cln1, Cln2, and Cln3, which form active protein kinase complexes with the cyclin dependent kinase (CDK) Cdc28 (Richardson, et al. 1989, Tyers, et al. 1992). Active Cln-Cdc28 kinase results in a G1-phase burst of transcription of about 100 genes driven by the SBF and MBF transcription factors (Andrews and Herskowitz 1989a, Andrews and Herskowitz 1989b, Bean, et al. 2005, Dirick, et al. 1992, Ferrezuelo, et al. 2010, Harrington and Andrews 1996, Koch, et al. 1993). These genes have functions in cell cycle control, budding, DNA synthesis, cell wall formation, and other processes (Charvin, et al. 2010, Spellman, et al. 1998).

A key feature of this process is a positive feedback loop (Skotheim, et al. 2008). The G1 cyclin genes *CLN1* and *CLN2*, but not *CLN3*, are themselves clients of the SBF transcription factor. When SBF target genes are transcriptionally induced, *CLN1* and *CLN2* are induced, and they in turn further activate SBF. This positive feedback loop between *CLN1* and *CLN2* on the one

hand, and SBF on the other, contributes to the rapid, switch-like onset of Start, and to the irreversibility (approximately) of Start (Charvin, et al. 2010, Skotheim, et al. 2008).

However, *CLN3* is not a client of either SBF nor MBF. It is thought that Cln3-Cdc28 may provide much of the initial kinase activity that leads to activation of the feedback loop (Cross and Tinkelenberg 1991, Tyers, et al. 1993). Exactly how it does this, and how Cln3-Cdc28 activity becomes elevated to turn the feedback loop from "off" to "on", are unclear.

SBF- and MBF-dependent transcription is opposed by several transcriptionally repressive proteins. These include proteins that are transcriptionally-repressive for many classes of genes, such as the histone deacetylase Rpd3, and its targeting subunit Sin3 (de Bruin, et al. 2008, Huang, et al. 2009, Kasten, et al. 1997, Wang, et al. 2009). There are also at least two proteins, Whi5 and Stb1, that seem to be specifically involved in repressing transcription from SBF and MBF genes—that is, Whi5 and Stb1 are SBF and MBF-specific transcriptional repressors (Costanzo, et al. 2004, Costanzo, et al. 2003, de Bruin, et al. 2008, de Bruin, et al. 2004, Ho, et al. 1999, Kasten and Stillman 1997, Takahata, et al. 2009). In a general sense, Start is opposed by repressors such as Whi5 and Stb1, and promoted by activators such as Cln1, Cln2, Cln3, and Bck2, and occurs when the activators somehow overwhelm the repressors. This occurs in a cell size and growth dependent way.

One kind of experiment that shows the balance between the activators and repressors is that the double mutant *cln3 bck2*, which lacks two major activators, is inviable with an arrest at Start (Epstein and Cross 1994). However, inviability can be suppressed either by a *whi5* deletion, or an *stb1* deletion (that is, both the *cln3 bck2 whi5*, and the *cln3 bck2 stb1* triple mutants are alive) (Wang, et al. 2009). This result suggests that to some extent, Whi5 and Stb1 are similarly-potent repressors of Start.

A striking feature of both Whi5 and Stb1 is that they contain a large number (12 or 18, respectively) and very high density of putative sites for phosphorylation by CDK. This suggests that activators such as Cln1-, Cln2-, and Cln3-Cdc28 may inactivate Whi5 and Stb1 by phosphorylating them. Whi5 mutants lacking phosphorylation sites have been studied, and there is good evidence that Whi5 is inactivated by phosphorylation (Wagner, et al. 2009), and that the phosphorylated Whi5 leaves SBF/MBF promoters and moves to the cytoplasm (Costanzo, et al. 2004, de Bruin, et al. 2004). A plausible and popular model is that Whi5 is initially phosphorylated by Cln3-Cdc28 (de Bruin, et al. 2004), but there is no direct evidence for this, and indeed Koiviomagi et al. (Koivomagi, et al. 2021) have recently argued against this view.

Stb1 is a second repressive modulator of Start (Costanzo, et al. 2003, de Bruin, et al. 2008, Ho, et al. 1999, Kasten and Stillman 1997, Takahata, et al. 2009, Wang, et al. 2009). It is somewhat less studied, and has a more complex phenotype than Whi5, in that, depending on the situation and on what is being assayed, it has an ability to activate Start as well as to repress it (Costanzo, et al. 2003, de Bruin, et al. 2008, Ho, et al. 1999). *stb1* mutants have less of a peak of SBF/MBF transcription, consistent with reduced repression pre-Start, and reduced induction post-Start

(de Bruin, et al. 2008). Exactly like Whi5, Stb1 is nuclear in G1 before Start, and then moves to the cytoplasm after Start (Youn, et al. 2017). Exactly like *whi5*, an *stb1* deletion suppresses inviability of *cln3 bck2* (Wang, et al. 2009*)*. The relationship between the repressive activities of Whi5 and Stb1 is unclear. Phosphorylation site mutants of Stb1 have not been studied.

