Hcm1 integrates signals from Cdk1 and calcineurin to control cell proliferation

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ABSTRACT **Cyclin-dependent kinase (Cdk1) orchestrates progression through the cell cycle by coordinating the activities of cell-cycle regulators. Although phosphatases that oppose Cdk1 are likely to be necessary to establish dynamic phosphorylation, specific phosphatases that target most Cdk1 substrates have not been identified. In budding yeast, the transcription factor Hcm1 activates expression of genes that regulate chromosome segregation and is critical for maintaining genome stability. Previously we found that Hcm1 activity and degradation are stimulated by Cdk1 phosphorylation of distinct clusters of sites. Here we show that, upon exposure to environmental stress, the phosphatase calcineurin inhibits Hcm1 by specifically removing activating phosphorylations and that this regulation is important for cells to delay proliferation when they encounter stress. Our work identifies a mechanism by which proliferative signals from Cdk1 are removed in response to stress and suggests that Hcm1 functions as a rheostat that integrates stimulatory and inhibitory signals to control cell proliferation.**

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INTRODUCTION

Cyclin-dependent kinases (Cdks) orchestrate progression through the cell division cycle by phosphorylating hundreds of regulatory proteins in a defined order (Ubersax *et al.*, 2003; Holt *et al.*, 2009). Disruption of this ordered phosphorylation can result in errors in DNA replication and chromosome segregation that can be lethal or result in genomic instability that can lead to cancer (Hanahan and Weinberg, 2011). The activities of Cdks are opposed by phosphatases, which are equally important for establishing the cyclical phosphorylation patterns that drive the cell cycle. However, whereas a great deal is known about how Cdks recognize and target substrates, much less is known about the phosphatases that antagonize Cdk function (Mochida and Hunt, 2012). The best-understood phosphatases that function in cell cycle control are those that counteract Cdk1 to regulate mitosis. These include Cdc14, PP1, and PP2A (Visintin *et al.*, 1998; Domingo-Sananes *et al.*, 2011; Wurzenberger and Gerlich, 2011; Grallert *et al.*, 2015). In addition, a few phosphatases, including PP2A (Mochida and Hunt, 2007; Alvarez-Fernández *et al.*, 2011), PP4 (Toyo-oka *et al.*, 2008), and *Caenorhabditis elegans* CDC-14 (Saito *et al.*, 2004), have been found to remove Cdk-catalyzed phosphorylations in interphase. However, for the majority of Cdk substrates, their cognate phosphatases remain to be identified, and the extent to which phosphatases play direct roles in controlling progression through interphase is not known.

The calcium-activated phosphatase PP2B/calcineurin (CN) has also been implicated in cell cycle control. CN is required for progression through meiosis in *Xenopus* and *Drosophila* (Cao *et al.*, 2006; Mochida and Hunt, 2007; Nishiyama *et al.*, 2007; Takeo *et al.*, 2010). In *Xenopus* oocytes, CN directly removes Cdk1-catalyzed phosphorylations from substrates to trigger cell cycle reeentry (Cao *et al.*, 2006; Mochida and Hunt, 2007). The role of CN in controlling the mitotic cell cycle is less clear. Positive genetic interactions between Cdk1 (*cdc28*) and CN (*cnb1*) mutants in budding yeast suggest that CN may antagonize Cdk1 phosphorylation, and CN targets include known Cdk1 substrates (Fiedler *et al.*, 2009;

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Abbreviations used: Cdk, cyclin-dependent kinase; CN, calcineurin.

