Regulation of a transcription factor network by Cdk1 coordinates late cell cycle gene expression

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Abstract

To maintain genome stability, regulators of chromosome segregation must be expressed in coordination with mitotic events. Expression of these late cell cycle genes is regulated by cyclin-dependent kinase (Cdk1), which phosphorylates a network of conserved transcription factors (TFs). However, the effects of Cdk1 phosphorylation on many key TFs are not known. We find that elimination of Cdk1-mediated phosphorylation of four S-phase TFs decreases expression of many late cell cycle genes, delays mitotic progression, and reduces fitness in budding yeast. Blocking phosphorylation impairs degradation of all four TFs. Consequently, phosphorylation-deficient mutants of the repressors Yox1 and Yhp1 exhibit increased promoter occupancy and decreased expression of their target genes. Interestingly, although phosphorylation of the transcriptional activator Hcm1 on its N-terminus promotes its degradation, phosphorylation on its C-terminus is required for its activity, indicating that Cdk1 both activates and inhibits a single TF. We conclude that Cdk1 promotes gene expression by both activating transcriptional activators and inactivating transcriptional repressors. Furthermore, our data suggest that coordinated regulation of the TF network by Cdk1 is necessary for faithful cell division.

Keywords  Cdk1; cell cycle; mitosis; proteolysis; transcription

Introduction

Progression through the cell cycle depends upon the orchestrated expression of hundreds of genes. This cyclical transcription is critical for accurate cell division, as it ensures the proper order of cell cycle events (Haase & Wittenberg, 2014) and prevents uncontrolled proliferation that can lead to the development of cancer (Massagué, 2004). Networks of transcription factors (TFs) control cell cycle-regulated transcription by similar mechanisms in all eukaryotes (Morgan, 2007). Changes in cell cycle-regulated gene expression are further modulated by the activities of cyclin-dependent kinases (CDKs), which can affect both the activity and expression of cell cycle-regulatory TFs.

Cyclin-dependent kinase regulation of transcription is best understood at the G1/S transition, where the properties of transcriptional regulation are conserved from yeast to humans (Cross et al, 2011). In late G1, CDK triggers the expression of G1/S genes by directly phosphorylating and inactivating transcriptional repressors (Rb family members in mammals, Whi5 in yeast), which promotes the function of transcriptional activators (E2F in mammals, SBF and MBF in yeast) that stimulate expression of G1/S genes (de Bruin et al, 2004; Costanzo et al, 2004; van den Heuvel & Dyson, 2008; Henley & Dick, 2012). Later in S-phase, phosphorylation of G1/S activators by S-phase cyclin/CDKs inhibits their function and shuts off gene expression (Bertoli et al, 2013). Although CDK also impacts the expression of late cell cycle genes, less is known about the details of this regulation. In budding yeast, genes that peak at the G2/M transition are activated by the forkhead family TF Fkh2 bound to its co-activator Ndd1 and the MADS box protein Mcm1 (Koranda et al, 2000; Kumar et al, 2000). CDK phosphorylates both Fkh2 and Ndd1 and is required for their interaction and recruitment to target gene promoters (Reynolds, 2003; Pic-Taylor et al, 2004). In mammals, the related forkhead protein FoxM1 regulates expression of a similar group of mitotic genes (Laoukili et al, 2005), and phosphorylation of the FoxM1 C-terminal transactivation domain by Cdk2 is required for its activity (Major et al, 2004; Lüscher-Firzlaff et al, 2006; Wierstra & Alves, 2006; Laoukili et al, 2008). Thus, the general principles of CDK-regulated transcription are conserved between yeast and mammals throughout the cell cycle.

For many cell cycle-regulatory proteins, CDK phosphorylation not only regulates their activity, but also modulates their expression by stimulating or blocking their ubiquitination and degradation in the proteasome. The activities of the two major families of cell cycle-regulatory ubiquitin ligases, the Skp1-Cullin-F-box (SCF) and anaphase-promoting complex (APC) are both impacted by CDK (Benanti, 2012). Phosphorylation of many cell cycle regulators promotes their interaction with F-box protein subunits of SCF ubiquitin ligases, which leads to their ubiquitination and destruction (Willems et al, 2004; Skaar et al, 2013). In contrast, CDK phosphorylation of some APC targets near degron motifs inhibits their ubiquitination by the APC (Littlepage & Ruderman,
following DNA damage (de Oliveira et al., 2012) and was found to interact with the Rpd3L and Set1c histone deacetylase complexes (Shevchenko et al., 2008; de Oliveira et al., 2012), suggesting that it acts as a transcriptional repressor, although its molecular function is not known.

To inhibit the phosphorylation of each S-phase TF, we constructed mutants that lack all Cdk1 consensus sites (referred to as Cdk- TFs, see Supplementary Fig S1A for specific mutations). When expressed from a constitutive promoter, these mutations led to increased protein levels of all four TFs (Fig 1A; HA, light exposure) and increased the electrophoretic mobility in a gel (most dramatic for Tos4 and Yhp1), consistent with a loss of phosphorylation (Fig 1A; HA, dark exposure). The observed shifts were similarly reduced upon inhibition of Cdk1, confirming that these shifts are the result of Cdk1 phosphorylation in vivo. Moreover, upon comparison to the wild-type TFs, which are all established substrates of Clb2/Cdk1 in vitro (Loog & Morgan, 2005; Köövomägi et al., 2011b), we found that the Cdk- TFs showed reduced phosphorylation by Clb2/Cdk1 (Fig 1B, Supplementary Fig S1B), confirming that these mutations eliminate Cdk1 phosphosites.

Each Cdk- TF was then integrated into its endogenous locus and expressed as the sole copy of that TF in the cell. In the course of integrating the stable YOXL allele, we found that a more conservative mutation that includes mutations in only the C-terminal S/T-P sites, yox1-9A, exhibited phenotypes identical to that of yox1-13A (Supplementary Fig S6C). In addition, mutation of this group of C-terminal sites reduced phosphorylation by Cdk1 in vitro (Supplementary Fig S1B), confirming that these sites are indeed targeted by Cdk1. Therefore, we integrated this more conservative allele at the endogenous YOXL locus. As expected, expression of each wild-type TF increased in S-phase and dropped in mitosis (Fig 1C, Supplementary Fig S2). Notably, expression of each of the Cdk- TFs was prolonged over the course of the cell cycle. This change was most dramatic for Tos4-9A and Hcm1-15A, although Yox1-9A and Yhp1-13A were also expressed at higher levels during G1 and mitosis, as compared to the WT proteins (Fig 1C, see 0 and 60 min time points). We also examined the timing of cell cycle progression in cells expressing each of the Cdk- TFs. None of the mutations significantly altered cell cycle progression under optimal growth conditions, although we noted a subtle, but reproducible, delay in S-phase progression in cells expressing Yox1-9A, compared to WT cells (Supplementary Fig S2).

