Recruitment of Cln3 Cyclin to Promoters Controls Cell Cycle Entry via Histone Deacetylase and Other Targets

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Abstract

In yeast, the G1 cyclin Cln3 promotes cell cycle entry by activating the transcription factor SBF. In mammals, there is a parallel system for cell cycle entry in which cyclin dependent kinase (CDK) activates transcription factor E2F/Dp. Here we show that Cln3 regulates SBF by at least two different pathways, one involving the repressive protein Whi5, and the second involving Stb1. The Rpd3 histone deacetylase complex is also involved. Cln3 binds to SBF at the CLN2 promoter, and removes previously bound Whi5 and histone deacetylase. Adding extra copies of the SBF binding site to the cell delays Start, possibly by titrating Cln3. Since Rpd3 is the yeast ortholog of mammalian HDAC1, there is now a virtually complete analogy between the proteins regulating cell cycle entry in yeast (SBF, Cln3, Whi5 and Stb1, Rpd3) and mammals (E2F, Cyclin D, Rb, HDAC1). The cell may titrate Cln3 molecules against the number of SBF binding sites, and this could be the underlying basis of the size-control mechanism for Start.

Citation: Wang H, Carey LB, Cai Y, Wijnen H, Futcher B (2009) Recruitment of Cln3 Cyclin to Promoters Controls Cell Cycle Entry via Histone Deacetylase and Other Targets. PLoS Biol 7(9): e1000189. doi:10.1371/journal.pbio.1000189

Academic Editor: Mark Solomon, Yale University, United States of America

Received February 24, 2009; Accepted July 30, 2009; Published September 8, 2009

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Funding: This work was funded by grant RO1 GM039978 from the National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: CDK, cyclin dependent kinase; ChIP, chromatin immunoprecipitation; qPCR, quantitative PCR.

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Introduction

The budding yeast Saccharomyces cerevisiae commits to cell-cycle entry at a point called “Start,” equivalent to the restriction point in animals. Start depends on cell growth to critical size [1–5]. At the molecular level, Start coincides with and depends on a Start-specific burst of transcription of over 100 genes including the G1 cyclins CLN1 and CLN2, the S-phase cyclins CLB5 and CLB6, and many genes for budding and DNA synthesis [6,7]. The burst of transcription at Start depends on two closely related transcription factors SBF and MBF, each of which contains the transcriptional modulator Swi6, and a sequence-specific DNA binding protein, Swi4 in SBF, and Mbp1 in MBF [8]. SBF is most important for the transcription of CLN1 and CLN2, and these G1 cyclins are most important for propelling the cell cycle forward. SBF is found bound to the CLN1 and CLN2 promoters in early G1, well before Start, but at this time does not induce any transcription [9–12]. Indeed, in early G1, SBF may repress transcription. When cells have grown to critical size, the SBF is somehow converted to a transcriptional activator, and induces transcription of many genes including CLN1 and CLN2.

The G1 cyclin Cln3, in combination with the cyclin dependent kinase Cdc28 (or Cdk1), is a key regulator of Start [13,14], and is critical for the size-dependent activation of SBF and MBF [7], converting them from their early G1 repressive forms into the late G1 activating forms. Consistent with the idea that CLN3 is a critical activator of Start, hyperactive alleles of CLN3 (e.g., WHI1-1), and over-expression of CLN3, accelerate Start to smaller cell sizes, whereas deletion of CLN3 delays Start to much larger cell sizes [13,14]. A cln3 null mutant, despite having a delayed Start and large cells, is viable because there are alternative pathways of inducing transcription of CLN1 and CLN2. The most important alternative route depends on the mysterious gene BCK2. The mechanism by which the Bck2 protein activates CLN1 and CLN2 transcription is still largely unknown [15,16]. A cln3 bck2 mutant is inviable in most genetic backgrounds precisely because it does not express sufficient amounts of CLN1 or CLN2, and inviability can be suppressed by the expression of CLN2 from a heterologous promoter [17,18].

An obvious model for the CLN3-dependent activation of SBF is that the Cln3-Cdc28 kinase complex might phosphorylate SBF, thus activating it. However, no evidence for this model has been found [19]. Instead, there has been an accumulation of evidence that Cln3 works, at least in part, by inhibiting a repressor of SBF. Costanzo et al. and de Bruin et al. have identified Whi5 as one such repressor [20,21]. The Whi5 protein associates with SBF on the CLN2 promoter to repress transcription, and Cln3-Cdc28 phosphorylates and antagonizes Whi5 [20,21]. Furthermore, deletion of WHI5, like over-expression of CLN3, accelerates Start to smaller cell sizes [22], and the whi5 null mutant suppresses the inviability of the cln3 bck2 double mutant [20,21].

Although Whi5 is clearly an important target of Cln3, and an important regulator of SBF, it may not be the only target.
Costanzo et al. found some evidence that *whi5* null mutants were still responsive to *CLN3*, suggesting that *CLN3* was also acting by at least one alternative pathway.

An enduring mystery has been the link between cell size and the activation of SBF. *Cln3-Cdc28* activates SBF only when cells have grown to a critical size. But *Cln3-Cdc28* is present even in very small cells. At least in slowly growing G1 cells, *Cln3* abundance increases through G1 as the cell grows more-or-less in proportion to cell size and total cell protein. That is, its absolute abundance increases, but its relative abundance (relative to cell volume, or relative to protein content) does not, or at least not by very much [7,23]. How does a small increase in abundance trigger Start at a critical size? One possibility is that *Cln3* is titrated against something that is constant per cell. Here, we suggest that increasing amounts of *Cln3* are titrated directly against the SBF bindings sites in genomic DNA, which are of course constant in increasing amounts of Cln3 are titrated directly against the SBF relative to protein content) does not, or at least not by very much increases, but its relative abundance (relative to cell volume, or relative to protein content) does not, or at least not by very much increases through G1 as the cell grows more-or-less in proportion to cell size. At least in slowly growing G1 cells, *Cln3* abundance grown to a critical size. But *Cln3-Cdc28* is present even in very small cells. At least in slowly growing G1 cells, *Cln3* abundance increases through G1 as the cell grows more-or-less in proportion to cell size and total cell protein. That is, its absolute abundance increases, but its relative abundance (relative to cell volume, or relative to protein content) does not, or at least not by very much [7,23]. How does a small increase in abundance trigger Start at a critical size? One possibility is that *Cln3* is titrated against something that is constant per cell. Here, we suggest that increasing amounts of *Cln3* are titrated directly against the SBF bindings sites in genomic DNA, which are of course constant in number through G1 phase. At a sufficiently high *Cln3/SBF* site ratio, SBF is activated, and Start ensues.

Finally, it is remarkable how well eukaryotic cell cycle control mechanisms have been conserved, with the functional replacement of fission yeast *cld2* by human *cl2* (*CDK1*) [24] an early and striking example. The yeast system for promoting Start is analogous and perhaps homologous to the mammalian system, with SBF, *Cln3*, and Whi5 playing roles similar to those of E2F-Dp, Cyclin D, and Rb, respectively [25]. Our results suggest that the analogy goes even deeper, with both the yeast and mammalian system making critical use of the Rpd3 histone deacetylase to repress transcription of S-phase genes.

**Results**

**Whi5 Is Not the Sole Target of Cln3**

The only known role of *Cln3* is to activate SBF and MBF; evidence for this is that *swe6* mutants (which lack both SBF and MBF) are completely nonresponsive to *CLN3* [19]. That is, the cell size of *swe6* mutants is unaffected by over-expression or under-expression of *CLN3*. If Whi5 is the one and only target of *Cln3*, then the size of *whi5* mutant cells, like that of *swe6* mutant cells, should also be nonresponsive to *CLN3*. Whether or not this is true is unclear, although Costanzo et al. [20] found some evidence that *whi5* mutants did respond to *CLN3*.