Here, we make phosphorylation site mutants of Stb1, and combine them with mutants of Whi5. We find that the signaling pathway from Cln3 to Start is intact if at least one of these two proteins has phosphorylation sites. However, Cln3 is impotent to induce either Start, or SBF/MBF transcription, if both Whi5 and Stb1 lack phosphorylation sites. As far as we have observed, Cln3 has little effect of any kind in a strain where both Whi5 and Stb1 lack their phosphorylation sites.

Materials and Methods.

Yeast strains and media

Strains used in this study were derived from BY4741 and BY4742 strains and are listed in Table 1. Cells were grown in standard YEPD medium containing yeast extract (1%), peptone (2%), and glucose (2%). For galactose-induced experiments, cells were grown in media containing 2% raffinose and then inoculated into 1% galactose 1% raffinose media.

*whi5** (phosphorylation site mutant allele of *WHI5*) was generated by transforming *whi5Δ::KanMX6* with plasmid pGZ110-G418 (expressing both Cas9 nuclease and guide RNA targeting the Kan locus) in the presence of a linear PCR product *whi5-18Ala* (repair DNA for indigenous homologous recombination) amplified from pRS413 *MET3-whi5-18Ala* plasmid (a gift from Steven F. Dowdy). To construct *stb1** (phosphorylation site mutant allele of *STB1*), strain *stb1Δ::KanMX6* was co-transformed with plasmid pGZ110-G418 and a BglII cut linear plasmid providing the donor DNA, *stb1-18Ala*, for the repair of double strand break caused by Cas9. Stb1-18Ala is a synthetic DNA constructed by Genscript, which replaces all Ser and Thr residues shown in yellow in Fig. 1 with Ala. Presence of all phosphorylation site mutants in both *whi5** and *stb1** alleles was confirmed by sequencing. The *whi5* stb1** strain was generated by crossing *MATα whi5** with *MATa stb1**. *GAL-CLN3* alleles were constructed by integration of pFP2-2 plasmid into *CLN3*, which marks the locus with *URA3* marker and simultaneously inserts the *GAL1-10* promoter in front of *CLN3*.

We have no direct evidence as to whether the phenotypes of *whi5** or *stb1** are dominant or recessive to wild-type (though they are dominant over a null). *whi5** has very little phenotype of any kind on its own, and *stb1** could possibly have both dominant and recessive phenotypes. In the absence of direct evidence, we have named them with lower case letters.

Cell Size Measurements

Yeast cells were grown in YEPD overnight at 30°C. Saturated cultures were diluted (1:50 v/v) with fresh medium and incubated again at 30°C until cells reached early log phase ($1*10^6$ to $2*10^7$ cells/ml, but generally the higher end of that range) in about 6-7 hours. Cells were diluted (1:100 v/v) in Isoton buffer, then briefly sonicated. Cell size analysis was performed on

asynchronous cultures with a particle size counter (Coulter Z2, Beckman Coulter) running Z2 AccuComp software. In Fig. 2 and Table S1, we report the mean cell volume, as determined by the AccuComp software.

Elutriation

Cells with inducible *GAL-CLN3* allele were grown in YEP with 2% filter-sterilized raffinose to early exponential phase. Cells were harvested by spinning at 3000 rpm and briefly sonicated. Small unbudded G1 cells were isolated by centrifugal elutriation. Elutriated cells were allowed to recover for about 15 min at room temperature, harvested, resuspended in fresh YEP, and split into two halves. To one half of the culture in YEP, raffinose was added to 2% final concentration and to the other half both 1% raffinose and 1% galactose (final concentration) were added. Both cultures were incubated at 30°C in a shaking water bath and samples were collected at 15 min intervals (with a few additional samples at 7.5 min intervals). Cell size distributions were determined on a Z2 Coulter Counter. Cells for budding index calculations were kept on ice, then the percentage of budded cells was determined by counting the cells with visible buds in a minimum of 200 cells under microscope. Samples for the RNA-Seq experiment were chilled to 0°C, centrifuged at 3000 rpm at 4°C and the resulting cell pellets were snap frozen in liquid nitrogen prior to storing at -70°C until processing. Elutriation experiments were done two to four times with each strain, but the RNA-seq experiment was done only once, on the "best" experiment (see text).