Phosphorylation by Cdk1 regulates the ubiquitination and degradation of many cell cycle regulators (Benanti, 2012), so we compared the half-lives of wild-type and Cdk- TFs to determine whether phosphorylation affected their stabilities. Each Cdk- TF was more stable than the corresponding WT protein (Fig 1D–G), which accounts for their persistence throughout the cell cycle. Moreover, direct inhibition of Cdk1 similarly stabilized Hcm1, Tos4, and Yox1 (Fig 2A–C), confirming that Cdk1 regulates their stabilities. Interestingly, although Cdk1 inhibition decreased phosphorylation of Yhp1 (Fig 1A), it did not appear to impair Yhp1 degradation (Fig 2D), which could be the result of incomplete Yhp1 dephosphorylation after Cdk1 inhibition. Additionally, we cannot rule out the possibility that some subset of S/T-P sites in each TF are phosphorylated by another kinase in vivo. However, our data clearly demonstrate that Cdk1 phosphorylates at least a subset of S/T-P sites in each TF and that phosphorylation of these sites promotes degradation of each factor.
In yeast, most protein degradation that depends upon phosphorylation is triggered by SCF family E3 ubiquitin ligases, especially those containing the F-box proteins Cdc4 or Grr1 that recognize Cdk1-phosphorylated epitopes on substrates (Lanker et al., 1996; Nash et al., 2001; Kõivomägi et al., 2011a; Landry et al., 2012; Lyons et al., 2013). To determine whether an SCF E3 promotes the degradation of S-phase TFs, we expressed each in cells carrying a temperature-sensitive allele of the core SCF subunit cdc53-1 and analyzed their degradation upon Cdc53 inactivation. Interestingly, phosphorylated forms of Tos4, Yox1, and Yhp1 were each stabilized in cdc53-1
cells (Fig 2E–H), demonstrating that an SCF E3 regulates the degradation of the Cdk-phosphorylated forms of these TFs. Hcm1 was not stabilized in this assay, which could be due to the fact that inactivation of Cdc53 arrests cells in G1 (Supplementary Fig S4B). We subsequently found that Hcm1 degradation in G1 is independent of phosphorylation, but that Hcm1 is targeted by Cdc53 when cells arrested in mitosis (discussed below).

Interestingly, each TF was still degraded to some extent upon blocking phosphorylation (Figs 1D–G and 2A–D) and upon inactivation of the SCF (Fig 2E–H). In addition, Cdk- TFs still underwent modest cell cycle-regulated expression (Fig 1C), suggesting that Cdk-independent pathways also degrade these proteins. One possibility is that they may also be targeted by the APC, since their levels are low in mitosis and G1 when the APC is active. Additionally, some evidence suggests that Yhp1 and Tos4 can be targeted by the APC (Ostapenko & Solomon, 2011; Ostapenko et al., 2012), raising the possibility that the APC can promote Cdk-independent degradation of all of these TFs. However, we did not observe any stabilization of Hcm1, Yox1, or Yhp1 in cells lacking the APC activator Cdh1, or in cells in which all APC activity is inhibited by the spindle check point (Fig 2I). In fact, levels of these TFs were lower in cdc53A cells, most likely because a larger fraction of asynchronous cdc53A cells are in G2/M when these TFs are not transcribed (Supplementary Fig S4C). As reported previously (Ostapenko et al., 2012), Tos4 was partially stabilized in cells lacking the APC activator Cdh1 (Fig 2I). However, upon examination of Tos4 levels in previously described cells in which the core APC subunits are deleted (Thornton & Toczyski, 2003), or in cells in which the APC has been hyperactivated by expression of a constitutively active Cdh1 (Cdh1-m11) (Zachariae et al., 1998), we observed no change in Tos4 expression (Fig 2J). This suggests that the Cdk-independent degradation of S-phase TFs is not driven by the APC in vivo.

Phosphorylation of S-phase TFs promotes expression of late cell cycle genes

Since all four S-phase TFs are expressed during the same window of the cell cycle (Fig 1C) and are predicted to impact expression of overlapping groups of genes, we reasoned that Cdk1 phosphorylation of these TFs may redundantly regulate gene expression and therefore simultaneous mutation of these TFs may have a larger impact on the cell cycle than any individual allele. For this reason, we introduced all four Cdk- TFs into one strain (referred to as 4P, Fig 3) and analyzed cell cycle progression. In the course of constructing this strain, we found that all combinations of Cdk- TF alleles were viable, including the 4P mutant. The 4P strain expressed each of the four Cdk- TFs at higher levels than its WT counterpart across the cell cycle (Fig 3A), confirming that the Cdk- proteins are expressed similarly when they are integrated individually and all together (compared to Fig 1C). However, in contrast to the single mutant strains, the 4P strain exhibited a delay in progression through mitosis (see 75 and 90 min time points, Fig 3B), consistent with the possibility that expression of mitotic regulatory genes was either reduced or delayed. Interestingly, a similar delay in mitotic progression was also observed in cells lacking all four transcriptional regulators (Supplementary Fig S5B), suggesting that some or all of the phosphosite mutations may impair protein function.