To address this issue in a more sensitive way, we used strains containing a *bck2* mutation. Since Bck2 is a redundant with *Cln3* for expression of *CLN1*, *CLN2*, and other genes [17,18], *bck2* mutants are even more sensitive than wild-type cells to the effects of *CLN3*. Thus we compared *bck2 Whi5* cells with *bck2 whi5* cells with respect to the effect of *CLN3* on cell size. Results (Figure 1, top two panels) show clearly that both genotypes are still responsive to *CLN3*. Thus, *Cln3* can affect cell size, and presumably SBF/MBF activation, even in a *whi5* null strain, suggesting it has some target in addition to Whi5.

**A Screen for Additional Repressors of SBF**

As described above, a *cln3 bck2* double mutant is inviable in many strains, because SBF cannot be activated, and so *CLN1*, *CLN2*, *CLB3*, and *CLB6* cannot be expressed at sufficient levels. However, a *whi5* mutation relieves some of the repression of SBF, and so a *cln3 bck2 whi5* mutant is viable.

Mutations in other putative repressors of SBF might also suppress the inviability of a *cln3 bck2* strain. Thus we constructed a *cln3 bck2* strain kept alive by plasmid-borne *MET-CLN2* (a construct where *CLN2* expression is repressed by methionine). This strain is viable in the absence of methionine, but dies with a G1 arrest in the presence of methionine. The strain was mutagenized using a transposon library (so that mutant genes could be identified), and spread on *met* plates to select suppressors.

This screen yielded two classes of mutants irrelevant to our studies. First, there were a variety of mutants (many co-acting) that de-repressed the *MET* promoter, and thus mis-expressed the plasmid-borne *MET-CLN2*. Second, there were mutants that by one means or another increased the expression of the *RME1* gene, which encodes a transcription factor that, among other things, binds directly to the *CLN2* promoter and increases *CLN2* expression [26]. We identified these irrelevant mutants using secondary screens; they were not further analyzed.

The screen also yielded four complementation groups that may be of direct relevance: *chd1*, *hda2*, *pho23*, and *stb1*. The *chd1* and *stb1* mutations were obtained many times each, whereas *hda2* and *pho23* were obtained only once each. No *whi5* mutation was obtained, but subsequent examination of the mutagenic transposon library by PCR showed that this library did not contain even one disrupted copy of *WHI5*.

**CHD1** is CHromoDomain 1, a nucleosome remodeling factor containing a chromodomain, (which can mediate binding to histones bearing methylated lysines), a helicase domain, and a DNA binding domain [27]. It is a component of both the SAGA and SILK complexes [27]. It is a likely mediator of SBF activity, but its relevance will be considered in a separate report. Interestingly, Rb-binding protein 1 (RBP1), a mediator of E2F repression in mammalian cells, also contains a chromodomain.

**HDA2** is Histone DeAcetylase 2, a member of the Hda1 histone deacetylase complex [27]. Its function is poorly understood. Although we do not further consider *Hda2* here, it could well be a repressor at the *CLN2* promoter.

**PHO23** encodes a component of the Rpd3 histone deacetylase complex [28]. The Rpd3 histone deacetylase is a major histone deacetylase activity in yeast [29,30], and moreover is the yeast ortholog of mammalian HDAC1, the histone deacetylase that interacts with E2F and Rb.

Finally, **STB1** (Sin Three Binder 1) was originally isolated as an interactor with Sin3 [31], and Sin3 is a targeting subunit for the Rpd3 histone deacetylase [32]. Stb1 has also been isolated as a protein binding to the Swi6 component of SBF and MBF, and modulating transcription [33,34]. Thus Stb1 could be a link between SBF and the Rpd3 histone deacetylase complex.

The involvement of both Stb1 and Pho23 implicated the Rpd3 histone deacetylase complex at the *CLN2* promoter. Furthermore...
Figure 1. Responsiveness to CLN3. Various mutants containing bck2 and CLN3 under the control of the GAL promoter (GAL-CLN3) as the only allele of CLN3 were grown in YEP raffinose medium (CLN3 off, solid grey line), split into two aliquots, and galactose was added to one aliquot (CLN3 on, dotted black line). Cell volume distributions were measured with a Coulter Channelizer (left column), and photomicrographs were taken (right two columns). The median cell size (fi) as measured by the Coulter Channelizer is shown in the bottom left corner of each photograph, and the percentage budding is shown in the bottom right corner. A shift to smaller cell sizes and to higher percent budding in galactose shows responsiveness to CLN3. The changes in budding are statistically significant at the $p<0.01$ level for all strains except the whi5 stb1 strain (4th from top) and the whi5 sin3 strain (5th from top). The cell size distribution is not shown for GAL-CLN3 bck2 cells in raffinose (top panel) because these extremely large cells are off-scale. Strains used, from top to bottom, are: LC517, LC520, LC518, LC504, LC524, LC521, and LC523.

doi:10.1371/journal.pbio.1000189.g001
the Rpd3 complex has previously been implicated in the repression of various cell cycle genes, especially SBF or MBF dependent genes [35,36]. Therefore we asked whether mutations in RPD3 (encoding the catalytic subunit) or SIN3 (encoding the targeting subunit) could, like mutations in STB1, PHO23, or WHI5, suppress the inviability of the cln3 bck2 double mutant. Indeed, both rpd3 and sin3 did suppress the inviability of the cln3 bck2 mutant (Figure 2). Consistent with this, D. Huang, S. Kaluarachchi, and B. Andrews (personal communication) have also found that rpd3 can suppress the cln3 bck2 double mutant. As judged by growth rate of the various mutants (Figure 2), whi5 is the strongest suppressor.

Because RME1 slightly activates CLN2 transcription directly, and because one class of suppressor over-expressed RME1, we wondered about the relationship, if any, between suppression. First, it could be that some or all of the suppressors CLN2 of these genotypes could not lose the MET-CLN2 plasmid. In the rme1 background, all the suppressors could suppress inviability, and could lose the MET-CLN2 plasmid. In the rme1 background, the suppressors could again suppress inviability of the cln3 bck2 MET-CLN2 strain on +met plates; however, strains of these genotypes could not lose the MET-CLN2 plasmid (with the whi5 strain being an exception, and able to lose the plasmid). This result suggested that the slight, residual expression from the repressed MET-CLN2 construct was important for viability.

The inability of the suppressed rme1 strains to lose the MET-CLN2 plasmid meant there were two possible explanations for the suppression. First, it could be that some or all of the suppressors de-repressed the native, genomic CLN2 locus, allowing viability, but that the degree of de-repressed CLN2 expression was modest, and viability also required a trace of additional expression, which could come either from RME1 (driving genomic CLN2), or from repressed MET-CLN2 (expressing low, residual levels of CLN2). Second, it could be that some or all of the suppressors were activating RME1 (thereby inducing native CLN2) and also de-repressing MET-CLN2.

Two lines of experimentation showed that the first possibility is correct. First, the transcript from the native CLN2 locus differs in length from the MET-CLN2 transcript. We used quantitative (q) PCR to show that the suppressors increase transcription of the native CLN2 locus, but have no effect on transcription of MET-CLN2 (Figure 3). The two strongest suppressors, stb1 and whi5, activated CLN2 transcription to similar extents (Figure 3). Expression of CLN1 was also increased. Second, we integrated a second copy of CLN2 at the CLN2 locus, using a large restriction fragment that included sequences up to and including the flanking genes. The tested suppressors (sin3, stb1, and whi5) were able to suppress inviability of the resulting cln3 bck2 rme1 2 × CLN2 strain, and these strains were able to lose the MET-CLN2 plasmid (unpublished data). Thus, in a 2 × CLN2 strain, neither RME1 nor MET-CLN2 is required for suppression; the suppressors must act by de-repressing the native CLN2 locus.