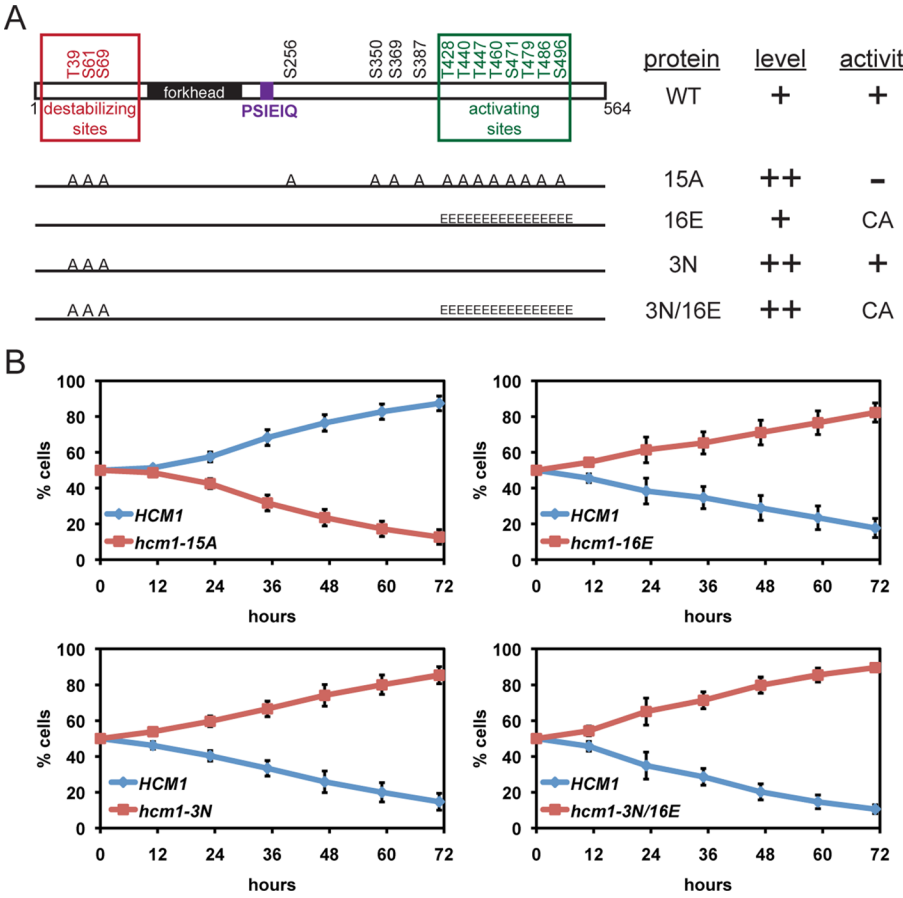

FIGURE 1: Hcm1 phosphorylation affects cellular fitness. (A) Positions of Cdk1 phosphosites that activate (green) and destabilize Hcm1 (red). Mutations that block degradation lead to an increase in protein level (15A, 3N, 3N/16E); phosphomimetic mutations (16E, 3N/16E) lead to constitutive activity (CA). (B) Strains expressing *PGK1-URA3* (blue lines) and *PGK1-GFP-URA3* (red lines) were cocultured and the percentage of each strain in the population determined at the indicated time points. Average values ±1 SD of five to eight replicates.

Goldman *et al.*, 2014). Moreover, CN activation delays the cell cycle in yeast strains lacking Zds1 and Zds2 (Miyakawa and Mizunuma, 2007), which are regulatory subunits of the phosphatase PP2A (Rossio and Yoshida, 2011), suggesting that CN and PP2A may act redundantly to arrest the cell cycle in some contexts. In addition, our recent work identified a number of CN targets involved in polarized growth, which is coordinated with the cell cycle (Goldman *et al.*, 2014). Thus, although a role for yeast CN in mitotic cell cycle regulation seems likely, this function has yet to be elucidated.

Among the key targets of Cdks in all eukaryotes are cell cycle–regulatory transcription factors, which coordinate gene expression with the cell cycle (Bertoli *et al.*, 2013; Sadasivam and DeCaprio, 2013). In budding yeast, Cdk1 phosphorylation regulates the activity of the forkhead family transcription factor Hcm1, which is a homologue of the human oncogene FoxM1 (Myatt and Lam, 2007). Like FoxM1, Hcm1 activates expression of genes that are required for accurate chromosome segregation, including molecular motors, components of the spindle pole body, and the kinetochores (Laoukili *et al.*, 2005; Pramila *et al.*, 2006; Grant *et al.*, 2013). Consistent with the functions of these target genes, mutations that inactivate Hcm1 sensitize cells to microtubule poisons that destabilize the mitotic spindle (Horak *et al.*, 2002; Daniel *et al.*, 2006), cause an increased rate of chromosome loss (Pramila *et al.*, 2006), and are lethal when combined with mutations in spindle checkpoint genes (Sarin *et al.*, 2004; Tong *et al.*, 2004; Daniel *et al.*, 2006). Thus proper regulation of Hcm1 activity is critical for cells to maintain a stable genome.

We recently found that Cdk1 phosphorylation of Hcm1 is required for both its activation, by promoting chromatin binding, and its degradation via the ubiquitin-proteasome system (Landry *et al.*, 2014). Of interest, these opposing outcomes are mediated by phosphorylation of distinct clusters of Cdk1 phosphosites. It is not known whether or how these antagonistic phosphosites are differentially regulated in order to establish a window of Hcm1 activity during the cell cycle. One possibility is that the two clusters of sites are phosphorylated by Cdk1 with different efficiencies and/or by distinct cyclin/Cdk1 complexes. A second possibility is that the activating and destabilizing sites are phosphorylated simultaneously but with one group of sites being differentially regulated by a phosphatase. Here we provide evidence that activating phosphorylations on Hcm1 are specifically removed by the phosphatase CN. We find that by removing activating phosphorylations but not targeting the destabilizing phosphosites, CN inactivates Hcm1 without disrupting ubiquitin-mediated degradation. This decrease in Hcm1 activity contributes to decreased proliferation in response to stress, since blocking Hcm1 dephosphorylation allows cells to proliferate better in response to CN-activating stresses. In addition, cells expressing a CN-resistant mutant of Hcm1 are fitter than wild type cells in the absence of stress, demonstrating that CN directly antagonizes Cdk1 activity to control proliferation.

RESULTS AND DISCUSSION

Phosphorylation of Hcm1 regulates proliferation

Hcm1 activates expression of genes that regulate chromosome segregation and mitosis (Pramila *et al.*, 2006). As a result, deletion or inactivation of *HCM1* affects progression through the cell cycle. This cell cycle defect is easily observed when mutant cells are cocultured with wild-type cells in a competitive fitness assay, which is a sensitive growth assay that reveals differences in proliferation rate. Previously we found that blocking all phosphorylation of Hcm1 by Cdk1 led to a reduction in cellular fitness (*hcm1-15A*; Figure 1B; Landry *et al.*, 2014), thus demonstrating that regulation of Hcm1 by Cdk1 is critical for robust proliferation.

Here we wanted to address whether phosphoregulation limits Hcm1 activity during the cell cycle. To answer this question, we examined the fitness of cells expressing Hcm1 phosphosite mutants that have increased activity. Strains expressing a mutant that is highly expressed because it cannot be phosphorylated on destabilizing sites (*hcm1-3N*) and a mutant that is constitutively active because it has mutations that mimic phosphorylation at activating Cdk1 sites (*hcm1-16E*; Figure 1A; Landry *et al.*, 2014) were tested in coculture assays with wild-type cells. Remarkably, increasing Hcm1