RNA-Seq

Time course samples from the elutriation experiment were spun down, washed twice with ice cold water and frozen at -70C. Prior to RNA extraction, the frozen pellets of cells were resuspended in TES buffer and processed with hot phenol RNA extraction method described in Collart and Oliviero (Collart and Oliviero 2001). RNA-seq libraries were prepared with the Ovation Universal RNA-Seq System (NuGEN). The quality control analysis of libraries was performed on an Agilent Bioanalyzer. The libraries were multiplexed and sequenced by standard methods on an Illumina sequencer.

Results.

Creation of an allele of *STB1* lacking all 18 SP/TP putative CDK consensus phosphorylation sites.

Cyclin-dependent kinases typically phosphorylate serine or threonine followed by proline (i.e., SP or TP). Preferred sites have a basic residue at the +4 position (e.g., TPxK), while a basic residue at the 3 or 5 position is also somewhat favorable. Stb1 has 18 SP or TP motifs, a large number of sites for a 420 aa protein. Of these 18, 5 are preferred sites (S/T-P-x-K/R), while an additional 6 have a basic residue at the 3 or 5 position. We created a synthetic gene that encodes alanine instead of serine or threonine at all 18 of these putative CDK sites. We call this allele "*stb1**" ("stb1 Star") (Fig. 1). In this work, we use three alleles of *STB1*: the wild-type

(*STB1*), the null (*stb1*, or *stb1Δ*), and *stb1**, the phosphorylation site mutant. This work uses a genetic approach—we made mutations, and assayed their phenotypes. We did not use biochemical assays of phosphorylation, so we make no claims about the actual phosphorylation, or lack thereof, of any protein.

An allele of *WHI5* lacking CDK consensus phosphorylation sites.

Whi5 (296 amino acids) contains 12 SP or TP sites, 4 of which have the full consensus (S/T-P-x-K/R), and a further 4 of which have a basic residue at the 3 or 5 position. In overall density of putative CDK sites, Whi5 is very similar to Stb1. Wagner et al. (Wagner, et al. 2009) have studied the phosphorylation of Whi5, and find that an additional 6 serine residues may be phosphorylated in a CDK-dependent way i*n vitro*, even though they are not in a CDK consensus sequence. For our studies, we used an allele of *WHI5* created by and obtained from Wagner et al. in which all 18 putative phosphorylation sites (that is, all 12 in a CDK consensus, plus 6 not in a consensus) were destroyed by substituting the phosphorylatable residues with alanine. Wagner et al. refer to this allele as *WHI518A*; in congruence with our *STB1* nomenclature, we refer to this same allele as *whi5** ("WHI5 Star") (Fig. 1).

Genetic interactions between alleles of *CLN3*, *WHI5*, and *STB1* for cell size.

We created and sequence-validated yeast strains carrying *whi5Δ*, *whi5**, *stb1Δ*, and *stb1**, and crossed these strains with each other and with strains carrying *CLN3* or *cln3Δ*. Initially, we used cell size (as measured with a Beckman-Coulter Channelyzer) to assay ability of cells to undergo Start. The simple preliminary expectation is that deletion of an inhibitor of Start (putatively, *WHI5* or *STB1*) should result in small cell size, while creating a non-phosphorylatable allele of an inhibitor (putatively, *whi5** or *stb1**) should result in large cell size, because the inhibitor cannot be inactivated. Results are shown graphically in Fig. 2, and full numerical results are presented in Table S1.

Consistent with expectations and published results, *cln3Δ* cells are very large (67 fL) compared to WT (42.5 fL) (Nash, et al. 1988), while *whi5Δ* cells are small (34 fL) (Costanzo, et al. 2004, de Bruin, et al. 2004). Also largely consistent with the literature (de Bruin, et al. 2008), *stb1Δ* cells are slightly larger than WT (45.9 fL vs 42.5 fL, p-value = 0.007). Although consistent with the literature, the slightly large size of *stb1Δ* cells is surprising for a gene thought to be an inhibitor of Start; deletion of an inhibitor ought to make cells small, as in the case of *whi5Δ*. In agreement with de Bruin et al., we believe the solution to this paradox is that Stb1 is both an inhibitor (perhaps in the unphosphorylated form) and also an activator (perhaps in the phosphorylated form), and in the *stb1Δ* strain, these two effects largely cancel, yielding an almost wild-type size (Discussion). Consistent with the idea that unphosphorylated Stb1 is an inhibitor, the *stb1** strain has large cells (53.25 fL).