These results establish that Stb1, Sin3, and Rpd3, like Whi5, play a role in the repression of SBF target genes. However, they do not establish whether Stb1, Sin3, and Rpd3 are additional components of the Whi5 pathway (i.e., Whi5 might act by attracting the Rpd3 complex), or whether some or all of these new repressors constitute the second pathway that allows whi5 mutant cells to respond to CLN3. To address this, we did epistasis analysis. We constructed double mutants with whi5 (i.e., stb1 whi5, sin3 whi5, rpd3 whi5), and asked whether any of these double mutants would reduce or eliminate responsiveness to CLN3 (which would indicate that the new repressors are in the second new pathway). Unlike either of the single mutants, a whi5 stb1 double mutant is almost nonresponsive to CLN3 (Figure 1). Thus STB1 likely defines a second pathway by which CLN3 controls activity of SBF.

Epistasis analysis of rpd3 and its targeting subunit sin3 with whi5 and with stb1 gave complex results. The whi5 sin3 and the whi5 rpd3 mutants are still responsive to CLN3 with respect to size (Figure 1, fifth and sixth panels), although the whi5 sin3 mutant does not show any responsiveness with respect to budding. This suggests that sin3 (in particular) and rpd3 may be partially but not fully blocking the Stb1 pathway. But stb1 sin3 double mutants are responsive to CLN3 (unpublished data), suggesting that the sin3 mutation is not fully blocking the Whi5 pathway. Previous experiments have established links between Sin3, Rpd3, and Stb1 [29,31]. We feel there are several alternative interpretations of these data (see Discussion), the most likely being that Whi5, Stb1, and Swi6 all interact to some extent with the Rpd3 histone deacetylase complex. Consistent with this, Huang, Kaluarachchi and Andrews have recently found an association between Whi5 and Rpd3 by co-immunoprecipitation (personal communication).
Rpd3, Sin3, and Stb1 Are Found at the CLN2 Promoter, and Rpd3 and Sin3 Are Removed in a CLN3-Dependent Fashion

To further characterize the mechanisms by which Cln3 promotes transcription of SBF target genes, we used chromatin immunoprecipitation (ChIP) to build on the earlier work of Cosma, Nasmyth, and coworkers [9,10] and observe events at the CLN2 promoter [an important SBF target gene] as a function of Cln3 abundance. This is a challenging goal, since once cells have passed through Start, they repress transcription of SBF target genes by additional mechanisms [37]. Thus activation of SBF target genes under normal conditions is transient and difficult to characterize. Therefore, we constructed a strain with genotype GAL-CLN3 bck2 cdc34-2 (i.e., CLN3 is expressed from the GAL promoter). This strain can be synchronized in G1 before Start by growing in raffinose (i.e., without galactose, CLN3 expression is off, its target G1 cyclins CLN1 and CLN2 are not expressed, and Start does not occur). When these G1 cells are then switched to raffinose plus galactose medium at 37°C (the restrictive temperature for the cdc34-2 mutation), CLN3 is turned on, SBF targets are transcribed, but progress through the cell cycle (and the consequent repression of SBF targets) does not occur because of the cdc34-2 defect. Thus we can follow a cell population from a state where SBF genes are fully repressed in all cells (in raffinose medium) to a state where SBF genes are fully induced in all cells (in galactose medium at 37°C).

Figure 4 shows the fate of some relevant proteins at the CLN2 promoter as a function of CLN3 expression. As expected from previous work, Swi4 is at the SBF binding sites of the CLN2 promoter at all times [10–12], regardless of the presence or absence of CLN3. RNA polymerase II is initially absent, but is recruited to the TATA box of CLN2 (and to the TATA box of BBP1), the divergently transcribed, SBF-controlled gene at the other end of the intergenic region) 5 to 10 min after induction of CLN3. Northern analysis shows that the production of CLN2 mRNA almost exactly coincides with recruitment of RNA pol II (Figure 4). Previous studies have shown that this recruitment of RNA pol II depends on Cdc28 kinase [9]. Stb1 is also found near the SBF binding sites, and its presence is not affected by induction of Cln3. Finally, Whi5, Sin3, and Rpd3 are all initially present near the SBF binding sites on the CLN2 promoter, and each of these proteins is lost after CLN3 is induced. Consistent with the relative early and CLN3-dependent loss of Rpd3, Huang, Kaluarachchi and Andrews have recently found that CLN2 can reduce the amount of the Whi5-Rpd3 complex seen by co-immunoprecipitation (personal communication).

Several of these results were also repeated, with the same results, on the YOX1 promoter, which is also regulated by CLN3 and is coregulated with CLN2 (Figure 4C).

Surprisingly, the loss of the repressive proteins is not obvious until 5 to 15 min (for Sin3 and Rpd3), or 15 to 25 min (for Whi5) after CLN3 induction; that is, recruitment of RNA pol II, and the appearance of CLN2 transcript, occur before all the repressive proteins are lost. There are at least three nonequivalent explanations for these kinetics: first, it could be that the repressive proteins are quickly phosphorylated and thereby inactivated as repressors by the Cln3-Cdc28 kinase complex; loss of the proteins from the promoter could be a secondary event. Second, it could be that Cln3-Cdc28 is promoting some positive event that directly induces transcription, and this precedes full loss of the repressive activities. Perhaps phosphorylation of Stb1 or Swi6 or Swi6, for instance, could directly promote transcription even in the presence of repressors. Third, there could be some systematic bias in our ChIP assay such that it is easier to see new proteins arriving at CLN2 than to see old proteins leaving. Additional experiments will be required to distinguish these possibilities.
The GAL-CLN3 bck2 cdc34-2 strain used in the experiments above allowed us to look at events at the CLN2 promoter in a way that is powerful and sensitive, but also contrived. Therefore, we repeated some of the experiments in a different genetic background. We used a strain carrying a cdc20 mutation and a galactose inducible CDC20 gene (GAL-CDC20) to arrest cells at the cdc20 block (mitosis; pre-anaphase), then release them synchronously. Although this approach is less sensitive than the GAL-CLN3 bck2 cdc34-2 method, we were able to reproduce several of the main results. For example, Figure 4E shows Sin3 being recruited to the CLN2 promoter early in G1, then leaving as cells exit G1 (in this experiment, budding begins at about 50 min).

We began to characterize the binding dependencies of some of the proteins at the CLN2 promoter (Figure 5; Table 1); one obvious question is whether binding of the Sin3-Rpd3 complex depends on Whi5 or Stb1. Sin3 still binds to the CLN2 promoter in a whi5 mutant and also in an stb1 mutant, and also in a whi5 stb1 double mutant. To see if Sin3 binding was SBF dependent, we used both swi4 and swi6 mutations, and found that the association of Sin3 with the CLN2 promoter is dependent on SWI6 (p = 4 × 10^-3), but only slightly if at all dependent on SWI4 (Figure 5; Table 1). Presumably in the absence of Swi4, MBF (Mbp1+Swi6) is binding to the CLN2 promoter and recruiting Sin3-Rpd3. The dependence of Rpd3 binding on Swi6 correlates with previous findings that Swi6 contains Cln3-modulated repressive domains [19]; these could be the regions responsible (directly or indirectly) for recruiting the Rpd3 complex. Stb1 and perhaps Whi5 could further influence the recruitment or activity of the Sin3-Rpd3 complex.

Robert et al. [35] previously found that Rpd3 associates with the promoters of CLB6 and PCL1, which are regulated by SBF and MBF. In contrast to our finding of Swi6 but not Swi4 dependence at the CLN2 promoter, Robert et al. found that the association of Rpd3 with PCL1 required both Swi6 and Swi4. The reason for the difference between the studies with respect to the requirement for Swi4 is unclear, but in any case both studies agree that SBF is involved in the recruitment of the Rpd3 complex.