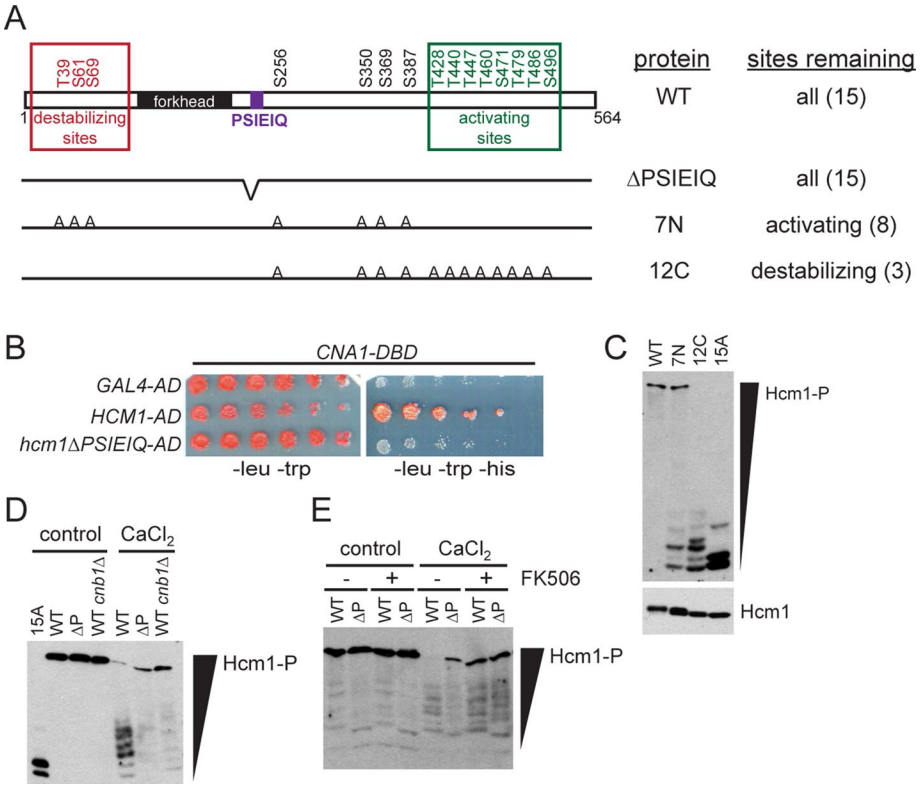

FIGURE 2: Hcm1 is dephosphorylated by calcineurin in vivo. (A) Diagram of Hcm1 mutants assayed in B–D, along with the number of Cdk1 (S/T-P) sites retained in each protein. (B) Yeast two-hybrid assay showing interaction between Hcm1 and Cna1. Deletion of the docking site (*hcm1ΔPSIEIQ*) disrupts the interaction. (C) Western blots comparing separation of Hcm1 proteins that contain different numbers of Cdk1 sites on a Phos-tag gel (top) and standard SDS–PAGE (bottom). (D) Phos-tag Western blot of strains expressing Hcm1-15A (15A, no Cdk1 sites), wild-type Hcm1 (WT), Hcm1ΔPSIEIQ (ΔP), or wild-type Hcm1 in a *cnb1Δ* strain assayed after the addition of $CaCl_2$ for 10 min and compared with untreated control cells. (E) Hcm1 phosphorylation assayed as in D, except that cells were treated with FK506 or ET buffer for 1 h and then $CaCl_2$ was added for 15 min.

activity by either mechanism caused cells to outcompete wild-type cells to a similar extent, as did combining both sets of mutations (Figure 1B). These results indicate that Hcm1 activity is normally limiting for maximal proliferation. Moreover, because the phosphomimetic mutant showed a dramatic fitness advantage, this suggested that the activating sites on Hcm1 are not always fully phosphorylated in wild-type cells and that a phosphatase may critically regulate Hcm1 activity in vivo.

Hcm1 is a target of the phosphatase calcineurin

In a previous yeast two-hybrid screen, we identified Hcm1 as a candidate substrate of the conserved Ca^{2+}-activated phosphatase CN (unpublished data). Hcm1 interacted with the CN-regulatory subunit Cna1 in this assay, and the interaction was eliminated upon deletion of a six–amino acid sequence on Hcm1 (PSIEIQ; Figure 2, A and B) that matches the consensus for the previously identified CN docking-site sequence (Roy *et al.*, 2007). In addition, we found that Hcm1 associated with Cna2 in vivo, and this interaction was inhibited by deletion of the docking site (Supplemental Figure S1A). These data suggest that CN might antagonize Hcm1 phosphorylation by Cdk1 to regulate its activity and affect proliferation.

The best-understood role of CN in yeast is to promote cell survival in response to environmental stresses that signal through an increase in intracellular Ca^{2+} (Cyert and Philpott, 2013). Although CN was found to target several Cdk1 substrates in a recent proteomic screen (Goldman *et al.*, 2014), it is not known whether it directly regulates proliferation. To determine whether CN regulates proliferation via dephosphorylation of Hcm1, we examined Hcm1 phosphorylation in vivo after CN activation. We first established a Western blotting assay using Phos-tag reagent, which enables the resolution of individual phosphosites upon its addition to a polyacrylamide gel (Kinoshita *et al.*, 2006). Under these gel conditions, the phosphorylation status of a panel of Hcm1 mutants with different numbers of Cdk1 phosphosites is clearly distinguishable (Figure 2, A and C). In addition, we found that within 10 min of direct CN activation by the addition of $CaCl_2$ to the growth medium, Hcm1 was dramatically dephosphorylated (Figure 2D). This dephosphorylation was blocked by deletion of the CN docking site in Hcm1 (ΔP; Figure 2D), by deletion of the regulatory subunit of CN, Cnb1 (Figure 2D), or upon addition of the CN inhibitor FK506 (Figure 2E). These data demonstrate that Hcm1 is dephosphorylated by CN in vivo.