We next examined cell cycle-regulated mRNA levels in the 4P mutant directly, using gene expression microarrays. WT and 4P cells were synchronized in G1 phase, released into the cell cycle, and samples for RNA analysis were collected at 15-min intervals. Work from several laboratories has generated a consensus list of 930 genes that undergo cell cycle-regulated transcription (Cho et al., 1998; Spellman et al., 1998; Pramila et al., 2006; Lu et al., 2007). Interestingly, we found that the average expression of these cell cycle-regulated genes was downregulated in S through M phases in 4P cells, while expression during G1 was relatively unaffected (Fig 3C and D, compare 30–75 min time points to 0–15 min time points). We then divided the 930 genes into six groups, based on the timing of their peak expression (Pramila et al., 2006), and analyzed average expression of each of these groups of genes at each time point. Peak expression of genes that peak in S-phase and mitosis was downregulated in the first cell cycle after release (Supplementary Fig S5C). Moreover, the average expression of genes that peak in S-phase and are predicted to be targets of Hcm1 was modestly downregulated in 4P cells (Fig 3E), to a similar extent as what has been observed in hcm1A cells (Pramila et al., 2006). Additionally, genes that peak at the M/G1 transition and include Yox1/ Yhp1 target genes (Spellman et al., 1998; Pramila et al., 2002) were similarly downregulated over the cell cycle (Fig 3F). Importantly, although expression of individual genes in these classes continued to cycle (Supplementary Fig S5C and D), their peak expression was reproducibly decreased (Fig 3H). In contrast, CLB2 cluster genes (Spellman et al., 1998), which are regulated by Fkh2/Ndd1 and peak at the G2/M transition (Loy et al., 1999; Koranda et al., 2000; Kumar et al., 2000; Pic et al., 2000; Zhu et al., 2000), were not significantly altered during the first cell cycle after release (Fig 3G). Decreased expression of CLB2 cluster genes was observed from 90 to 105 min.
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after release, but this is likely due to the fact that 4P cells are delayed in progression through the cell cycle at this time (Fig 3B).

Together, this analysis suggests that blocking Cdk1 phosphorylation inhibits the function of the activator Hcm1, and/or increases the activity of the repressors Yox1 and Yhp1, leading to decreased expression of cyclical genes late in the cell cycle.

**The repressors Yox1 and Yhp1 are inactivated by phosphorylation**

Next, we sought to understand how phosphorylation alters the functions of each individual TF. We first analyzed the consequences of overexpressing each WT and Cdk1- TF. Among the four TFs, overexpression of Tos4 or Yox1 has been reported to slow progression through the cell cycle (Pramila et al., 2002; de Oliveira et al., 2012). However, we found that constitutive overexpression of Tos4 or Tos4-9A had only a very minor effect on growth (Supplementary Fig S6A). Tos4 has also been implicated in the DNA damage response due to the fact that deletion of Tos4 and the checkpoint kinase DUN1 was found to be synthetic lethal in the presence of the replication inhibitor hydroxyurea (HU) (de Oliveira et al., 2012). We attempted to reproduce this finding, using tos4A cells, in order to test whether tos4-9A showed a similar genetic interaction with dun1A, but could not replicate the reported result for tos4A (Supplementary Fig S6B). Because of the lack of phenotypes in these assays, we did not investigate the molecular consequences of phosphorylation on Tos4 further.

In contrast to Tos4, YOX1 overexpression severely impaired growth and, interestingly, overexpression of yox1-13A was even more deleterious, suggesting that it is a hyperactive allele (Supplementary Fig S6A). To determine whether Yox1 or Yox1-13A arrested cells in a specific cell cycle phase, cells were arrested in G1 and overexpression was induced as cells were released from the arrest. (Supplementary Fig S6A). To determine whether Yox1 or Yox1-13A arrested cells in a specific cell cycle phase, cells were arrested in G1 and overexpression was induced as cells were released from the arrest. (Supplementary Fig S6A). To determine whether Yox1 or Yox1-13A arrested cells in a specific cell cycle phase, cells were arrested in G1 and overexpression was induced as cells were released from the arrest. Notably, yox1-13A overexpression led to a mitotic arrest, which did not occur upon overexpression of WT YOX1 (Fig 4A). Since Yox1-13A was expressed at higher levels than WT Yox1 (Fig 4B), this raised the possibility that it blocked cell cycle progression due to increased repression of target genes. Indeed, expression of four targets (DBF2, HST4, MCM3, and CDC20) was reduced following overexpression of WT Yox1, and to a greater extent following overexpression of Yox1-13A (Fig 4C). Interestingly, the differences in target gene expression between WT and 13A-overexpressing cells were relatively modest, yet only the 13A-overexpressing cells arrested in mitosis. This suggests that a small increase in expression of these mitotic genes is sufficient to promote cell cycle progression.

We next examined whether blocking phosphorylation of endogenous Yox1 had a similar effect on gene expression. Since mutation of 9 C-terminal sites of Yox1 (Supplementary Fig S1A) was sufficient to confer both cell cycle arrest and decreased target gene expression upon overexpression (Supplementary Fig S6C, unpublished observations), the yox1-9A allele was integrated into the genomic locus and used for further analysis. Additionally, since Yhp1 and Yox1 are related proteins that regulate overlapping sets of genes (Pramila et al., 2002), we also analyzed yhp1-13A. Notably, Yox1/Yhp1 target genes that peak in M/G1, including DBF2, HST4, KIN3, and MCM3, were downregulated in yox1-9A, yhp1-13A, and double-mutant cells (Fig 4D). In contrast, the Yox1 target genes CDC20 and SPO12, which peak earlier in G2/M (Pramila et al., 2002; Darieva et al., 2010), were not greatly affected. Consistent with the downregulation of M/G1 genes, Yhp1-13A and Yox1-9A also associated with the promoters of these genes at higher levels than the wild-type proteins (Fig 4E). Interestingly, mutation of Cdk1 sites had greater effects on DNA binding of Yox1 than on Yhp1, which could be due to the larger increase in Yox1 protein levels that are observed upon mutation of Cdk sites (Supplementary Fig S7B). Together, these data further support the model that Cdk1 phosphorylation normally promotes degradation of Yox1 and Yhp1, thereby restricting the activity of these transcriptional repressors to S-phase.