Cln3 Is Found at the CLN2 Promoter

While it is clear that Cln3-Cdc28 protein kinase complex somehow promotes the loss of Sin3, Rpd3, and Whi5 from the CLN2 promoter, it is not clear how directly Cln3 acts, or exactly what proteins the Cln3-Cdc28 complex phosphorylates. We found that Cln3 co-immunoprecipitates with the Swi6 component of SBF, and that this co-immunoprecipitation depends on Swi4 (Figure 6). Thus, there is a relatively direct interaction between Cln3 and SBF. ChIP showed that Cln3 is found on the CLN2 promoter close to the SBF binding sites (Figure 6), the same location as SBF, Whi3, Sin3, Stb1, and Rpd3. Cln3-Cdc28 is thus in a location suitable for the direct phosphorylation of these and other associated proteins. Both Whi5 and Stb1 have a very high density of consensus phosphorylation sites for the Cdc28 kinase (2.7 or 2.6 consensus and near-consensus sites per 100 amino
of the upper two bands ("dyn" plus "up") is shown at the bottom of the intensity of the SBF band (arrowhead) to the sum of the intensities experiments for each of the four genotypes are shown here. The ratio of statistically after "blinding" the samples (Table 1). The two median-most genotypes. Results for all 40 experiments were obtained and tested strains. Ten independent experiments were done for each of the four Figure 5. Dependency analysis. (A) ChIP analysis of proteins at the CLN2 promoter in various mutants. Cells (left panel: S288c, HWL99, HWL110, OBS1, and HWL117; right panel S288c, HWL110, HWL119, and OBS5) were CLN3 BCK2 CDC34 in exponential growth. The TAP-tagged protein being assayed is indicated, as is any additional mutation in the strain. The arrowhead (>) indicates the band containing the SBF binding sites. Other bands are as drawn in Figure 4A. (B) ChIP analysis of proteins at the CLN2 promoter in various mutants. As in Figure 4A, but with a different selection of mutants. The arrowhead indicates the band containing the SBF binding sites. (C) ChIP analysis of Sin3-TAP at the CLN2 promoter in wild-type, swi4, swi6, and untagged (negative control) strains. Ten independent experiments were done for each of the four genotypes. Results for all 40 experiments were obtained and tested statistically after “binding” the samples (Table 1). The two median-most experiments for each of the four genotypes are shown here. The ratio of the intensity of the SBF band (arrowhead) to the sum of the intensities of the upper two bands ("dyn" plus "up") is shown at the bottom of each gel lane.

doi:10.1371/journal.pbio.1000189.g005

### Table 1. Binding of Sin3 to the CLN2 promoter in swi4 and swi6 mutants: p-values.

| Strain | WT | swi4 | swi6 | Negative Control |
|--------|----|------|------|------------------|
| WT     | 0.5| 0.004| 0.0003|                   |
| swi4   | 0.0008| 0.00001|       |                   |
| swi6   | 0.1|      |      |                   |

p-Values for a test of a difference of means for ChIP analysis of the CLN2 promoter fragment containing the SBF sites in strains containing tagged Sin3. The strains are wild-type (WT), swi4, and swi6 strains all carrying tagged Sin3, and an untagged Sin3 strain as a control. The experiment was done ten independent times in each strain. For each experiment, PCR-amplified DNA from ChIP was placed in a tube with a coded label by one investigator (HYW), and given to a second investigator (LBC). PCR fragments were separated by gel electrophoresis, and the ratio of the "sbf" band to the mean of the "up" and "dyn" bands (see Figures 4 and 5) was determined using image analysis software. Once ratios were determined, the label code was broken, and ratios were assigned to their genotypes. One-tailed t-tests were done to calculate p-values of the differences of the means of the logarithms of the ratios (Log ratios were used so as to produce a normal distribution). These p-values are shown above. For example, Sin3 does not appear to ChIP to the CLN2 promoter in a swi6 mutant, and the difference between the swi6 mutant and the wild-type in this regard has a p-value of $4 \times 10^{-7}$. Two typical (median) experiments were chosen from each of the ten sets of experiments and shown in Figure 5. 
doi:10.1371/journal.pbio.1000189.t001

acids, respectively), and previous work suggests that they are very likely substrates for Cln-Cdc28 kinase [20,21,34]. Swi6 and Swi4 also have multiple potential Cdc28 phosphorylation sites, and could be Cln3-Cdc28 substrates in vivo.

#### Cln3 May Be Titrated by SBF and Its Binding Sites

The fact that Cln3 is at the CLN2 promoter raises another issue. Yeast has 100 to 200 genes under the control of SBF and the related transcription factor MBF, and these genes typically have two, three, or more SBF/MBF binding sites each. Thus the total number of functional SBF and MBF binding sites in the cell is in the vicinity of 400. But the average number of Cln3 molecules in a haploid cell is only about 100 [38]. Of course there is considerable uncertainty in these measurements, but nevertheless it is likely that cellular SBF/MBF binding sites are in excess over Cln3.

This excess of binding sites could provide a basis for the critical size requirement for Start. As cells grow in mass, ribosome content, and protein synthetic capacity, they contain increasing numbers of Cln3 molecules [7]. Indeed, growth in the number of Cln3 molecules may be faster than the growth in mass [39]. Yet the number of SBF binding sites is fixed by DNA content. Thus, as the cell grows, it could titrate an increasing number of Cln3 molecules against a fixed number of SBF binding sites, which are initially in excess. At some ratio, the bound Cln3 could activate SBF, resulting in Start.

If this model were correct, then an increase in the number of SBF sites in the cell would increase the requirement for Cln3, and so would cause an increase in cell size at Start. We transformed otherwise wild-type cells with a high copy number plasmid containing four tandem, perfect SBF binding sites (an SBF binding site is called an “SCB”). Since the plasmid has a copy number of about 30, this provides about 120 extra sites, or roughly a 20% increase over the wild-type number of sites. We used elutriation to collect small G1 phase cells carrying the 4×SB7 plasmid (or a control plasmid lacking the 4×SB7 insert), then let these cells grow. We assayed cell volume, and the percentage of budded cells as an assay of Start. As shown in Figure 7, cells lacking the 4×SB7 insert went through Start at about 32 fl, while cells containing the 4×SB7 insert went through Start at about 38 fl, roughly a 20% increase. This experiment is consistent with the idea that the cell is
setting the critical size for Start by titrating some molecule against
the number of available SBF binding sites.

This experiment was done using several independent pairs of
transformants a total of five times (i.e., five pair-wise comparisons). In
every case, the strain with the $4\times$SCB plasmid had a larger critical
size than the strain with the control plasmid. The differences in
critical size in the five experiments were 2.6, 3.7, 6.1, 9.2, and 10.6 fl,
with a mean of 6.4 fl ($p<0.005$ for a test of the hypothesis that the
difference is 0 using a paired sample one-tailed Student's $t$-test; also
statistically significant by nonparametric tests). The experiment
shown in Figure 7 is the median experiment, with a 6.1 fl difference.

One issue with this titration experiment is that the $4\times$SCB plasmid
might increase critical size through some irrelevant
pathology. If this were so, then the $4\times$SCB plasmid would cause
roughly the same percentage increase in size regardless of any
changes we might make to the $CLN3/WHI5/SBF$ system. A second
issue is that even if the activator titration model is correct, it is not clear what activator is being titrated; it might be Cln3, but
Swi4, Swi6, and even Stb1 are also possibilities.