Surprisingly, though in good agreement with previous work (Wagner, et al. 2009), *whi5** cells are not significantly different from wild-type in size (WT mean 42.5 fL, *whi5** mean 43.1 fL, p-

value = 0.30). This is unexpected in terms of a theory suggesting that the Whi5 inhibitor is inactivated by phosphorylation, as the Whi5* protein ought to be permanently inhibitory, yielding large cells. Superficially, this result challenges the theory that unphosphorylated Whi5 is an inhibitor of Start. However, this particular protein is not wild-type Whi5—it is a mutant with 18 ser or thr to ala substitutions, and as such may be defective in Whi5 function. For instance, it may have a short half-life (Discussion). Alternatively, it could be true that inactivation of the inhibitory effect of Whi5 does not require phosphorylation of Whi5, at least on these sites.

In current models of Start, Cln3 initiates a positive feedback loop for expression of Cln1 and Cln2, and the resulting G1 CDK activity causes phosphorylation of Whi5, and perhaps Stb1, relieving their repressive effects on downstream target genes. In this model, one expects a *cln3* null mutant to be largely epistatic to *whi5* stb1** mutations (but not necessarily perfectly epistatic: a *cln3* null is alive, implying that Cln1 and Cln2 can also inactivate Whi5 and Stb1). These predictions are also supported by our data, in that the *cln3 whi5**, *whi5* stb1**, and *cln3 stb1**, are all similar in size to the *cln3Δ* mutant. They are not exactly the same, however; *cln3 stb1** cells are distinctly larger than *cln3Δ* cells, suggesting that inactivation of Stb1* is a challenge for the cell. The *cln3 whi5** cells are distinctly smaller than *cln3Δ* cells, again consistent with the idea that Whi5* is a defective protein, or that phosphorylation is not required to inactivate Whi5.

Overall, these results are consistent with previous results and existing models, but provide extra support for the idea that, while unphosphorylated Stb1 is repressive, phosphorylated Stb1 may an activator of gene expression (otherwise the *stb1Δ* cells would be small). Also, the fact that Whi5* cells have size indistinguishable from WT requires explanation.

Whi5 and Stb1 provide redundant, alternative routes to Start.

To look more directly at the signaling pathway from Cln3, through Whi5 and Stb1, to Start, we assayed the ability of an inducible allele of *CLN3* to promote budding as a function of *WHI5* and *STB1* genotype. In these experiments, we used cells containing *GAL-CLN3*. Elutriation was used to obtain ~95% pure cultures of G1 cells in the absence of *GAL-CLN3* expression. After elutriation, galactose was added to induce expression of *GAL-CLN3*, and budding was assayed as a function of time and cell size.

The elutriation experiments were done two to four times for the different strains. Such elutriation experiments vary in quality, in that there is always some percentage of either budded, or dead, cells in the "unbudded" fraction, and in different experiments the co-efficient of variation for cell size can be larger or smaller in the unbudded fraction. For each genotype, we chose the "best" experiment (in terms of a low percentage of dead or budded cells in the "unbudded" fraction; and a small co-efficient of variation of cell size) of two to four elutriation experiments for the most extensive analysis (Fig. 3, 4). However, results appeared similar for all repeats of an experiment for a given genotype. Different genotypes necessarily have different starting cell sizes, and Figure 3 is drawn so as to align cells of similar size.

As previously shown, *GAL-CLN3* cells are exquisitely sensitive to galactose; exposure to galactose induces expression of *CLN3*, and cells bud very quickly afterwards, with minimal need for growth (Schneider, et al. 2004). The same is true of *GAL-CLN3 whi5** cells, and *GAL-CLN3 stb1** cells. That is, neither the *whi5** mutation, nor the *stb1** mutation (on their own) significantly decrease signaling from CLN3 to Start, fully consistent with the cell sizes of these mutants. The inducibility of Start in the top three genotypes of Fig. 3 (WT; *whi5**; *stb1**) appears similar by eye; it is perhaps not valid to make very quantitative comparisons, because the starting cell sizes for the three genotypes are necessarily different, and so the activities of other important regulators such as *CLN1*, *CLN2*, and *BCK2* are likely different.

In striking contrast, the *whi5* stb1** double mutation almost completely abrogates the ability of *GAL-CLN3* to induce Start. There is a period (about 4 fL of cell growth, about 15 minutes) in which galactose fails to induce any budding; and even when budding does occur, it is only slightly to modestly higher in the cells treated with galactose than in the untreated cells. The galactose induces slightly faster cell growth, and this could itself be favorable for Start, and could possibly be sufficient to explain the difference between the +Gal and -Gal curves.

The x-axis on Fig. 3 is cell size. All eight cell cultures are adding biomass at about the same rate (though slightly faster for the +Gal cultures than the -Gal cultures), and when budding is plotted against time instead of size (not shown), results appear very similar.

Thus, the phosphorylation sites on Whi5 and Stb1 appear to be redundant, alternative routes by which *CLN3* can induce Start. There is no indication that one route is preferred over the other. However, when the phosphorylation sites on both proteins are simultaneously absent, *CLN3* has little (if any) ability to induce Start.