**Phosphorylation of the Hcm1 N-terminus promotes its degradation**

Surprisingly, although the Hcm1-15A mutant was more stable than WT Hcm1 (Fig 1C), Hcm1 target genes were downregulated in 4P cells (Fig 3E), suggesting that Hcm1-15A might be less active than WT Hcm1. To determine whether these opposing effects of phosphorylation on Hcm1 could be uncoupled, we mutated different subsets of its phosphosites. The 15 Cdk1 consensus sites in Hcm1 primarily cluster in two groups outside of the DNA-binding domain, with three sites in the N-terminus and eight in the C-terminus (Supplementary Fig S1A). To dissect the functions of each cluster, we first examined whether each can be targeted by Cdk1. Importantly, Hcm1 proteins containing only the 3 N-terminal S/T-P sites (Supplementary Fig S1B, Hcm1-12C), or only the 8 C-terminal sites (Hcm1-7N), were phosphorylated by Cdk1 in vitro, but to lesser degrees than the wild-type protein that contains all sites, confirming that some sites in each cluster are targeted. We then constructed two additional mutants that contained Ser/Thr to Ala mutations in only the three N-terminal sites (referred to as 3N) or the 8 C-terminal sites.
Figure 3. Simultaneous mutation of S-phase transcription factors (TFs) delays mitotic progression.

A, B Cells expressing differentially tagged WT TFs (TOS4-3FLAG HCM1-3HA YOX1-3V5 YHP1-13MYC), or phosphomutant TFs (4P, tos4-9A-3FLAG hcm1-15A-3HA yox1-9A-3V5 yhp1-13A-13MYC), were arrested in G1, released, and collected at 15-min intervals for Western blot (A) and flow cytometry (B). Levels of TFs and cyclin-dependent kinase 1 (Cdk1) are shown in (A). Quantification of proteins levels are shown in Supplementary Fig S5A. DNA content at each time point is shown (B, top). Overlay of WT (blue) and 4P (red) at 75 and 90 min highlight the mitotic delay in 4P cells (B, bottom). A representative of three replicate experiments is shown.

C–G mRNA from WT and 4P cells collected as in (A) were compared to mRNA from asynchronous WT cells. Data from one of two biological replicates are shown. Average expression of all 6,237 genes at each time point in WT (blue) and 4P (red) cells (C). Average expression of 930 cell cycle-regulated genes (D). For further explanation and breakdown of individual groups, see Supplementary Fig S5C. Average expression of 97 genes that have Hcm1-binding motifs in their promoters and whose expression peaks during S-phase (E) (Pramila et al, 2006). Average expression of 39 MCM cluster genes (F) (Spellman et al, 1998). Average expression of 31 CLB2 cluster genes (G) (Spellman et al, 1998). Data from biological replicates of all cell cycle-regulated genes, and lists of genes in each cluster, are included in Supplementary Dataset S1.

H RT-qPCR of representative Hcm1 targets (CIN8, HTZ1) and MCM cluster genes (DBF2, KIN3) at the indicated time points after release from G1. For each gene, mean expression from three biological replicates, with standard deviations, is plotted. Asterisks indicate that all four comparisons are statistically significant with a P-value of ≤ 0.01. (CIN8, P = 0.01; HTZ1, P = 0.005; DBF2, P = 0.007; KIN3, P = 0.003).

Data information: In all parts, wild-type cells are graphed in blue, 4P cells are red.

Source data are available online for this figure.
Figure 4. Phosphorylation inactivates Yox1 and Yhp1.

A–C Wild-type cells carrying plasmids expressing YOX1 or yox1-13A from the GAL1 promoter, or an empty vector control (EV), were grown in raffinose and arrested in G1. Cells were then released into medium containing raffinose and galactose, to induce overexpression of YOX1 or yox1-13A. DNA content 5 h after release from G1 is shown in (A). Western blots for Yox1-3HA, Clb2 (a marker of mitosis), and cyclin-dependent kinase 1 (Cdk1; loading control) as cells progressed through mitosis (90, 120, 150, and 180 min after release from G1) are shown in (B). All values were normalized to ACT1. Mean and standard deviations from technical replicates of a representative experiment are shown. See Supplementary Fig S7A and B for corresponding Western blots and cell cycle positions.

D Expression of Yox1/Yhp1 targets genes in asynchronous yhp1-13A, yox1-9A, or yhp1-13A yox1-9A cells compared to wild-type. All values were normalized to ACT1. Mean and standard deviations from technical replicates of a representative experiment are shown. See Supplementary Fig S7B and C for corresponding Western blots and cell cycle positions.

E ChIP-qPCR of 3V5-tagged Yhp1, Yhp1-13A, Yox1, and Yox1-9A, compared to an untagged control. Mean and standard deviations for three biological replicates are shown. For each primer set, binding is shown relative to Yhp1. See Supplementary Fig S7C and D for corresponding Western blots and cell cycle positions from a representative experiment.

Source data are available online for this figure.
Figure 5. Phosphorylation of the Hcm1 N-terminus promotes SCF-dependent degradation.

A Expression of Hcm1-3N-3HA and Hcm1-8C-3HA over the cell cycle. Cells were arrested in G1, released into the cell cycle, and samples taken for Western blot and flow cytometry (Supplementary Fig S8A) at 15-min time points.

B Cells expressing Hcm1-GFP or Hcm1-3N-GFP from the TEF1 promoter were arrested in G1 with alpha-factor, S-phase with HU, or mitosis with nocodazole and half-lives compared by cycloheximide-chase assay. Levels of Hcm1, Clb2, and cyclin-dependent kinase 1 (Cdk1) are shown. Cell cycle arrest was confirmed by flow cytometry (Supplementary Fig S8B).

C Cells expressing the indicated Hcm1 mutants from the TEF1 promoter were arrested in mitosis (Supplementary Fig S8C) and half-lives compared by cycloheximide-chase assay. Two exposures of Hcm1 blots are shown to highlight differences in stability between the double phosphomutants.

D Cycloheximide-chase assay of Hcm1(1-107)-GFP and Hcm1(1-107)3N-GFP fusion proteins in asynchronous cells.

E CDC53, cdc53-1, sic1Δ CDC53, and sic1Δ cdc53-1 cells expressing Hcm1(1-107)-GFP were arrested in mitosis (Supplementary Fig S8D) at the permissive temperature, shifted to 37°C for 15 min, and half-lives compared by cycloheximide-chase assay.