To address these issues, we first repeated the experiment in a
strain carrying two copies of $CLN3$ ($2\times CLN3$; a second copy is
tandemly integrated at the wild-type $CLN3$ locus). If the titration
hypothesis is correct, then the second copy of $CLN3$ should largely
compensate for the $\sim 20\%$ increase in SCB sites. Indeed, the
increased size caused by the $4\times$SCB plasmid in a $2\times CLN3$
background is only 0.6 fl (Figure 7). A $2\times CLN3$ $4\times$SCB strain had
almost exactly the same size as a wild-type (i.e., $1\times CLN3$)
strain bearing the control plasmid. (We note that a second copy of $CLN3$
causes only $\sim 10\%$ decrease in critical size in a wild-type strain
[38]. Presumably when Cln3 is sufficiently abundant, some other
molecule becomes limiting for Start.)

In addition, we did the titration experiment in a $cln3$ deletion
strain. If the effect of the $4\times$SCB plasmid is irrelevant pathology,
then in the $cln3$ strain, the same irrelevant pathology should occur,
and the $4\times$SCB plasmid should again increase critical size. On the
other hand, if Cln3 is the activator being titrated, then in the $cln3$
strain, the $4\times$SCB plasmid should have no effect on critical size,
since it has nothing to titrate. In fact, to our great surprise, we got
neither of these results. Instead, $cln3$ cells actually got smaller when
we added the $4\times$SCB plasmid (Figure 7). This surprising result
was confirmed with two additional experiments (unpublished
data), using independently constructed strains. (The experiment
shown has the median difference of the three experiments.) This
result tells us two things: first, the results are not irrelevant
pathology, because the results change in a specific way with
changes in the $CLN3/WHI5/SBF$ system. Second, a likely

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**Figure 6. Cln3 associates with SBF and ChIPs to the $CLN2$ promoter.**

(A) Cln3 co-immunoprecipitates with Swi6. Strains (HWL72, HWL112,
HWL130) with various combinations of $CLN3$ or $CLN3$-FLAG (pCM273), $SWI6$ or $SWI6$-Myc, or $SWI4$ or $swi4$, were grown and extracts made. In the left
half of (A, these extracts were tested for the presence of the FLAG-tagged Cln3 and the Myc-tagged Swi6 by Western blotting with Anti-FLAG or Anti-
Myc antibody. In the right half, proteins were immunoprecipitated with Anti-Myc antibody (directed against Swi6-Myc), and then these
immunoprecipitates were tested by Western blotting for the presence of Swi6-Myc and Cln3-FLAG. (B) As 6A, except that the immunoprecipitation is
done with the anti-FLAG antibody, followed by Western analysis with the anti-Myc antibody. (C) A ChIP experiment as in Figure 4B, for association of
Cln3-TAP (HWL49, HWL63) with the $CLN2$ promoter.

doi:10.1371/journal.pbio.1000189.g006
interpretation is that Cln3 is indeed the activator being titrated (otherwise a deletion of CLN3 would make no difference), but that the 4×SCB plasmid is also titrating repressors. That is, Cln3 is most limiting (so high copy 4×SCB causes bigger cells in a wild-type background), and repressors are next most limiting (so when there is no Cln3 anyway, then the high copy 4×SCB plasmid decreases size by titrating repressors).

If this is true, then a strain that lacks both Cln3 and also the repressors (Whi5 and Stb1) should not be affected by high copy 4×SCB. And this proves to be the case (Figure 7, bottom right); a whi5 stb1 cln3 strain is not affected by the high copy 4×SCB plasmid. Note that the whi5 stb1 mutant is not responsive to CLN3 (Figure 1); the fact that it is also not responsive to extra SCBs is the expectation from the titration model.

The whi5 stb1 cln3 strains shown in Figure 7 lack all known regulation of the Cln3 size control pathway. Yet, these strains have a size at budding similar to that of wild-type, and have a sigmoidal budding curve suggesting a dependence of budding on size. That is, although the best-characterized size control mechanism is missing, the cells apparently exhibit some form of size control. This suggests the existence of a redundant size control mechanism. The same phenomenon has been observed previously in different circumstances [39], where the redundant size control was attributed to a translational mechanism. In addition, Jørgensen et al. [22] found many size control mutants that were not in the CLN3 pathway.

Discussion

Here we have found that the Whi5 pathway is not the sole link between Cln3-Cdc28 and SBF activity. We have found several mutants that, like whi5, relieve the repression of SBF, and render its activity somewhat independent of Cln3-Cdc28. These mutants include chd1, hda2, pho23, sin3, rpd3, and stb1. Of these, pho23, sin3, stb1, and rpd3, are members of, or have been physically linked to, the Rpd3 histone deacetylase complex, a repressive histone deacetylase orthologous to mammalian HDAC1.

Although we do not know the exact relationship between these proteins and Whi5, we have found that the stb1 mutation is synergistic with whi5; that is, in the context of a bck2 mutation, the stb1 whi5 double mutant, unlike either single mutant, has little ability to respond to Cln3-Cdc28. Thus in some sense Stb1 identifies a pathway for regulating SBF that is separate from the Whi5 pathway.

While we have identified STB1 in a screen for repressors of SBF, others have previously identified STB1 as an activator of SBF or MBF [33,34]. While paradoxical at first sight, it is quite common for transcription factors to have both positive and negative roles in transcription. An example is Fkh2, which collaborates with Mcm1 and Ndd1 transcription factors, and with Cbk-Cdc28 kinase activity, to regulate mitotic genes. In this context, Fkh2 appears to be an activator in late G2 and mitosis, but a repressor at other times [40–44]. Similarly, we imagine that Stb1 helps repress SBF in the absence of Cln3 and Bck2 (the situation in which we found it as a repressor), but helps activate SBF in the presence of Cln3 or Bck2 (the situation in which it was characterized as an activator). Consistent with this, cell cycle expression analysis of stb1 mutants shows that target genes are less repressed at troughs, and less induced at peaks; i.e., they are less regulated and more constitutive (e.g., Figure 3 in [34]). The fact that CLN3 can induce expression of CLN2 even before Sin3, Rpd3, and Whi5 are lost from the
promoter (Figure 4) is consistent with the idea that the initial expression of CLN2 depends on activation, perhaps via Stb1, rather than on loss of repression.

If Stb1 is both a repressor and an activator, then some of our assays may preferentially see one of these activities, and some may see the other. Presumably it is the lack of repression by Stb1 that allows the stb1 mutation to suppress the lethality of the cln3 bck2 mutant. But the cell size assay for responsiveness to CLN3 (Figure 1) may be more sensitive to Stb1 as an activator; in particular, the synergistic defect between whi5 and stb1 may be due to the lack of repression in the whi5 mutant, plus the lack of activation in the stb1 mutant. We note that the combinations of mutations that include stb1 tend to have relatively large cell sizes after induction of GAL-CLN3 (Figure 1), perhaps showing that STB1 is needed for full induction of CLN2.

While Whi5 and Stb1 seem to define two pathways of regulation of SBF, it is still unclear how the Sin3-Rpd3 histone deacetylase complex is recruited to the CLN2 promoter. Previously, the Rpd3 complex has been linked to Stb1 [29,31]. More recently, Huang, Kaluarachchi and Andrews have found evidence for an association between Rpd3 and Whi5 (personal communication). Despite these associations, we found that even whi5 stb1 double mutants had at least some Sin3 (and so presumably Rpd3) at the CLN2 promoter, whereas swi6 mutants had little or no Sin3. Thus although one could imagine various relationships between these proteins, one model is that SBF has some ability to recruit each of Whi5, Stb1, and Sin3-Rpd3, but that these proteins in addition interact with each other (Figure 8). Later, in a size- and growth-dependent fashion, Cln3-Cdc28 also joins the complex, and phosphorylates Whi5 and Stb1 and probably Swi6 and possibly Swi4. This causes the loss of the Rpd3 complex; a somewhat slower loss of Whi5 (Figure 4); and perhaps allows phosphorylated Stb1 to help activate transcription (Figure 8). That Swi6, along with Whi5 and Stb1, is probably a target of Cln3-Cdc28 phosphorylation is strongly suggested by the fact that over-expression of a mutant Whi5 lacking CDK phosphorylation sites is lethal in a mutant where Swi6 is likewise lacking CDK phosphorylation sites [20,45]. The involvement of Swi6 as a likely target of Cln3-Cdc28, and as a recruiter of Sin3-Rpd3, may explain why even whi5 stb1 double mutants seem to have some slight residual Cln3-responsiveness (Figure 1); that is, this residual responsiveness could be through direct phosphorylation of Swi6.