Whi5 and Stb1 provide redundant, alternative routes to *CLN3*-induced expression of SBF and MBF genes.

CLN3 induces Start by inducing expression of genes that are under control of the SBF and MBF transcription factors. Using RNA-Seq, we looked directly at the expression of these genes in the elutriated cells shown in Fig. 3. For each pair of +Gal and -Gal cultures in Fig. 3, we chose the first sample of +Gal cells that had at least 50% budding, and compared it to the equivalent -Gal sample (equivalent in terms of time). mRNAs were quantitated by RNA-Seq. We concentrated on genes thought to be controlled by SBF or MBF. Data for all such genes were gathered and analysed. For each SBF or MBF gene, and for control galactose-inducible genes (*GAL1*, 10, 7, 2, and 3), we took the normalized read count for the +Gal culture, and divided by the normalized read count for the -Gal culture. The log2 of this ratio was then expressed as a color (i.e., as a heat map), with positive scores red, and negative scores green. Because it has been suggested that Stb1 might be specific for MBF genes (Costanzo, et al. 2003), we considered SBF-specific genes, MBF-specific genes, and SBF and MBF shared genes separately. Results are shown graphically in Fig. 4 and summarized numerically in Table 2, and full numerical results are given in Tables S2 and S3.

Essentially all the SBF, MBF, and shared SBF/MBF genes were strongly induced by *CLN3* in the wild-type culture. They were also strongly induced in the *whi5** culture, and also in the *stb1** culture. In particular, the SBF genes behaved similarly to the MBF genes, and there was no indication that *STB1* is specific for MBF genes, consistent with de Bruin et al. (de Bruin, et al. 2008) (Table 2).

We could see no clear difference between the WT, *whi5**, and *stb1** cultures, either in the strength of gene induction, or in which genes were induced. After quantitation, it initially appeared that the MBF genes were more highly induced in WT cells (mean log2 = 1.49) than in the *whi5** cells (mean log2 = 0.79) (Table 2). However, there was also a difference in the induction of the control *GAL* genes in the WT and whi5* experiments (mean log2 = 4.0 vs mean log2 = 1.8) (Table 2). After normalizing for induction of the *GAL* genes, the MBF genes now appear slightly less induced in the WT than in the *whi5** (mean normalized log2 WT = 0.37 vs mean normalized log2 *whi5** = 0.43) (Table 2). The elutriation experiment is technically challenging and some variation is to be expected. In particular, the elutriated cell fraction used for the *whi5** experiment contained exceptionally small cells (Fig. 3), and this could reduce gene induction. Given the fact of variation, we cannot conclude that there is any difference between the induction of any of these genes between these three genotypes.

In contrast to these results with the WT, *whi5** and *stb1** genotypes, we could not see significant induction of these genes in the *whi5* stb1** double mutant genotype. In fact, after quantitation, whether normalized by the *GAL* genes or not, it appears that the SBF and MBF genes are slightly but significantly repressed when *CLN3* is turned on (Table 2). It is difficult to interpret this apparent repression, but certainly there is much less induction in the *whi5*stb1** genotype than in any of the other genotypes, consistent with the failure of galactose to significantly induce Start in *whi5* stb1** (Fig. 3). Thus it appears that in the simultaneous absence of these putative CDK phosphorylation sites from both Whi5 and Stb1, that Cln3 is unable to induce gene expression.

Discussion.

Stb1 is likely an actipressor.

Stb1 has been studied less than Whi5. This may be partly because the phenotype of *stb1* is mild, with only a small effect on cell size. In addition, its phenotype is confusing, in that some assays show evidence that Stb1 is an activator of Start, while others show evidence that it is an inhibitor (Costanzo, et al. 2003, de Bruin, et al. 2004, Ho, et al. 1999, Wang, et al. 2009). In contrast, *whi5* cells have a clear small-cell phenotype, entirely consistent with a role as an inhibitor of Start. On the basis of our results here, combined with the many previous results cited above, we believe Stb1 is a more-or-less equal partner to Whi5 as a potent inhibitor of Start. Its more complex phenotype may be because Stb1 has other functions. In particular, like de Bruin et al. (de Bruin, et al. 2008), we suggest that after Start (and, presumably, after phosphorylation of Stb1), it changes from being an inhibitor to being an activator (an

"actipressor" (Leatherwood and Futcher 2010)), and can help activate expression of SBF and MBF genes.

The signaling pathway from Cln3 to Start requires the putative CDK phosphorylation sites on Whi5 or Stb1.

The results here suggest that Whi5 and Stb1 define are two different, parallel, redundant, roughly equal pathways by which a signal can be transmitted from Cln3 to Start. Cells with wild-type *WHI5*, but *stb1**, can respond to Cln3 both by budding and by gene expression; cells with *whi5** but wild-type *STB1* can likewise respond to Cln3 both by budding and by gene expression; while double mutant *whi5* stb1** mutants seem to be incapable of responding to *CLN3* at all.