F Fivefold dilutions of cells with the indicated genotypes were spotted onto rich medium plates (YPD), or plates containing the indicated concentrations of benomyl. Source data are available online for this figure.
sites (referred to as 8C). Interestingly, Hcm1-3N expression was increased over the cell cycle, as with the Hcm1-15A allele, whereas Hcm1-8C was expressed in a pattern similar to WT Hcm1 (Fig 5A, compared to Fig 1C). To gain further evidence that Cdk1 mediates Hcm1 degradation by phosphorylating these three N-terminal sites, we examined turnover of Hcm1 in cell cycle phases that have different levels of Cdk1 activity. Because HCM1 is only transcribed in S-phase, the constitutive TEF1 promoter was introduced upstream of the HCM1 gene, so that it would be expressed throughout the cell cycle. Notably, in S and M phases, when Cdk1 activity is high (as confirmed by high Cib2 levels, Fig 5B), Hcm1 was rapidly degraded in a manner dependent upon the three Cdk1 consensus sites in the N-terminus (Fig 5B). In contrast, the half-life of Hcm1 was longer in G1-arrested cells that lack Cdk activity, and the degradation that did occur was independent of the three phosphosites (Fig 5B). This confirms that Hcm1 is degraded by a Cdk1-dependent pathway.

To gain a better understanding of how Hcm1 degradation is regulated, we next sought to identify the specific sites within the N-terminus that are required for its degradation. Interestingly, mutation of no single site stabilized Hcm1; however, mutation of T39 and either of the remaining two sites (S61 or S66) was sufficient to almost completely stabilize the protein (Fig 5C), supporting the model that multiple phosphorylations are required for optimal degradation. Additionally, we found that the first 107 amino acids of Hcm1 constitutes a transferrable degron: This sequence promoted degradation of GFP when fused to its N-terminus, in a manner dependent upon the three Cdk1 consensus sites (Fig 5D).

Interestingly, although earlier experiments examining full-length Hcm1 (Fig 2F) suggested that Hcm1 degradation was not altered upon inactivation of the core SCF subunit Cdc53, we noted that the arrangement of Cdk1 consensus sites in the Hcm1 N-terminus resembled a phosphodegron recognized by the F-box protein Cdc4 (Hao et al, 2007; Köivomägi et al, 2011a). We considered the possibility that SCF-mediated degradation was masked in earlier experiments due to the fact that cdc53-1 cells arrest in G1, when degradation occurs independent of N-terminal phosphorylation (Fig 5B). Consistent with this possibility, Hcm1(1-107)-GFP was stabilized in cdc53-1 cells that were arrested in mitosis (Fig 5E). Moreover, this stabilization was also observed in cells lacking the Cdk1 inhibitor Sis1, confirming that the stabilization was not an indirect consequence elevated Sis1 and inhibition of Cdk1 activity. Together, these data support the model that, like the other S-phase TFs, the Cdk1-dependent degradation of Hcm1 is mediated by an SCF ubiquitin ligase.

Next, we tested whether Hcm1-3N or Hcm1-8C showed reduced activity as a transcriptional activator, as we observed for Hcm1-15A (Fig 5A). First, we examined the growth of each strain on media containing the microtubule destabilizing drug benomyl. Although benomyl sensitivity may be an indirect consequence that results from a general disruption of mitotic gene expression, earlier studies found that hcm1Δ cells are benomyl-sensitive, which makes this a straightforward measure of Hcm1 activity (Pramila et al, 2002; Daniel et al, 2006). Cells expressing Hcm1-15A or Hcm1-8C were similarly sensitive to benomyl (Fig 5F), consistent with the fact that these cells have increased Hcm1 activity. Interestingly, overexpression of stable Hcm1 (TEF1p-hcm1-3N) had the opposite effect compared to overexpression or stabilization alone, resulting in benomyl hypersensitivity. Thus, just as having too little Hcm1 sensitizes cells to benomyl, overproduction of Hcm1 (and/or misexpression at inappropriate times in the cell cycle) is detrimental when spindle function is compromised. These data suggest that the transcriptional program that is driven by Hcm1 is important for fine-tuning the sensitivity of the spindle checkpoint response.

Phosphorylation of the C-terminus of Hcm1 is required for its activity

Since Hcm1 targets were downregulated in 4P cells (Fig 3E), and cells expressing either Hcm1-15A or Hcm1-8C were sensitive to benomyl (Fig 5F), this suggested that Cdk1-dependent phosphorylation of the C-terminus of Hcm1 may be required for its activity. To test this directly, we examined expression of several Hcm1 target genes during S-phase and found that they were downregulated in hcm1-15A and hcm1-8C cells, as in hcm1Δ cells (Fig 6A). One trivial possibility is that changing Ser/Thr residues in the C-terminus leads to misfolding of Hcm1 and that the 8C mutant is non-functional for this reason. Therefore, to provide further evidence that phosphorylation of the Hcm1 C-terminus is important for its function, we attempted to construct a phosphomimetic version of the protein. First, all 8 C-terminal Ser/Thr residues were changed to Glu (Hcm1-8E). Cells expressing this mutant grew slightly better than hcm1-8C cells on benomyl plates, but were more sensitive than WT cells (Fig 6B). One possible explanation for this partial effect may be that a phosphorylated Ser or Thr introduces a change in net charge of −2, whereas the replacement of a Ser/Thr with a single Glu only changes the net charge by −1. Previously, substitution with two Glu residues was shown to more closely mimic the phosphorylated state than individual Glu replacements (Strickfaden et al, 2007), so we used a similar strategy and replaced Ser/Thr-Pro motifs with Glu-Glu in the Hcm1 C-terminus (Hcm1-16E). Interestingly, hcm1-16E cells were substantially healthier on benomyl plates than hcm1-8C cells and were comparable to wild-type cells (Fig 6B). We then examined target gene expression in hcm1-16E cells and found only modest changes in gene expression, compared to the hcm1-8C mutant (Fig 6A). These data support the model that increasing the negative charge of the Hcm1 C-terminus through phosphorylation is important for its activity, perhaps by mediating interactions with other regulatory proteins.

Next, we investigated how phosphorylation regulates Hcm1 activity. First, we examined Hcm1 localization, since the subcellular localization of some forkhead TFs is regulated by phosphorylation (Myatt & Lam, 2007). However, nuclear localization of Hcm1 was not disrupted by any of the Cdk1 site mutations, although the stabilized forms (Hcm1-15A and Hcm1-3N) were detectable throughout the cell cycle instead of being restricted to S-phase cells (Supplementary Fig S9). We then tested whether modulating the phosphorylation of the Hcm1 C-terminus affected its recruitment to target gene promoters. Interestingly, the association of Hcm1-8C with several target promoters was strongly reduced relative to WT, whereas the Hcm1-16E phosphomimetic mutant was recruited slightly better than wild-type (Fig 6C). Thus, Cdk1 phosphorylation appears to promote the activity of Hcm1 by stimulating its association with chromatin. Altogether, our data indicate that Cdk1 phosphorylation has two opposing effects on Hcm1: Phosphorylation of the C-terminus...
promotes its DNA binding activity, leading to activation of its target genes, while phosphorylation of the N-terminus leads to its degradation.