Results reminiscent of ours with regard to Sin3 and Rpd3 were previously obtained by Veis et al. [36], who found that Sin3 and Rpd3 associate with the promoter of the CLB2 gene, which encodes a mitotic cyclin. Although CLB2 is most highly expressed in G2/M, the association of Sin3 and Rpd3 with the CLB2 promoter was lost in late G1, at about the same time we see loss of Sin3 and Rpd3 from the CLN2 promoter. Veis et al. interpreted their results in terms of the association between Sin3/Rpd3 and the Fkh2 (forkhead) transcription factor, and suggested that this association was sensitive to Start. However, we note that CLB2, despite being most strongly up-regulated in G2/M, is a client of SBF as well as a client of Fkh2. The CLB2 promoter contains at least three clustered SBF/MBF binding sites, at least two of which are conserved in other species of yeast [46]. In ChIP experiments, CLB2 is a target of SBF or MBF binding [47,48]. Thus the loss of Sin3/Rpd3 from the CLB2 promoter in late G1 as seen by Veis and coworkers could involve SBF at the CLB2 promoter, and so could be related to the phenomenon we see at the CLN2 promoter.

Another protein we find at the CLN2 promoter is Cln3. However, demonstrating this association was difficult, and required a special genetic background and over-expression of Cln3. Part of the difficulty in ChIPing Cln3 to the CLN2 promoter is the loss of the Rpd3 complex; a somewhat slower loss of Whi5. The transcriptional activation domain of Swi6 is revealed, possibly aided by an activating function of phospho-Stb1. doi:10.1371/journal.pbio.1000189.g008

is presumably because Cln3 is a nonabundant protein, and does not bind DNA directly. But in addition, Cln3 may not be a stoichiometric member of the complex. Instead, it may bind weakly and transiently, phosphorylate its substrate(s), and leave. The two proteins we find to be essential for Cln3 responsiveness, Whi5 and Stb1, are both very likely substrates of Cln-Cdc28 [20,21,34].

Cln3 is present at only about 100 molecules of protein per cell, and yet there are in the vicinity of 400 functional binding sites for SBF and the related factor MBF. The fact that Cln3 is sub-stoichiometric with respect to binding sites could provide a partial solution to the size control problem: Perhaps the amount of Cln3 in the cell, which is a function of cell size and growth rate, is titrated against the number of binding sites. And indeed we found that cells containing extra SCBs had to grow to a larger size to accomplish Start, and this effect could be compensated by one extra dose of CLN3. Extra SCBs did not enlarge a cln3 null mutant, and extra SCBs had no effect whatever on cln3 stb1 whi5 triple mutants. These findings are all supportive of the titration model.

Even though larger G1 cells contain more Cln3 molecules than smaller cells, the increase in Cln3 content with size is probably quite moderate, possibly only linearly correlated with cell size. Thus even at cell sizes adequate for Start, Cln3 may still be sub-stoichiometric with respect to binding sites. Thus we imagine that...
at any and all physiologically reasonable concentrations of Cln3, there will only be fractional occupancy of SBF sites, especially if Cln3 is a weak and transient binder. But as the amounts of Cln3 rise, and are titrated against a fixed number of SBF sites, that fractional occupancy will rise, until at some occupancy (i.e., at some critical cell size), CLN2 and other targets are expressed, and the cell passes through Start. The issue is, how to convert a relatively small change in total Cln3 into a large change in fractional occupancy, or, alternatively, how to convert a small change in occupancy into a large effect?

Although we do not know the answers to either of these questions, Ferrell and coworkers have described many mechanisms by which such “super-sensitivity” can occur [49–56]. One mechanism would use the fact that SBF target genes have multiple SBF binding sites. Perhaps the binding of Cln3 to SBF is cooperative; or perhaps the Cln3 molecules, once bound, cooperate to do something else, such as phosphorylate a substrate. Cooperativity of any kind between multiple sites will give exponential sensitivity to Cln3 amounts, so this is one possible mechanism. A second mechanism is multistate phosphorylation. That is, perhaps the substrates of Cln3-Cdc28 have to be phosphorylated at multiple sites, and this can only happen when fractional occupancy of SBF sites by Cln3 is relatively high. Since phosphorylation is in a dynamic equilibrium with dephosphorylation, a requirement for multistate phosphorylation (at, say, five sites) imposes a super-sensitive threshold on the amount of kinase required [52,57,58]. Multistate phosphorylation can give extreme sensitivity to the amounts of a protein kinase [52,57,58]. A third possible mechanism is to consider the relationship between the complexes at the multiple SBF sites. There are three sites at CLN2; if all three have repressive proteins, is enough Cln3 needed to fill all three sites simultaneously, even though occupancy of any one site is always transient? At any rate, although we do not know how supersensitivity works in this situation, there are lots of ways it could work in theory, as cited above.

There are remarkable parallels between the SBF/Cln3/Whi5,Stb1/Rpd3 regulatory module in yeast, and the E2F-Dp/Cyclin D1/Rb/HDAC1 regulatory module in mammalian cells. To begin with, the cluster of regulated genes is highly conserved: In S. cerevisiae [6], in the distantly related yeast S. pombe [59], in mammalian cells [60], and probably in most or all other eukaryotes, there is a highly conserved cluster of genes needed for DNA replication, and expressed around the G1/S transition. In both yeasts and mammals, the motifs regulating these genes contain a core “CGGG” element. In both yeasts and mammals, the transcription factors recognizing this element (SBF/MBF in the yeasts, E2F-Dp in mammals) contain a DNA binding domain with a “winged helix” fold [61–63]. There is no apparent sequence homology between the yeast and mammalian DNA binding domains, but the domain is small, the evolutionary distance vast, and there are other examples where structure but not sequence has been preserved across time.

In E2F-Dp, the transactivator is repressed by binding of Rb and its family members. There are two mechanisms of repression [64–70]. First, the transactivation domain is masked. Second, Rb family members [but possibly not Rb itself—[69]] recruit mSin3B and HDAC1 which deacetylate and otherwise modify chromatin so as to be inhospitable towards expression. Here, we likewise show that there are at least two pathways of regulation, one of them involving recruitment of a histone deacetylase. In mammals, the transactivation domain is unmasked when a cyclin-CDK complex such as cyclin D-CDK4 phosphorylates Rb and family members, disrupting binding to E2F-Dp, and allowing Sin3m and HDAC1 to leave the chromatin. Similarly, in yeast, Cln3-CDK phosphorylates Whi5 and probably Stb1. Whi5, Sin3, and Rpd3 all leave the chromatin. Interestingly, expression of the target gene Cln2 precedes the loss of the repressive proteins, consistent with a dominant activation, possibly due to Stb1. In any case, it is clear that there are deep, well-conserved parallels between the SBF/Cln3/Whi5,Stb1/Rpd3 regulatory module in yeast, and the E2F-Dp/Cyclin D1/Rb/HDAC1 regulatory module in mammals. It is possible that these modules have regulated the cluster of genes for DNA synthesis since early in eukaryotic evolution.

Materials and Methods

Strains

Strains are shown in Table 2.