As discussed above, it is surprising that *whi5** mutant cells have the same size as WT cells (Fig. 2; (Wagner, et al. 2009)). Possibly the Whi5* protein turns over rapidly. However, these results have been gathered in an *STB1* WT background, where the signaling pathway from Cln3 via *STB1* is intact. Possibly *whi5** mutants would have the expected large cell phenotype in an *stb1*Δ background.

Mechanistically, how does Cln3 work? Does Cln3 phosphorylate Whi5 and/or Stb1?

An obvious and popular model is that the Cln3-Cdc28 kinase complex phosphorylates Whi5 to inactivate it and relieve transcriptional repression (de Bruin, et al. 2004), just as Cyclin D-CDK4 is thought to phosphorylate Rb in mammalian cells. However there is no direct evidence for this—Whi5 has never been shown to be a substrate of Cln3-Cdc28.

Indeed, the kinase activity of the Cln3-Cdc28 complex has always been problematic. Cln3 is a very non-abundant protein (about 100 molecules per cell (Cross, et al. 2002), with a very short half-life (Tyers, et al. 1992), so biochemistry is difficult under the best circumstances. In a fully wild-type setting, it has been difficult to show Cln3-Cdc28 kinase activity at all in yeast extracts. However, when *CLN3* is over-expressed from the *GAL* promoter, or when the protein is stabilized using the *CLN3-1* mutant (which removes the C-terminal, destabilizing region), a *CDC28*-dependent kinase activity can be seen (Tyers, et al. 1992). Strangely, a *cdc34* mutation (a ubiquitin-conjugating enzyme) increases kinase activity about 10-fold, while increasing protein abundance only about two-fold. The Cln3-Cdc28 kinase has a co-precipitated substrate of about 45 kDa, not seen with other Cdc28 complexes (Tyers, et al. 1992). 45 kDa is roughly the size expected of phosphorylated Whi5 (33 kDa, unphosphorylated), but preliminary results suggest p45 is still present in Cln3 immunoprecipitates even in a *whi5*Δ strain (BF, unpublished). Unphosphorylated Stb1 is 46 kDa, but whether p45 could be Stb1 has not been tested.

Because the kinase activity of Cln3-Cdc28 is apparently weak, it has been worth considering other models for activation of gene expression via Cln3. Two-hybrid fusions showed that Cln3 is a powerful transcriptional transactivator. This is partly due to the PEST-rich C-terminal tail of Cln3, and partly due to other sequences. Wijnen et al. (Wijnen, et al. 2002) created mutants of *CLN3* that lacked transcriptional transactivating activity. These mutants were still capable of rescuing the viability of a *cln3 bck2* mutant, though they were not quite wild-type in this regard. This result is somewhat ambiguous: it suggests the transactivation activity of Cln3 is not essential for Cln3 function, but does not show whether the transactivation activity might nevertheless contribute to the ability of Cln3 to induce gene expression.

Recently, Koivomagi et al. (Koivomagi, et al. 2021) using biochemical approaches, failed to find *in vitro* evidence that Cln3-Cdc28 could directly and significantly phosphorylate Whi5. Instead, they found by screening candidates that Cln3-Cdc28 could phosphorylate serine 5 of the C-Terminal Domain (CTD) of RNA polymerase II. This CTD is essential, and consists of about 25 repeats of the sequence YSPTSPS. Serines 2 and 5 are frequently phosphorylated, and the key protein kinases phosphorylating them are likewise essential. The cyclin-CDK complex Ccl1-Kin28 is important for phosphorylating Serine 5, a serine in a context favorable for phosphorylation by a CDK (. . . SP . . .). Thus Koivomagi et al. suggest that Cln3-Cdc28 can "replace" Ccl1-Kin28 at SBF-regulated promoters by phosphorylating Ser5, and that this is the mechanism by which Cln3 activates gene expression. We note, however, that in a biochemical screen for substrates, the RNA polymerase II CTD has a built-in advantage, in that there are ~25 copies of the repeat.

The role of RNA pol II CTD phospho-Ser5 appears to be to promote 5' capping of mRNA (Cho, et al. 1997, McCracken, et al. 1997). That is, phosphorylation of Ser5 may not promote transcription as such—instead it promotes translation. Although Koivomagi et al. do not discuss this in detail, a fuller version of their model might be that Cln3-Cdc28 phosphorylates Ser5 of the RNA polymerase CTD at SBF-regulated genes, and in that way promotes the translation of the few constitutive transcripts. This would include transcripts of *CLN1* and *CLN2*; newly translated Cln1 and Cln2 could then phosphorylate Whi5, which would then allow transcriptional up-regulation.