Of note, Hcm1 retained some phosphorylation after CN activation (Figure 2, D and E), suggesting that CN might target only a subset of Hcm1 phosphosites. To test this, we examined CN-dependent dephosphorylation of previously characterized mutants in which all Cdk1 sites were mutated except for the eight activating Cdk1 sites (Hcm1-7N, with 7 N-terminal S/T-P sites mutated to A-P; Figure 2A) or the three destabilizing Cdk1 sites (Hcm1-12C, with 12 C-terminal S/T-P sites mutated to A-P; Figure 2A; Landry *et al.*, 2014). Of interest, Hcm1-7N was almost completely dephosphorylated after CN activation, whereas Hcm1-12C phosphorylation was largely unchanged (Figure 3A). This specificity was also observed in vitro. Recombinant Hcm1 proteins were phosphorylated with Clb2/Cdk1 and then incubated with or without purified CN. Consistent with the in vivo results, CN dephosphorylated wild-type Hcm1 and Hcm1-7N but had no effect on Hcm1-12C (Figure 3B and Supplemental Figure S1B). In addition, deletion of the PSIEIQ motif impaired Hcm1 dephosphorylation in vitro (Figure 3B and Supplemental Figure S1B), although it was not completely blocked. This partial dephosphorylation of the ΔPSIEIQ mutant suggests that this motif is less important to mediate the interaction between CN and Hcm1 in vitro than in vivo, where there are hundreds of other CN targets present that reduce the concentration of available CN. These results show that Hcm1 can be directly dephosphorylated by CN and demonstrate that CN has specificity for C-terminal Cdk1 sites that are required for Hcm1 activity.

Calcineurin inactivates Hcm1

To determine the consequences of Hcm1 dephosphorylation, we first examined whether CN regulates Hcm1 expression. If CN does not target N-terminal Cdk1 sites that promote phosphorylation-mediated degradation of Hcm1, as our data suggest (Figure 3, A and B), then CN activation should not stabilize Hcm1. Consistent

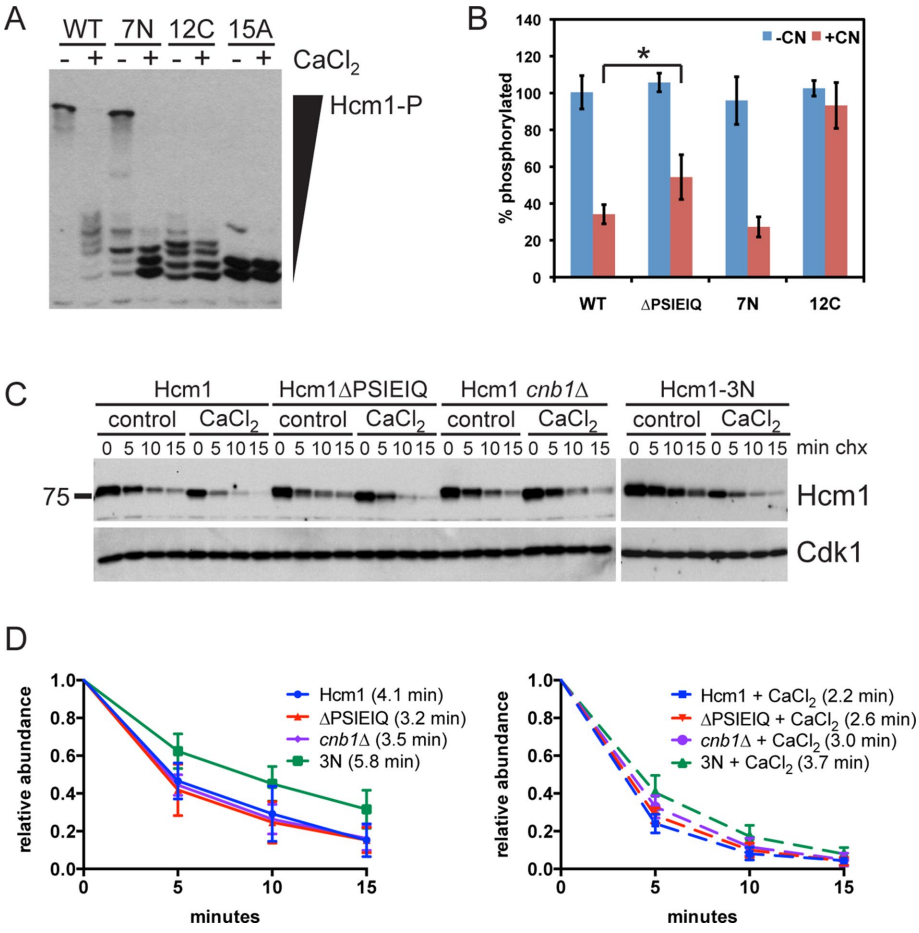

FIGURE 3: Calcineurin removes activating phosphorylations from Hcm1. (A) Strains expressing indicated Hcm1 proteins were treated with $CaCl_2$ for 10 min and phosphorylation assayed by Phos-tag Western blot. Note that the top band in the 12C sample is also present in the 15A sample, suggesting that it is the result of Cdk1-independent phosphorylation. (B) Cdk1-phosphorylated Hcm1 proteins were incubated with or without CN for 45 min. Average percentage phosphorylation remaining is shown ± 1 SD ($n = 3$). Hcm1ΔPSIEIQ is significantly different from wild-type Hcm1 (*$p < 0.05$, Student's t test). Also see Supplemental Figure S1B. (C) Cycloheximide-chase assays comparing the stability of Hcm1 proteins with or without $CaCl_2$ treatment. (D) Average data from five experiments were used to calculate half-life values (indicated in parentheses) of each protein with or without $CaCl_2$.

with this prediction, $CaCl_2$ treatment did not lengthen Hcm1 half-life (Figure 3, C and D). In fact, Hcm1 half-life was modestly decreased after $CaCl_2$ treatment. Of importance, deletion of the CN docking site or expression of wild-type Hcm1 in a CN-deficient strain also did not lengthen Hcm1 half-life (Figure 3, C and D), and deletion of the CN docking site did not affect cell cycle–regulated expression of Hcm1 (Supplemental Figure S2A). Together these data support the finding that the N-terminal phosphosites in Hcm1 are not targeted by CN. They also suggest that there may be an active mechanism to further destabilize Hcm1 when cells are exposed to stress. This destabilization appears to be independent of CN and Cdk1, since the half-life of all mutants were shortened. One possibility is that the previously identified Cdk1-independent mode of Hcm1 degradation that occurs in G1 phase (Landry *et al.*, 2014) may be enhanced to promote a further reduction in Hcm1 activity.