**Coordinated phosphorylation of S-phase TFs is important for cellular fitness**

Our analyses of individual TFs led to the model that Cdk1 promotes expression of late cell cycle genes by stimulating the activity of a transcriptional activator (Hcm1) and inactivating transcriptional repressors (Yox1, Yhp1) in S-phase. We hypothesized that phosphorylation by Cdk1 might be necessary to coordinate the activities of these TFs, in order to ensure that their target genes are expressed in the proper order as cells progress through S-phase and mitosis. To gain a better understanding of the importance of this regulation, we took a genetic approach and examined a panel of strains that includes every possible combination of Cdk- TF alleles.

First, we measured the median cell size of each strain in an asynchronous cell culture, since subtle differences in proliferation rates often lead to differences in the median size of a population (Jorgensen et al, 2002). Interestingly, the 4P strain (hcm1-15A tos4-9A yox1-9A yhp1-13A) was approximately 17% larger than a matched wild-type strain (48.5 fl compared to 41.45 fl, Fig 7A). The size of this strain is comparable to what have been classified as large mutants (3P, 33.6 fl) (Jorgensen et al, 2002). Among the single mutant Cdk-TF strains, hcm1-15A had the largest change in cell size, whereas the other single mutants were larger than wild-type, but smaller than hcm1-15A (Fig 7A). Notably, combining any second mutation with hcm1-15A increased the size to a level comparable to the 4P strain, and any subsequent mutations did not increase size further, suggesting that the other TFs may impinge upon the same cellular processes.

Since combining any second mutation with hcm1-15A led to a further increase in cell size, we hypothesized that the inclusion of other Cdk- TFs may also exacerbate the benomyl sensitivity of hcm1-15A cells. Surprisingly, in contrast to hcm1-15A, the 4P strain grew as well as wild-type on benomyl plates, as did a triple mutant that included both yox1-9A and yhp1-13A alleles (Fig 7B). However, yox1-9A or yhp1-13A alone could not rescue the hcm1-15A phenotype. These results suggest that hyperactivation of Yox1/Yhp1 counteracts the consequences of Hcm1 loss of function in this scenario. A likely possibility is that the decreased expression of Yox1/Yhp1 target genes slows progression through mitosis, thereby allowing cells to cope with the compromised spindle function that occurs in hcm1-15A cells when they are challenged with benomyl (see further discussion below).

We observed a similar relationship between hcm1-15A and the other Cdk- TFs in a fitness assay. Strains of different genotypes were co-cultured and the fraction of each strain in the culture was quantified over time. In this assay, hcm1-15A mutants exhibited a strong fitness defect, whereas a strain carrying the other three Cdk- alleles (3P, tos4-9A yhp1-13A yox1-9A) was as fit as wild-type (Fig 7C). Interestingly, similar to the benomyl result, the hcm1-15A fitness defect was partially rescued in the 4P strain that included all four Cdk- TFs. However, it is important to note that although it was healthier than the hcm1-15A strain, the 4P mutant was less fit than wild-type. This is consistent with the observation that 4P cells have
a delay in mitosis when growing exponentially (Fig 3B) and confirms that Cdk1 phosphorylation of these TFs is important for overall fitness. Importantly, losing phosphorylation of these TFs is not equivalent to deleting all four TFs, since a more severe defect was observed in a quadruple delete strain (Fig 7C). These results highlight the utility of dissecting phosphorylation networks using a combinatorial genetic analysis. Additionally, these findings illustrate how Cdk1 acts as a master regulator of S/M-phase transcription, by coordinately regulating the activities of TFs that collectively control the expression of late cell cycle genes.

**Discussion**

Although Cdk1 is known to be required for robust regulation of cell cycle-regulated gene expression (Orlando et al, 2008; Simmons Kovacs et al, 2012), the mechanism by which it controls the TF network is not well understood. Here, we have elucidated this regulation by directly eliminating phosphorylation of four TFs that are co-expressed in S-phase. Interestingly, mutation of no individual S-phase TF significantly impacted the cell cycle (Supplementary Fig S2A, C, E and G). However, when Cdk1 phosphorylation of all four TFs was eliminated simultaneously, cells showed decreased expression of late cell cycle genes (Fig 3C–H), progressed through mitosis at a slower rate (Fig 3B), and had reduced fitness (Fig 7C), confirming the overall importance of Cdk1 regulation of this group of S-phase TFs.

The mitotic delay that we observed in 4P cells is most likely due to the widespread reduction of genes that are required for progression through mitosis. Interestingly, most cell cycle-regulated genes continue to cycle in 4P cells, similar to what is observed in cells that lack all Cdk1 activity (Haase & Reed, 1999; Orlando et al, 2008).
However, we find that many late cell cycle genes never reach maximal expression (Fig 3, Supplementary Fig S5C and D). During S-phase, Hcm1 activates transcription of a large number of genes that impact chromosome segregation and the mitotic spindle (Pramila et al., 2006). Conversely, Yox1 and Yhp1 act in S-phase to prevent premature expression of genes that promote the M/G1 transition. Thus, the combination of reduced Hcm1 activity and increased Yox1/Yhp1 function in 4P cells appears to lead to reduced expression of both groups of genes, and slower progression through mitosis. Interestingly, while this combination of mutations slows the cell cycle and leads to decreased fitness under optimal growth conditions, the coordinate downregulation of both groups of target genes may actually be beneficial to cells when they are challenged with spindle disruption. By slowing progression through mitosis, the hyperactivation of Yox1 and Yhp1 may help cells cope with the compromised spindle function that occurs in the absence of Hcm1 activity.