Our results are highly consistent with the older model in which Cln3-Cdc28 phosphorylates and inactivates Whi5, except that we would extend this phosphorylation to Stb1. In the case of Stb1, we suggest the phosphorylation might convert Stb1 from a repressor to an activator. However, our results are also consistent with the model of Kovoimagi et al., again with the addition of the idea that either Cln3-Cdc28 or Ccl1-Kin28 must also phosphorylate Stb1.

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Table 1. Plasmids and strains

Name	Genotype	Source
Plasmids		
pFP2-2	*GAL::CLN3 URA3*	B. Futcher
pGZ110-G418	pRS425 CRISPR-Cas9 G418 gRNA	G. Zhao and B. Futcher (Unpublished data)
pSH150	pRS413 *MET3-WHI5*-18Ala	Steven F. Dowdy
pSTB1*-180-CSBN	*stb1**, S and T to A substitutions	Genscript
Strains BY 4741/4742		
YSH 226	BY4741 *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
YSH 227	BY4742 *MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
YSH 571 P4/1	*MATa stb1**	This study
YSH 571 P4/3	*MATa stb1**	This study
YSH 573	*MATα whi5**	This study
YSH 574/1A	*MATa/MATα whi5* stb1**	This study
YSH 574/3B	*MATa/MATα WHI5* STB1**	This study
YSH 574/P4/1-3A	*MATa/MATα WHI5* STB1**	This study
YSH 578	*MATα* cln3Δ::kanMX6	Deletion collection
YSH 585/C2	*MATa WHI5**	This study
YSH 587/B4	*MATa/MATα WHI5** cln3Δ::kanMX6	This study
YSH 587/C3	MATa/MATα *WHI5** cln3Δ::kanMX6	This study
YSH 588/P4/1-1A	*MATa/MATα STB1** cln3Δ::kanMX6	This study
YSH 588/C1	*MATa/MATα STB1** cln3Δ::kanMX6	This study
YSH 589	*MATa* whi5Δ::kanMX6	Deletion collection
YSH 591	*MATα* whi5Δ::kanMX6	Deletion collection
YSH 594	*MATa* stb1Δ::kanMX6	Deletion collection
YSH 595/1	*MATa GAL1-CLN3 URA3*	This study
YSH 597/3	*MATa STB1* GAL1-CLN3 URA3*	This study
YSH 599	*MATα WHI5* GAL1-CLN3 URA3*	This study
YSH 600/7	*MATa/MATα WHI5* STB1* GAL1-CLN3 URA3*	This study

Table 2. Summary Statistics on SBF/MBF Gene Expression

		WT	whi5*	stb1*	whi5*stb1*
SBF Genes	Mean	1.43	1.16	1.15	-0.30
	SD	0.99	1.36	0.72	0.50
	SE	0.10	0.14	0.07	0.05
	p-val(0)	10^{-24}	10^{-13}	10^{-27}	10^{-8}
MBF Genes	Mean	1.49	0.79	1.30	-0.36
	SD	0.75	1.46	0.64	0.41
	SE	0.07	0.14	0.06	0.04
	p-val(0)	10^{-40}	10^{-7}	10^{-40}	10^{-3}
GAL Genes	Mean	4.04	1.83	2.49	4.03
	SD	0.66	0.42	0.49	1.36
	SE	0.30	0.19	0.22	0.61
SBF Norm.		0.35	0.64	0.46	-0.08
MBF Norm.		0.37	0.43	0.52	-0.09

Summary statistics on SBF/MBF gene expression in four genotypes. Values are log2(Exptl./Control), where experimental is the galactose-induced culture. SD is the standard deviation of individual genes; SE is the standard error of the mean. P-val(0) is the p-value of the difference between mean induction or repression, and zero change. For example, the SBF genes in the WT culture have a p-value of about 10^{-24} for induction above 0. SBF Norm and MBF Norm are the mean induction of the SBF and MBF genes, respectively, divided by (i.e., normalized by) the mean induction of the GAL genes in the same culture. Negative values show repression.

Figure Legends.

Fig. 1. Mutation of putative CDK phosphorylation sites of Stb1 and Whi5.

The amino acid sequences of Stb1 and Whi5 are shown. All putative CDK consensus phosphorylation sites (SP or TP) are shown in yellow; other residues known to be phosphorylated in Whi5 are shown in green. In the STB1* mutant, all Ser and Thr residues in the yellow-colored regions were converted to Ala. Similarly, all putative phosphorylation sites in Whi5 were removed by Wagner et al.

Fig. 2. Cell Volumes of Mutants.