We next examined whether CN might affect Hcm1 activity, by analyzing previous genome-wide expression data (Yoshimoto *et al.*, 2002) to determine whether Hcm1 target genes change in expression in response to CN activation. Of interest, we found that more than half of predicted Hcm1 target genes were down-regulated after cells were treated with $CaCl_2$ and that this down-regulation was largely reversed when CN was inhibited by FK506 (Figure 4, A and B). We next confirmed these findings by examining expression of a subset of these genes that we previously demonstrated are direct Hcm1 targets (Landry *et al.*, 2014). Each of these targets was down-regulated after $CaCl_2$ treatment in wild-type cells (blue bars, Figure 4C). Of note, the extent of down-regulation observed was similar to the decrease in peak expression of these genes that occurs during a normal S phase when Hcm1 has been inactivated or deleted (Landry *et al.*, 2014), consistent with the prediction that dephosphorylation inactivates Hcm1. Moreover, target gene expression was largely unchanged in cells expressing the Hcm1 docking-site mutant (*hcm1ΔPSIEIQ*) or Hcm1-16E, which contains phosphomimetic mutations in activating Cdk1 sites and restores Hcm1 binding to each of these promoters (red and green bars, Figure 4C; Landry *et al.*, 2014). Of importance, wild-type and mutant cells displayed similar cell cycle distributions after $CaCl_2$ treatment (Supplemental Figure S2B), ruling out this potential indirect effect on expression of Hcm1 target genes. Moreover, *hcm1ΔPSIEIQ* cells did not exhibit sensitivity to the microtubule poison benomyl (Supplemental Figure S2C), confirming that Hcm1ΔPSIEIQ is a functional protein. These findings demonstrate that CN impairs Hcm1 activation and are consistent with our other data suggesting that activating sites on Hcm1 are selectively dephosphorylated by CN.

Dephosphorylation of Hcm1 by calcineurin impairs proliferation

Although no role for CN in control of an unperturbed cell cycle has been described, the fact that the *hcm1-16E* phosphomimetic mutant outcompetes a wild-type strain in coculture assays suggests that Hcm1 is not fully phosphorylated when cells are grown in rich medium without any additional stresses (Figure 1B) and that a phosphatase may actively remove phosphorylations at these sites. To determine whether CN plays a role in this regulation, we tested whether the Hcm1ΔPSIEIQ mutant, which severely impairs dephosphorylation of these same sites (Figure 3 and Supplemental Figure S1B), displays a similar increase in fitness. Indeed, *hcm1ΔPSIEIQ* cells consistently exhibited a modest proliferative advantage in this assay (Figure 5A), suggesting that there may be a low level of CN activity in normal growth conditions and that CN contributes to the phosphorylation status of Hcm1.

CN is strongly activated in response to several types of environmental stress, including alkaline pH (Viladevall *et al.*, 2004), toxic cations (Nakamura *et al.*, 1993; Matsumoto *et al.*, 2002), and cell wall