Cell Cycle Synchronization

For GAL-CLN3 cdc34-2 block and release experiments, cells growing in YEP with 2% raffinose+2% galactose (YEPRG) at 25°C were arrested in G1 by washing with YEP+2% raffinose (YPEP) and incubating in YEP for four hours at 25°C. Cells were then shifted to 37°C for 1 h, and then cultures were split in two; one half remained in YEPR and to the other half galactose was added to 2% final concentration. Both cultures were incubated at 37°C and samples were taken every 5 min.

For elutriations, cells containing plasmids (pBA70 and pMT3579) were grown in Synthetic Complete (SC) medium with 2% filter-sterilized sucrose as the carbon source. Small unbudded G1 cells were isolated by centrifugal elutriation and grown in preconditioned SC+2% sucrose at 30°C. Cell size distributions were obtained on a Z2 Coulter Counter and budding indexes were determined by counting cells. Bud counts were done “blind” on randomized samples.

Immunoprecipitations, Northern, and Western blots

Immunoprecipitations and Northern and Western blots were carried out essentially as described previously [71,72]. Cell lysates were obtained by vortexing cell suspensions in lysis buffer (0.1% NP40, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, and 50 mM Tris [pH 7.5]) in the presence of glass beads. Cell debris was pelleted by centrifugation for 10 min at 4°C. Protein concentration in the lysate was determined by the DC protein assay (BioRad). Lysates typically contained 20–50 mg/ml of total protein. For immunoprecipitation, 5 mg of cell lysate mixed with 2 μl of 9E10 anti-Myc ascites fluid or 5 μg of anti FLAG antibody (Sigma) were rotated for 1–2 h at 4°C. Immune complexes were collected on protein A-Sepharose beads by rocking at 4°C for 1 h. For detection of immunoprecipitated proteins, beads were pelleted by very gentle, brief, low-speed centrifugation, washed four times with lysis buffer, and boiled in protein sample buffer immediately before SDS-PAGE.

ChiPs

Early exponential phase cells were collected and formaldehyde was added to 1% final concentration. Cells were fixed at room temperature for 15 min. Cross-linking was quenched by the addition of glycine to 125 mM. Cells were pelleted at 3,000 g for 5 min and washed twice with ice-cold TBS (150 mM NaCl, 20 mM Tris-HCl [pH 7.6]). To break cells, cell suspensions in lysis buffer (50 mM HEPES-KOH [pH7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) were mixed with glass beads and vortexed at 4°C for 45 min. Chromatin was sheared by sonication at power 3 (W-380 Sonicator, Heat Systems-Ultrasound, INC) ten times, 10 s each time, and tubes were kept on ice throughout sonication. Cell debris was removed by maximal speed centrifugation for
15 min at 4°C. Whole-cell extracts were prepared for use in ChIPs. Protein concentration for each sample was determined by DC protein assay (Bio-Rad). Immunoprecipitations were performed with 1 mg of extract. Lysates were rotated with 25 μl IgG Sepharose beads at 4°C overnight. Immune complex beads were washed with lysis buffer, lysis buffer 500 (50 mM HEPES-KOH [pH 7.5],...
500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate), and LiCl/detergent (0.5% sodium deoxycholate, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 10 mM Tris [pH 8.0]) twice for each buffer and washed once with cold TE. Bead washing was performed at 4°C. DNA was eluted by incubating beads at 65°C with elution buffer (10 mM EDTA, 1% SDS, 50 mM Tris [pH 8.0]) for 10 min, and crosslinks were reversed by incubating samples at 65°C overnight. PCR was carried out for 30 cycles and products were separated using 2.4% agarose gels.

qReal Time-PCR
A SuperScript III Platinum SYBR green one-step q(real-time) RT-PCR kit (Invitrogen) was used for the detection and quantification of RNA. 5 ng RNA was used for the RT-PCR reaction. Total RNA were purified with RiboPure-Yeast kit (Ambion).

Asynchronous Cell Size
Cells were grown overnight in either YEPR or YEPRG so that cell densities were between 1 and 2×10^6 cells/mL. Cultures were placed on ice, sonicated to separate mothers from daughters, and cell sizes were measured on a Z2 Coulter Counter. Cells were then photographed at 40× and brightness and contrast adjusted in Adobe Photoshop. All data shown are from cells in the S288c genetic background; cells in both the W303 and BF305 backgrounds were also tested, and gave identical results.

Genetic Suppression
Cells were grown overnight in SC-Met, sonicated briefly, and 1:4 serial dilutions were plated onto either SC-Met or SC2r2M Met plates. Cells on SC-Met plates were grown for 3 d at 27°C before being photographed, whereas cells grown on SC2r2M Met plates were grown for 5 d at 27°C.

Acknowledgments
We thank M. Aldea, D. Stillman, N. Edgington, M. Snyder, B. Andrews, and M. Tyers for plasmids and strains; N. Edgington for additional experiments; and two anonymous reviewers for helpful suggestions.

Author Contributions
The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: LBC BF. Performed the experiments: HW LBC YC HW. Analyzed the data: LBC BF. Wrote the paper: BF.

References
1. Hartwell LH, Culotti J, Pringle JR, Reid BJ (1974) Genetic control of the cell division cycle in yeast. Science 185: 46–51.
2. Hartwell LH, Unger MW (1977) Unequal division in Saccharomyces cerevisiae and its implications for the control of cell division. J Cell Biol 75: 422–435.
3. Johnston GC, Pringle JR, Hartwell LH (1977) Coordination of growth with cell division in the yeast Saccharomyces cerevisiae. Exp Cell Res 105: 79–96.
4. Di Tella S, Skotheim JM, Bean JM, Sugia ED, Cross FR (2007) The effects of molecular noise and size control on viability in the budding yeast cell cycle. Nature 448: 947–951.
5. Skotheim JM, Di Tella S, Sugia ED, Cross FR (2008) Positive feedback of G1 cyclins ensures coherent cell cycle entry. Nature 454: 291–296.
6. Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, et al. (1998) The G1 and G2/M cyclins ensure coherent cell cycle entry. Mol Cell 2: 3273–3297.
7. Tyers M, Tokica G, Fucher B (1993) Comparison of the Saccharomyces cerevisiae G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. Embryo J 12: 1955–1968.
8. Koch C, Nasmyth K (1994) Cell cycle regulated transcription in yeast. Curr Opin Cell Biol 6: 451–459.
9. Cosma MP, Panizza S, Nasmyth K (2001) Cdk1 triggers association of RNA polymerase to cell cycle promoters only after recruitment of the mediator by SBF. Mol Cell 7: 1213–1220.
10. Cosma MP, Tanaka T, Nasmyth K (1999) Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. Cell 97: 299–311.
11. Harrington LA, Andrews BJ (1996) Binding to the yeast Swi4,6-dependent cell cycle box, CACGAAA, is cell cycle regulated in vivo. Nucleic Acids Res 24: 538–563.
12. Koch C, Schleiffer A, Ammerer G, Nasmyth K (1996) Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinase activated bound transcription factor SBF (Swhl/Sw6) at start, whereas Clb/Cdc28 kinase displaces it from the promoter in G2. Gen Dev 10: 129–141.
13. Cross FR (1988) DAF1, a mutant gene affecting size control, pheromone arrest, and its implications for the control of cell division. J Cell Biol 75: 422–435.
14. Lee MG, Nurse P (1987) Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdk2. Nature 327: 31–35.
15. Ferrezuelo F, Aldea M, Futcher B (2009) Bck2 is a phase-independent activator of Saccharomyces cerevisiae G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. Mol Cell 7: 1213–1220.
16. Robert F, Pokholok DK, Hannett NM, Rinaldi NJ, Chandy M, et al. (2004) Global position and recruitment of HATs and HDACs in the yeast genome. Mol Cell 7: 127–139.
17. Silbie HH, ter Schure EG, Rommets AJ, Hul P, Wohlingh RG, et al. (1997) Effect of different carbon fluxes on G1 phase duration, cyclin expression, and reserve carbohydrate metabolism in Saccharomyces cerevisiae. J Bacteriol 179: 6455–6456.
18. Lee MG, Nurse P (1987) Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdk2. Nature 327: 31–35.
19. Schaefer JB, Breden LL (2004) RB from a bud's eye view. Cell 117: 849–850.
20. Toone WM, Johnson AL, Banks GR, Toyn JH, Stuart D, et al. (1995) Rme1, a negative regulator of mitosis, is also a positive activator of G1 cyclin gene expression. Embryo J 14: 3024–3032.
21. Kasten MM, Dierland S, Stillman DJ (1997) A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. Mol Gen Genet 256: 376–386.
22. Kasten MM, Dierland S, Stillman DJ (1997) A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. Mol Gen Genet 256: 376–386.
23. Kasten MM, Dierland S, Stillman DJ (1997) A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. Mol Gen Genet 256: 376–386.
24. Kasten MM, Dierland S, Stillman DJ (1997) A large protein complex containing the yeast Sin3p and Rpd3p transcriptional regulators. Mol Gen Genet 256: 376–386.
37. Amnu A, Tyers M, Fletcher B, Nasmyth K (1993) Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. Cell 74: 993–1007.