The mean cell volumes of various mutants were measured with a Z2 Beckman-Coulter Channelyzer (see Methods and Materials). Relevant genotypes are indicated along the X-axis. Each vertical bar is an independent measurement of the mean cell volume of a single culture. Each "forest" of vertical bars is a set of independent measurements of independent cultures of the same starting strain (i.e., technical replicates). The mean cell volume (in fL) of each "forest" of measurements is given above the bars of the forest. Different "forests" of bars, but of the same genotype, represent measurements of independently-constructed strains (i.e., biological replicates). For example, two different *whi5* strains were constructed; in each case 6 cultures were measured; the mean of the six measurements of the first strain was 33.8 fL, while the mean of the six measurements of the second strain was 33.9 fL. Selected p-values (t-test) for relevant pair-wise differences are given in the text. Full numerical values are given in Table S1, so that other statistical comparisons can be made as desired.

Fig. 3. Ability of *CLN3* to promote budding in various genotypes.

Small, unbudded, G1-phase cells of four different genotypes were obtained by elutriation from 2% raffinose medium (Methods and Materials). After 15 minutes of recovery, the culture was split into two aliquots, and galactose was added to 1% to one aliquot (see Materials and Methods for details). Budding was then monitored as a function of both time and cell size (assayed using a Z2 Beckman-Coulter Channelyzer). Starting cell sizes were different for each genotype, partly as a result of genotype but also partly due to the outcome of the particular elutriation; results are plotted so as to align cells of similar sizes. In the *whi5* stb1** experiment, samples were taken at 0, 15, 30, 45, 52.5, 60, 75, 82.5, 90, 105, and 120 minutes.

Fig. 4. Ability of *CLN3* to promote SBF/MBF gene expression in various genotypes.

For each genotype (WT, *whi5**, *stb1**, *whi5* stb1**, sub-columns 1, 2, 3, and 4 respectively), the first galactose-induced sample in the elutriation of Fig. 3 to pass 50% budding was analyzed for gene expression using RNA seq. The equivalent (in time) un-induced sample was also analyzed.

Normalized read counts for the induced sample (E, experimental) were divided by normalized read counts of the uninduced sample (C, control), and log2 of the ratio was calculated. This log2(E/C) ratio is used in Table 1, and is expressed here in Fig. 4 as a color, with reddest red 4-fold up (log2 = 2) and greenest green 4-fold down (log2 = -2). SBF and MBF-regulated genes were selected from the genome, and shown here in super-columns as genes regulated by SBF, genes regulated by MBF, and genes regulated by both SBF and MBF (e.g., *POL30* is regulated by both SBF and MBF, so it appears in all three columns). At the bottom of each column are *GAL1*, *GAL10*, *GAL7*, *GAL2*, and *GAL3*, which serve as positive controls for galactose induction.

A. Stb1

MSQPQM**SP**EKEQELASKILHRAELAQMTRQLKLGLSNVPSTKRKQDSTTKKRSGEDAEDV
DEDHKTLLEAI**SP**AKKPLHDDTNKMTVI**SP**VKFVEKPN**TP**PSSRQRKAEDRSQQIKPRKD
TPS**TP**RASA**TP**IILPHASSHYQRPHDKNFM**TP**KRNNNNSSNHSNNNNNIKKKAAGSKDAP
QDSDNTAGADLLMYLAT**SP**YNKSSHHG**TP**MAVRMPT**TP**RSYHYASQLSLNGNTASTSNDA
VRFSHIKPSAS**SP**QSTFKSNLLPNFPDESLMD**SP**SLYLSNNNGSVQATL**SP**QQRRKPTTN
TLHPPSNVPT**TP**SRELNGTNFNLLR**TP**NFNMGDYLHNLF**SPSP**RVPAQQGASNTSASIPS
VPAMVPGSSSNTSAIATAAISSHTTNNFLDMNANGIPLIVGPGTDRIGEGESIDDKLTD

B. Whi5

MSLR**TPKR**SRTSDEQEQEQEQEQVQNPDTHVNNEHQQRPGPTTLLS**TPVR**LKNGFG**TPSP**
P**SP**PGITKSITKSRRRPSTTSLQGIFM**SP**VNKRRVGITAHGRVYDHNDDGHE**S**E**S**EDDEN
EEENENQKKYDGHVSMPLLPPT**TP**KSRR**S**EVFL**SPSP**RLR**SP**PTAARRSTGERPIREISH
TLRTRLNYALVKLQNGWTDKTLPELETELAPAVQ**TPPR**RYHNRFPDSADAGTSAHTAFLQ
ALGGHPPREEATAVETLMLLS**SPTK**KQQHRPVPAT**S**AGEP**T**DETEPE**S**DTEVETS

CDK Sites
Non-CDK Sites

Cell Volumes of Mutants