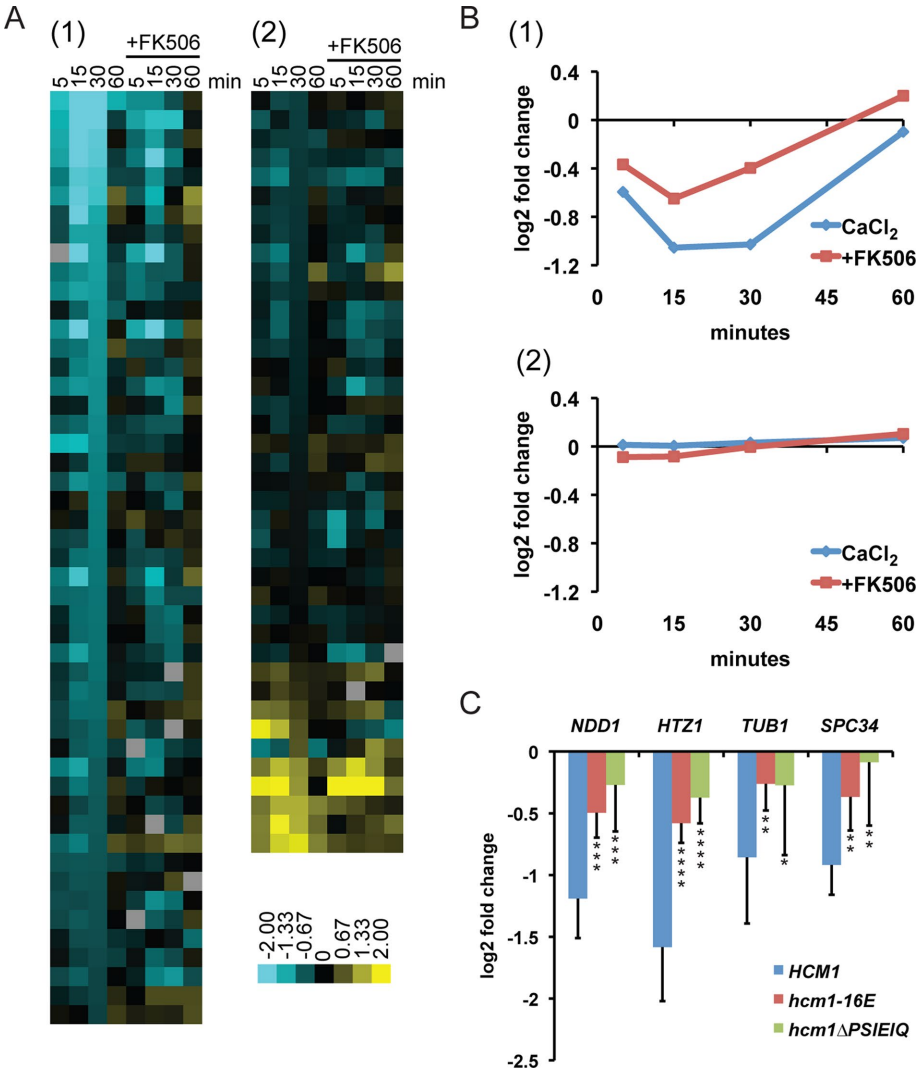

FIGURE 4: Calcineurin inactivates Hcm1. (A) Changes in expression of predicted Hcm1 target genes (Pramila *et al.*, 2006) in response to $CaCl_2$ treatment with or without the CN inhibitor FK506. Data are from Yoshimoto *et al.*, (2002). Cluster 1 includes 49 of 91 genes that are down-regulated (less than [–0.5 log 2]-fold change) at the 30-min time point. Cluster 2 includes the remaining 42 genes. (B) Average expression of genes in each cluster over time with or without FK506. For cluster 1, average values with or without FK506 are significantly different at each time point ($p < 0.0001$, paired *t* test); for cluster 2, average values with or without FK506 are not significantly different at any time point. (C) Reverse transcription quantitative PCR of Hcm1 target genes 30 min after the addition of $CaCl_2$. Fold change values were calculated by comparing expression after 30 min to expression in the same strain before treatment. Average log 2–fold change values ±1 SD ($n = 6$). Down-regulation was significantly impaired in each mutant strain compared with wild type. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

stress (Levin, 2011), so we next examined the importance of Hcm1 dephosphorylation for the cellular response to these stresses. Of interest, cells expressing Hcm1 mutants with varying amounts of activity responded differently to different classes of CN-activating stresses. The presence or absence of Hcm1 activity had no effect on survival when cells were exposed to alkaline stress (Supplemental Figure S3). However, Hcm1 activity was important for the response to cell wall stress and LiCl, both of which promote Hcm1 dephosphorylation (Figure 5B). Deletion of *HCM1* sensitized cells to both types of stress (Figure 5, C and D, and Supplemental Figure S3), and when Hcm1 dephosphorylation was impaired (by deletion of the docking site or expression of the phosphomimetic mutant), proliferation was enhanced (Figure 5, C and D). We also examined the *hcm1-3N* strain, which, unlike *hcm1-16E* and *hcm1ΔPSIEIQ*, is highly resistant to benomyl (Supplemental Figure S2C). Of note, *hcm1-3N* cells showed similar resistance to CN-activating stresses as cells expressing Hcm1ΔPSIEIQ, Hcm1-16E, or mutants that combined either the ΔPSIEIQ mutation or the 16E mutation with the 3N mutation (Figure 5, C and D). Thus Hcm1 activity limits proliferation during some stress conditions, and increasing either its level or activity through modulation of its phosphorylation state results in increased proliferation.

The wide array of CN targets that have been identified thus far suggests that CN orchestrates a global, integrated response to stress by affecting numerous regulatory pathways in order to promote cell survival in response to environmental stress (Heath *et al.*, 2004; Bultynck *et al.*, 2006; O'Donnell *et al.*, 2013; Alvaro *et al.*, 2014; Goldman *et al.*, 2014; Guiney *et al.*, 2015). In this study, we identify Hcm1 as an additional target and elucidate how CN connects this global stress response with changes in the cell cycle–regulatory network. In addition, we demonstrate that Hcm1 is a key node in the cell cycle network that relays signals from the extracellular environment to control proliferation. This is supported by our finding that increases or decreases in Hcm1 phosphorylation can positively or negatively affect fitness (Figure 1) and that in response to stress, CN targets a subset of Hcm1 phosphosites. Of importance, there are two groups of Cdk1 phosphorylation sites that have opposing effects on Hcm1 activity, and CN specifically targets the group of sites that promote Hcm1 function (Figure 3). By dephosphorylating activating sites while sites that promote degradation remain intact, CN rapidly extinguishes Hcm1 activity. Similarly, an additional phosphatase may regulate Hcm1 stability by dephosphorylating the N-terminal Cdk1 sites, thereby increasing Hcm1 levels. Thus Hcm1 activity is differentially modulated by multiple regulators, which likely respond to distinct signals. Because its activity is limiting for maximal proliferation, we propose that Hcm1 serves as a biological rheostat that integrates multiple signals from kinases and phosphatases to produce a broad continuum of proliferation rates. Further, this mode of cell cycle regulation might be conserved, as several transcription factors that control late–cell cycle gene expression in human cells are similarly regulated by Cdk phosphorylation (Sadasivam and DeCaprio, 2013). Understanding the role of phosphatases in transmitting signals to the cell cycle–regulatory network is key to elucidating how cell cycle progression is finely tuned to match the cellular environment.

MATERIALS AND METHODS

Yeast strains and growth conditions

A complete list of strains used in each experiment is given in Supplemental Table S1. All strains are in the S288c background