Previous results suggested that mutation of each of the four TFs would impact the expression and function of the downstream TFs Fkh2 and Ndd1 (Horak, 2002; Pramila et al., 2002, 2006; Darieva et al., 2010) and by extension affect expression of the CLB2 cluster genes that are regulated by Fkh2/Ndd1. To our surprise, expression of the CLB2 cluster was largely unaffected in cells carrying all 4 Cdk alleles (Fig 3G). The most likely explanation for this finding is that redundant factors enforce the expression of this group of genes. In support of this possibility, both Fkh2 and Ndd1 are themselves activated by Cdk1 (Reynolds, 2003; Pic-Taylor et al., 2004), and this activation still occurs in 4P cells. This regulation fits with our model of how Cdk1 promotes the expression of late cell cycle genes (Fig 8). In combination with previous work, our results suggest that from S-phase through the beginning of mitosis, Cdk1 stimulates the activity of transcriptional activators (Hcm1, Fkh2, Ndd1) and shuts off the activity of transcriptional repressors (Yox1, Yhp1), and together this coordinated regulation drives expression of genes with key mitotic functions. In the future, it will be of interest to determine whether this model applies to regulation by Cdk1 in other systems.

This theme of coordinated positive and negative regulation by Cdk1 not only occurs on the level of the TF network, but also happens on an individual protein. Remarkably, we find that separable Cdk1 sites on Hcm1 mediate its activity and degradation. This raises the possibility that these phosphorylation events may be separated in time: The C-terminal phosphosites of Hcm1 may be phosphorylated first, promoting its activity, followed by phosphorylation of the N-terminal sites to promote its degradation. Conceivably, this could be achieved if the S-phase cyclin/Cdk preferentially phosphorylates the C-terminal sites. Alternatively all sites might be phosphorylated simultaneously, which would result in a limited window of expression of active, but unstable, Hcm1. In either scenario, our data reveal the importance of regulating both the activation and degradation of Hcm1. We found that cells expressing Cdk- Hcm1 are hypersensitive to spindle poisons (Figs 5F and 6B) and are considerably less fit than wild-type cells (Fig 7C). Interestingly, we also found that cells constitutively expressing stable Hcm1 are also hypersensitive to spindle poisons (Fig 5F). Therefore, degradation of Hcm1 plays an important role in tuning the response of the cell to problems that arise with the mitotic spindle.

In contrast, Cdk1 phosphorylation leads to a decrease in the activities of the transcriptional repressors Yox1 and Yhp1. At M/G1 promoters, Yox1 and Yhp1 repress transcription by binding alongside Mcm1 at Early Cell cycle Box (ECB) elements. Interestingly, a recent study suggested that Yox1 and the transcriptional activator Bck2 compete for access to Mcm1 at these promoters (Bastajian et al., 2013). Cdk1-mediated phosphorylation of Yox1 might lead to its release from these sites, which could then allow Bck2 to bind and activate transcription. Although our data support the model that Cdk1 limits the activities of Yox1 and Yhp1 by simply promoting their degradation, we cannot rule out the possibility that phosphorylation may also regulate their DNA binding affinities or their interaction with co-regulatory proteins.

In conclusion, we have taken a genetic approach to understand how Cdk1 regulates S-phase TFs in vivo. Systematic combination of gene deletions in yeast has led to a wealth of information about how genes and proteins interact in cells (Tong et al., 2004; Costanzo et al., 2010). To our knowledge, no similar large-scale genetic analysis has been carried out using phospho-deficient alleles of proteins. Our work suggests that this type of approach could be extremely valuable when studying a kinase such as Cdk1 that targets a significant fraction of the proteome (Ubersax et al., 2003; Holt et al., 2009). By eliminating phosphorylation of a group of TFs that were predicted to act together in a common process, and to be coordinately phosphorylated, we were able to elucidate the specific role of Cdk1 in controlling the late cell cycle transcriptional network and to uncover the importance of this coordinate regulation. In the future, it will be of great interest to test whether unrelated Cdk1 targets with different cellular functions exhibit similar, coordinate regulation. In addition, it will be important to determine whether these general principles of regulation by Cdk1 are conserved in other systems. Coordinated positive and negative regulation by key regulatory kinases may be a conserved mechanism that functions to coordinate diverse processes in response to specific biological or environmental signals.

Materials and Methods

Yeast strains and plasmids

A complete list of strains, including the specific figures each was used in, is provided in Supplementary Table S1. 3HA-tagged

![Figure 8. Model of regulation of the late cell cycle gene regulatory network by Cdk1.](image-url)
Cdk- alleles and HCM1 phosphomimetic alleles were generated by gene synthesis (Biobasic; Invitrogen) and subcloned, along with wild-type counterparts, into the vector pRS413-GAL1p. Strains carrying mutations in phosphorylation sites were generated by integrating a PCR product containing the desired mutations, the 3HA tag, and the HIS3MX marker at the genomic locus. The integration of each mutation was then confirmed by sequencing. Alternate tags were then added to wild-type and Cdk- genes, as indicated, by replacing the 3HA-HIS3MX sequence with alternate tag-marker sequences. The 4P strain (containing all four Cdk- alleles) was generated by crossing strains that each carried two phosphomutant alleles and recovering strains carrying all combinations of alleles.

The TEF1 promoter was introduced upstream of HCM1 by integration of the Hyg-TEF1p cassette directly upstream of the HCM1 start codon. To construct the HCM1(1-107)-GFP strain, YDR071W (an uncharacterized, non-essential gene) was replaced in the YDR071W-GFP strain from the GFP collection (Huh et al., 2003) with the Hyg-TEF1p-HCM1(1-107)-GFP was then introduced into the cdc35-1 background by a genetic cross.

All strains were grown in YM-1 complete medium with 2% dextrose, with the exception of strains carrying plasmids, which were grown in C medium lacking histidine with 2% dextrose or raffinose (Benanti et al., 2007). To arrest cells in G1, 20 μg/ml alpha-factor (United Biochemical Research, Inc.) was added for 2–3 h. To arrest cells in mitosis, 10 μg/ml nocodazole (US Biological) was added to cells for 2 h. To arrest cells in early S-phase, 200 mM hydroxyurea (Sigma) was added to cells for 2 h. For G1 arrest-release experiments, cells were arrested in G1 as described above, pelleted, washed once with YM-1, and resuspended in YM-1 with 2% dextrose. Strains carrying temperature-sensitive CDC35 were grown at 23°C, then shifted to 37°C (Fig 2E–H), or arrested in nocodazole for 2 h and then shifted to 37°C for 15 min (Fig 5E). Cells carrying the cdc28-as1 were treated with 5 μM 1NM-PP1 for the indicated amount of time to inhibit Cdk1 activity.