38. Cross FR, Archambault V, Miller M, Klavestad M (2002) Testing a mathematical model of the yeast cell cycle. Mol Cell Biol 13: 52–70.

39. Schneider BL, Zhang J, Markwardt J, Tokiwa G, Volpe T, et al. (2004) Growth rate and cell size modulate the synthesis of, and requirement for, G1-phase cyclins at start. Mol Cell Biol 24: 10902–10913.

40. Darieva Z, Pic-Taylor A, Beros J, Spanos A, Geymonat M, et al. (2003) Cell cycle-regulated transcription through the FHA domain of Fkh2p and the coactivator Ndd1p. Curr Biol 13: 1740–1745.

41. Koranda M, Schleiffer A, Endler L, Ammerer G (2000) Forkhead-like transcription factors recruit Ndd1 to the chromatin of G2/M-specific promoters. Nature 406: 94–98.

42. Pic-Taylor A, Darieva Z, Morgan BA, Sharrocks AD (2004) Regulation of cell cycle-specific gene expression through cyclin-dependent kinase-mediated phosphorylation of the forkhead transcription factor Fkh2p. Mol Cell Biol 24: 10036–10046.

43. Reynolds D, Shi BJ, McLean C, Katis F, Kemp B, et al. (2003) Recruitment of Thr 319-phosphorylated Ndd1p to the FHA domain of Fkh2p requires Cdc2 kinase activity: a mechanism for Clb cluster gene activation. Genes Dev 17: 1789–1800.

44. Zhu G, Spellman PT, Volpe T, Bensen PO, Botstein D, et al. (2000) Two yeast forhead genes regulate the cell cycle and pseudohyphal growth. Nature 406: 90–94.

45. Wagner MV, Smolka MB, de Brun RA, Zhou H, Wittenberg C, et al. (2009) Whi3 regulation by site specific CDK-phosphorylation in Saccharomyces cerevisiae. PLoS ONE 4: e4300. doi:10.1371/journal.pone.0004300.

46. Harbison CT, Gordon DB, Lee TI, Rinaldi NJ, Macisaac KD, et al. (2004) Transcriptional regulatory code of a eukaryotic genome. Nature 431: 94–104.

47. Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M, et al. (2001) Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. Nature 409: 533–538.

48. Simon I, Barnett J, Hannett N, Harbison CT, Rinaldi NJ, et al. (2003) Serial regulation of transcriptional regulators in the yeast cell cycle. Cell 106: 697–708.

49. Bagowski CP, Besser J, Frey CR, Ferrell JE Jr (2005) The JNK cascade as a biochemical switch in mammalian cell: all-or-one responses. Curr Biol 13: 315–320.

50. Brandman O, Ferrell JE Jr, Li R, Meyer T (2002) Interlinked fast and slow transcriptional switches drive reliable cell decisions. Science 310: 496–498.

51. Ferrell JE Jr (1996) Tripping the switch fast: how a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem Sci 21: 460–466.

52. Ferrell JE Jr (1997) How responses get more switch-like as you move down a protein kinase cascade. Trends Biochem Sci 22: 288–298.

53. Ferrell JE Jr (1999) Building a cellular switch: more lessons from a good egg. Bioessays 21: 866–870.

54. Ferrell JE Jr (2002) Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr Opin Cell Biol 14: 140–148.

55. Ferrell JE Jr, Machleder EM (1998) The biochemical basis of an all-or-one cell fate switch in Xenopus oocytes. Science 280: 895–898.

56. Pomerening JR, Sontag ED, Ferrell JE Jr (2003) Building a cell cycle oscillator: hysteresis and bistability in the activation of Cdc2. Nat Cell Biol 5: 346–351.

57. Klein P, Lawson T, Tyers M (2003) Mathematical modeling suggests cooperative interactions between a disordered polypeptide ligand and a single receptor site. Curr Biol 13: 1669–1678.

58. Nash P, Tang X, Orlicky S, Chen Q, Gertler FB, et al. (2001) Multisite phosphorylation of a Cdk inhibitor sets a threshold for the onset of DNA replication. Nature 414: 514–521.

59. Obara A, Rosebrock A, Ferrezncl F, Pyne S, Chen H, et al. (2005) The cell cycle-regulated genes of Schizosaccharomyces pombe. PLoS Biol 3: e225. doi:10.1371/journal.pbio.0030225.

60. Whitfield ML, Sherlock G, Saldana AJ, Murray J, Ball CA, et al. (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol Cell Biol 13: 1977–2000.

61. Gajiwala KS, Barley SK (2000) Winged helix proteins. Curr Opin Struct Biol 10: 110–116.

62. Taylor IA, Treiber MK, Olivi L, Smerdon SJ (1997) The X-ray structure of the DNA-binding domain from the Saccharomyces cerevisiae cell-cycle transcription factor Mbp1 at 2.1 A resolution. J Mol Biol 272: 1–8.

63. St John EN, Sia XS, Cao Y, Chang CC, Sun X, et al. (2001) Phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature 414: 514–521.

64. Colominas D (2003) Pocket proteins and cell cycle control. Oncogene 24: 2796–2809.

65. DeGregori J (2004) The Rb network. J Cell Sci 117: 3411–3413.

66. Dimova DK, Dyon N (2005) The E2F transcriptional network: old acquaintances with new faces. Oncogene 24: 2810–2826.

67. Knudsen ES, Wang JY (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol Biol Cell 17: 5771–5783.

68. Lee C, Chang JH, Lee HS, Cho Y (2002) Structural basis for the recognition by the heterodimeric cell cycle transcription factor E2F-DB. Genes Dev 13: 666–674.

69. Knudsen ES, Wang JY (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol Biol Cell 13: 666–674.

70. Rubin SM, Gall AL, Zheng N, Pavletich NP (1999) Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DB. Genes Dev 13: 666–674.

71. Knudsen ES, Wang JY (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol Biol Cell 13: 666–674.

72. Rubin SM, Gall AL, Zheng N, Pavletich NP (1999) Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DB. Genes Dev 13: 666–674.

73. Knudsen ES, Wang JY (1997) Dual mechanisms for the inhibition of E2F binding to RB by cyclin-dependent kinase-mediated RB phosphorylation. Mol Biol Cell 13: 666–674.

74. Rubin SM, Gall AL, Zheng N, Pavletich NP (1999) Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DB. Genes Dev 13: 666–674.

75. Rubin SM, Gall AL, Zheng N, Pavletich NP (1999) Structural basis of DNA recognition by the heterodimeric cell cycle transcription factor E2F-DB. Genes Dev 13: 666–674.