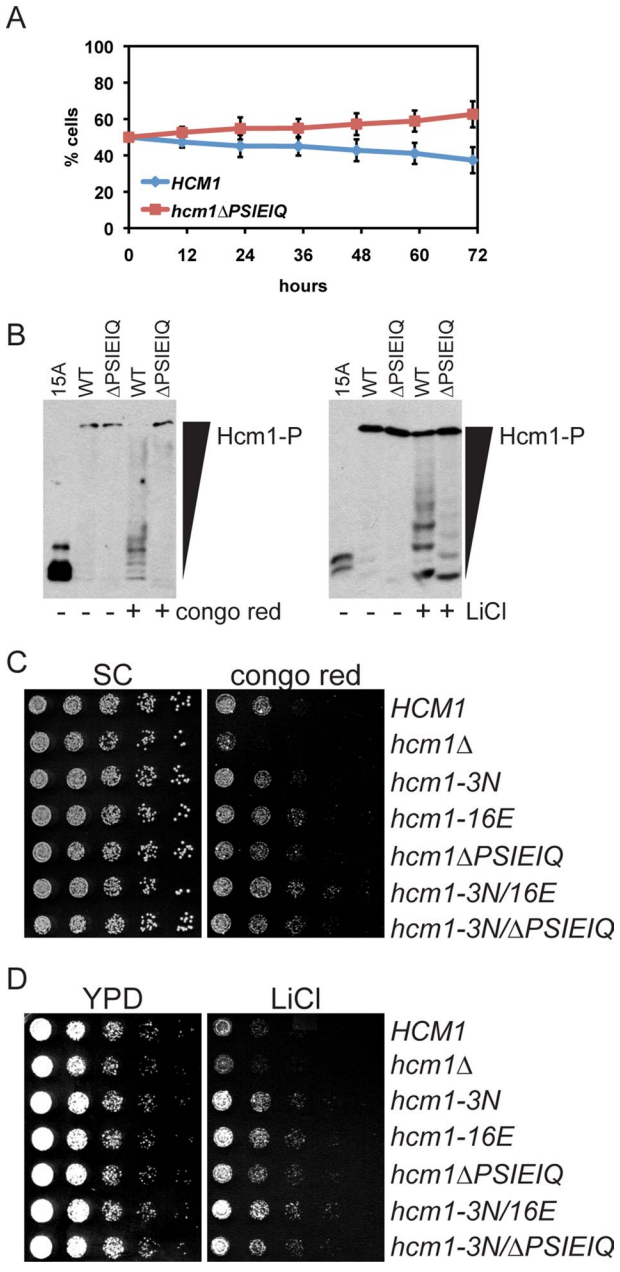

FIGURE 5: Hcm1 inactivation limits proliferation in response to environmental stress. (A) Indicated strains were cocultured and the percentage of each population at each time point quantified by flow cytometry. Average values ± 1 SD ($n = 13$). (B) Strains expressing the indicated Hcm1 proteins were treated with Congo red (cell wall stress) for 10 min or LiCl for 5 min and phosphorylation assayed by Phos-tag Western blot. (C) Fivefold dilutions of the indicated strains were spotted onto synthetic complete medium (SC) with or without Congo red. (D) Fivefold dilutions of the indicated strains were spotted onto rich medium (YPD) with or without LiCl.

except for PJ69-4A (which is W303). For all *HCM1* mutations, PCR products containing the desired mutations were integrated with a triple hemagglutinin epitope tag (3HA) and the *HIS3MX* cassette at the genomic locus using standard techniques. Each *HCM1* mutation was confirmed by sequencing. The *HCM1 cnb1Δ* strain was generated by replacing the *CNB1* gene with a *KanMX* cassette in the Hcm1-3HA–tagged strain.

Cells were grown in either rich medium (YM-1 complete medium with 2% dextrose; Benanti *et al.*, 2007) or synthetic medium (C medium with 1% ammonium chloride, 2% dextrose) at 30°C. For Ca^{2+} treatments, cells were grown overnight in rich medium and then transferred into synthetic medium and allowed to grow for at least one cell cycle. Cells were then treated with 200 mM $CaCl_2$ for the indicated times. Where indicated, cells were pretreated with 1 µg/ml FK506 (LC Laboratories, Woburn, MA) in ET buffer (90% ethanol and 10% Tween-20) or ET buffer alone for 1 h before $CaCl_2$ treatment. Where indicated, exponentially growing cells in rich medium were treated with 500 mM LiCl for 5 min or 250 µg/ml Congo red for 10 min. For cell cycle analysis, cells were arrested in G1 phase in rich medium containing 10 µg/ml α-factor for 2 h and then released into medium without α-factor. Flow cytometry to confirm cell cycle position was carried out as previously described (Landry *et al.*, 2014).

Yeast two-hybrid assay

Strain PJ69-4A was transformed with GAL4-DBD and GAL4-ACT fusion plasmids: BJP2014 (*CNA1-GAL4* activation domain fusion, *LEU2*), pACT2 (*GAL4* DNA-binding domain [DBD], *TRP1*), pACT2-HCM1 (*HCM1-GAL4* DBD fusion, *TRP1*), and pACT2-hcm1ΔPSIEIQ (*hcm1ΔPSIEIQ-GAL4* DBD fusion, *TRP1*). Cotransformants were scored for growth on synthetic media with or without histidine by dilution plating: from 0.5 to 1 OD_{600} unit of cells from each culture were serially diluted fivefold to sixfold in distilled water and spotted on plates. Plates were incubated at 30°C for 2–3 d.

In vivo CN interaction assay

Strain YEG2 (Guiney *et al.*, 2015) was transformed with plasmids pGAL-HCM1 (Landry *et al.*, 2014), pGAL-hcm1-3N (Landry *et al.*, 2014), and pGAL-hcm1ΔPSIEIQ (same as pGAL-HCM1 with *hcm1ΔPSIEIQ*) or vector (GAL-YEP352). Transformants were grown to mid log phase in synthetic media lacking histidine and containing 2% raffinose. Expression of the *GAL1* promoter was induced with 2% galactose for 4 h, and 200 mM $CaCl_2$ was added to the cultures for 15 min to activate CN. Cells were then pelleted, washed, and frozen at –80°C. Cell lysates were prepared as described (Goldman *et al.*, 2014), and 10 mg of cell lysates was incubated with 30 µl of immunoglobulin G–Sepharose (GE Healthcare, Pittsburgh, PA) for 3 h at 4°C. ZZ-tagged complexes were then precipitated and washed twice with buffer. Bound proteins were eluted by boiling in 2× SDS-dye and examined by SDS–PAGE and Western analysis with a LI-COR detection system. Monoclonal anti-HA antibody (12CA5) was used to detect HA-tagged proteins, and a polyclonal anti–S-tag antibody (kind gift from R. Kopito, Stanford University) was used to detect Cna2-S-ZZ.