Cell cycle analysis

In all experiments, cell cycle positions were confirmed by flow cytometry. Cells were fixed and labeled with Sytox Green (Invitrogen) as previously described (Landry et al., 2012). Samples were analyzed using a FACScan (Becton Dickinson) and data analyzed with FlowJo (Tree Star, Inc.) software.

Western blotting

Equivalent optical densities of cells were collected and lysed as previously described (Landry et al., 2012), with the exception of the experiments presented in Figs 1A and D–G, and 2A-I. In these experiments, cell pellets were lysed in cold TCA buffer (10 mM Tris pH 8.0, 10% trichloroacetic acid, 25 mM ammonium acetate, 1 mM EDTA). After incubation on ice, lysates were then centrifuged and the pellets resuspended in resuspension solution (0.1 M Tris pH 11.0, 3% SDS). Samples were heated to 95°C for 5 min, allowed to cool to room temperature, and clarified by centrifugation. Supernatants were added to 4× SDS–PAGE Sample Buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol) and heated to 95°C for 5 min. Western blotting was performed as previously described (Landry et al., 2012) with antibodies against Myc (Clone 9E10; Covance) Cdc28 (Cdk1) (sc-6709; Santa Cruz Biotechnology), Cdb2 (sc-9071; Santa Cruz Biotechnology), Flag (Clone M2; Sigma), HA (Clone 16B12; Covance), V5 (Invitrogen), GFP (Clone JL8; Clontech). Where indicated, protein levels were quantified using ImageJ and normalizing to Cdk1 as a loading control.

Kinase assays

Strains expressing 3HA-tagged WT or Cdk- TFs from the GAL1 promoter on a plasmid were arrested in G1 with alpha-factor (when Cdk1 activity is low), and expression was induced by the addition of galactose. TFs were immunoprecipitated with anti-HA antibody (clone 12CA5 or 16B12) bound to magnetic protein A or G beads and phosphorylated with Clb2/Cdk1 kinase that had been purified as previously described (Lopez-Mosqueda et al., 2010). Cold kinase reactions (Fig 1B) were carried out in reaction buffer (25 mM Hepes-Oh pH 8, 15 mM NaCl, 1 mM MgCl2, 1 mM DTT, 1 mM ATP) with Clb2/Cdk1 kinase for 45 min at room temperature, and phosphorylation was assayed by Western blot. Radioactive kinase assays (Supplementary Fig S1B) were carried out in similar buffer (25 mM Hepes-Oh pH 8, 15 mM NaCl, 1 mM MgCl2, 1 mM DTT, 20 μM ATP) supplemented with γ-32P-ATP, and assayed by SDS-PAGE followed by autoradiography. Phosphorylation of Yox1 and Hcm1 mutants were quantified using a GE Typhoon 9000 phosphor-imager and ImageJ software.

Cycloheximide-chase assays

Cells were grown to mid-log phase, or arrested as indicated, and assays carried out as previously described (Landry et al., 2012).

Microarray analysis

RNA was purified as described (Schmitt et al., 1990). One to 5 μg of total RNA was then subjected to RNA amplification and labeling using the Low Input Quick Amp Labeling Kit protocol (Agilent) with minor modifications. Briefly, cRNA was amplified by in vitro transcription with amino-allyl UTP (3:2 ratio for amino-allyl UTP: UTP) overnight at 37°C. Then, cRNA was purified using RNA Clean and Concentrator columns (Zymo Research) and labeled with Cy3 or Cy5 (GE Healthcare) at room temperature for 90 min in the dark. The fluorescence intensities of Cy3 and Cy5 were determined by NanoDrop, and 600 ng of each cRNA was used for fragmentation and hybridization on Agilent Yeast (V2) Gene Expression Microarrays, 8 × 15 K. Slides were scanned on Agilent DNA microarray scanner G2565CA, and fluorescence data were obtained using Agilent Feature Extraction software at the UMass Medical School genomics core facility. The microarray data from this publication have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) and assigned the identifier GSE55121.

RT-qPCR

RNA was digested with DNaseI (New England Biolabs) and precipitated. One to 2 μg of RNA was then reverse-transcribed using Random Primers (Invitrogen), followed by digestion with RNaseH.
Cell size measurements

Cells were grown to mid-log phase and measured on a Beckman Coulter Multisizer 3 Coulter Counter. Median size measurements were calculated using the Beckman Coulter Particle Characterization software version 3.53.

Competition assays

Competition assays were carried out as previously described (Torres et al., 2010). Equal numbers of cells with and without PGK1-GFP were co-cultured in 10 ml YM-1 with 20% dextrose and diluted every 12 h to keep cultures in continuous exponential growth phase. Cells were fixed in ethanol at the indicated time points and quantified after the completion of the experiment using a FACScan (Becton Dickinson).

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

JAB and BDL designed the experiments. BDL, HEA, CEM, and KEP carried out the experiments. BDL, HEA, and CEM analyzed the data. JAB wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

References


genome-wide transciptional analysis of the mitotic cell cycle. Mol Cell 2: 65 – 73
Littlepage LE, Ruderman JV (2002) Identification of a new APC/C recognition domain, the A box, which is required for the Cdh1-dependent destruction of the kinase Aurora-A during mitotic exit. Genes Dev 16: 2274 – 2285
Ma X, Defazio-Eli LG, Wohlschlegel J, Toczyski DP (2010) Damage-induced phosphorylation of Sld3 is important to block late origin firing, Nature 467: 479 – 483
S. cerevisiae. PLoS One 7: e45895. Available at: http://dx.plos.org/10.1371/journal.pone.0045895.g006


Pramila T, Miles S, GuhaThakurta D, Jemiolo D, Breeden LL (2002) Conserved homeodomain proteins interact with MADS box protein Mcm1 to restrict ECB-dependent transcription to the M/G1 phase of the cell cycle. Genes Dev 16: 3034–3045


Thornton BR, Toczyski DP (2003) Securin and B-cyclin/CDK are the only essential targets of the APC. Nat Cell Biol 5: 1090–1094