Competition assays

Competition assays were performed as previously described (Torres *et al.*, 2010; Landry *et al.*, 2014). An equal amount of cells with or without *PGK1-GFP* was mixed together and diluted every 12 h to maintain logarithmic-phase growth. Cells were fixed at each time point in 100% ethanol for analysis by flow cytometry using a FACScan (Becton Dickson, Franklin Lakes, NJ). Data were analyzed for GFP fluorescence using FlowJo (FLOWJO, LLC, Ashland, OR) software.

Western blotting

Cell pellets (1 $OD_{600\,nm}$) were lysed in trichloroacetic acid (TCA), and Western blotting was carried out as previously described (Landry *et al.*, 2014), with antibodies against HA (12CA5), Cdc28 (sc-6709; Santa Cruz Biotechnology, Santa Cruz, CA). For Phos-tag gels, the

resolving gel contained 6 or 7% acrylamide/bis solution 29:1 (Bio-Rad, Hercules, CA), 386 mM Tris-Cl, pH 8.8, 0.1% SDS, 0.2% ammonium persulfate (APS), 25 µM Phos-tag acrylamide, 50 µM manganese chloride, and 0.17% tetramethylethylenediamine (TEMED). The stacking gel contained 5% acrylamide/bis solution 37.5:1 (Bio-Rad), 126 mM Tris-Cl, pH 6.8, 0.1% SDS, 0.1% APS, and 0.04% TEMED. TCA-extracted samples were run on Phos-tag gels at 140 V for ~2 h, and then gels were washed twice in 100 ml of transfer buffer containing 10 mM EDTA for 15 min and once in 50 ml of transfer buffer for 10 min before being transferred to a nitrocellulose membrane for 2–3 h at 0.45 A.

In vitro dephosphorylation assays

Hcm1 and indicated mutants were cloned into the pET32mLic vector (hexahistidine-tagging vector), expressed in bacteria, and purified on Ni nitriloacetic acid agarose beads (Qiagen, Hilden, Germany) according to manufacturer's specifications. Proteins were eluted and stored in buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM β-mercaptoethanol, and 10% glycerol. From 20 to 30 µg of purified protein was phosphorylated by Clb2/Cdk1/Cks1 complex) a kind gift from M. Kõivomägi and J. Skotheim (Stanford University) in kinase buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 500 mM ATP) containing [γ-^{32}P]ATP as in Kõivomägi *et al.* (2011) for 16 h at room temperature. Phosphorylated proteins were purified from unincorporated ATP using a Centri-sep (Applied Biosystems, Waltham, MA) column and eluted into dephosphorylation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA). Phosphorylated proteins were then split into two reactions. Purified activated yeast calcineurin (Goldman *et al.*, 2014) was added to one reaction, and an equal volume of buffer was added to the other. Dephosphorylation was carried out at room temperature and samples withdrawn at 0, 45, and 90 min. Samples were boiled in SDS buffer and stored at –20°C. The extent of CN-mediated dephosphorylation was measured as in Goldman *et al*. (2014). Briefly, samples were subjected to SDS–PAGE, and the gels were stained by Gel Code blue reagent (Thermo Scientific, Waltham, MA) and then dried on a gel dryer. Total protein amounts were determined using ImageJ (National Institutes of Health, Bethesda, MD) software analysis of the stained protein bands, and [γ-^{32}P]ATP incorporation was measured by analysis of an exposed phosphorimager screen with the Typhoon Scanner and ImageJ software. Storage phosphor counts were normalized to protein levels to determine the extent of ATP incorporation in the presence or absence of CN.

Cycloheximide-chase assays

Cells were grown to mid log phase, and 50 µg/ml cycloheximide was added to block protein synthesis. Cells at 1 $OD_{600\ nm}$ were collected at the indicated time points and samples analyzed by Western blotting. Western blots from cycloheximide-chase assays were quantified using a ChemiDoc Touch imaging system (Bio-Rad). Hcm1-3HA signals were normalized to Cdk1 as a loading control, and half-life values from an average of five experiments were calculated using GraphPad Prism software.

Reverse transcription quantitative PCR

Total RNA was isolated from 5 $OD_{600\ nm}$ of pellets from asynchronous cells as described (Schmitt *et al.*, 1990), digested with DNaseI (New England BioLabs, Ipswich, MA), and ethanol precipitated. Reverse transcription was carried out using 2 µg of RNA and random primers (Invitrogen, Waltham, MA), then treated with RNaseH (New England BioLabs). Reverse transcription products were mixed with 2× SYBR Fast Master Mix Universal (Kapa Biosystems, Wilmington, MA) and the primers to the indicated genes (Landry *et al.*, 2014). Reactions were conducted on a Mastercycler ep realplex (Eppendorf, Hamburg, Germany). mRNA levels were normalized to *ACT1*. Fold change was calculated by comparing normalized expression of each target gene after 30 min of $CaCl_2$ treatment to the expression of the target in the same strain before $CaCl_2$ addition.

Serial dilution assays

Exponentially growing cells were serially diluted fivefold and spotted onto the indicated plates. Synthetic medium plates (SC with 1% ammonium chloride, 2% dextrose) contained 500 µg/ml Congo red and 188 µg/ml Fluorescent Brightener 28 (Calcofluor White; Sigma-Aldrich, St. Louis, MO), where indicated. Lithium chloride, 450 mM, or 15–20 µg/ml benomyl was added to rich media plates (yeast extract/peptone/2% dextrose [YPD]) where indicated. Plates were incubated at 30°C for 1–3 d.

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