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Identification of Cdk1 Phosphosites that are Required for Activation of the Transcription Factor Hcm1

A Major Qualifying Project Submitted to the Faculty of Worcester Polytechnic Institute in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Biology and Biotechnology

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Abstract

Hcm1 is a cell cycle-regulatory transcription factor in budding yeast, which activates several mitotic spindle regulatory genes. Its human homolog, FoxM1, is overexpressed in a number of human cancers. Preliminary data indicates that Cdk1 phosphorylation is required for Hcm1 activity but the consequences of this phosphorylation at the molecular level are not well understood. I developed a screen to identify the specific Cdk phosphorylation sites that are important for Hcm1 function, in order to understand how phosphorylation stimulates its activity.

Acknowledgments

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1. Introduction

Cell division is a fundamental process that drives development and reproduction, but its misregulation can cause a number of diseases, including cancer. The cell cycle is the process by which cells divide and replicate their DNA through a series of phases (G1, S, G2, and M) that must occur in the proper order. The cycle begins with a gap phase (G1) allowing for growth and control. Entry into S-phase is a critical regulatory transition, in that the cell commits to division or decides to exit the cycle, depending on nutrient availability and/or growth factor signaling. During S-phase DNA is replicated and chromosomes are duplicated. A second gap-phase, G2, occurs after S-phase to ensure that all genes necessary for the next part of the cycle are properly transcribed and that replication genes are turned off to prevent further DNA replication. Mitosis and cytokinesis, which comprise M phase, follow G2. It is in mitosis that the newly duplicated chromosomes are segregated into a pair of daughter nuclei and the cell divides itself into two daughter cells in cytokinesis (Morgan, 2007). Figure 1 details the events of the eukaryotic cell cycle. Control and regulation of the cycle is extremely important, as it enables cells to divide only when they receive the necessary signals. When cell cycle is misregulated, excessive cell proliferation can occur, leading to malignancies and many types of cancers. Ultimately, understanding the mechanisms of cell-cycle control will provide crucial information as to what goes wrong in cancer cells.

From **The Cell Cycle: Principles of Control** by David O Morgan

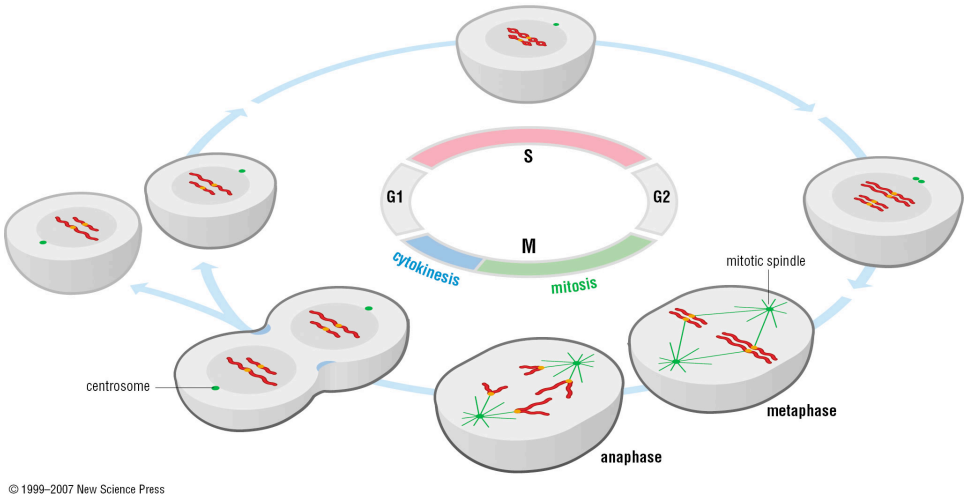

Figure 1: Stages of the eukaryotic cell cycle (Morgan, 2007).

The process of cell division and cell-cycle control is highly conserved among all eukaryotes. *Saccharomyces cerevisiae*, a single-celled eukaryote, is an excellent model organism for studying the cycle, and it was in budding yeast that many cell cycle genes were first identified (Hartwell et al., 1974). As a single-celled organism, the stages of the cell cycle can be easily visualized from the appearance and size of the budding cell. Additionally, the yeast genome has been fully sequenced and can be easily manipulated for genetic analysis (Morgan, 2007).

Of the 6,000 genes comprising the yeast genome, the transcription of about 1000 genes is cell cycle-regulated (Pramilla, 2006; Spellman, 1998; Cho, 1998). In combination with regulated protein degradation, cyclical transcription helps to ensure that proteins are expressed only when they are needed and are eliminated after their function is complete. This coordinated and periodic expression ensures that cell-cycle events happen unidirectionally and in the proper order. Different gene clusters peak in the different phases of the cycle, and this cell cycle-regulated transcription is controlled by cyclin dependent kinases (Cdks) (Dynlacht, 1997).

Cyclin-dependent kinases are serine/threonine protein kinases that coordinate all cell-cycle events. Budding yeast have only one, Cdk1, where humans have four, Cdk1, 2, 4 and 6, which are active in different phases of the cell cycle. Each Cdk pairs with a cyclin to phosphorylate its targets at serine/threonine residues. Binding to a cyclin exposes the active site of the Cdk and directs it to a particular set of targets. In yeast, despite there being only one Cdk, there are 9 cyclins that bind sequentially throughout the cell cycle. The particular cyclin that binds the Cdk confers its specificity. The G1 cyclin that is involved in cell growth and commitment to the cell cycle is Cln3. Cln3/Cdk1 promotes expression of Cln1 and 2, which facilitate the G1/S transition. Clb5 and 6 are expressed in S-phase and stimulate DNA replication. In M-phase, Clb1, 2, 3, and 4 are necessary for spindle formation along with chromosome alignment and segregation (Bloom and Cross, 2007). The activity of Cdks is thus required for progress through cell-cycle transitions (G1/S and G2/M), and their activity must be extinguished for cells to complete mitosis. Cdk1 targets hundreds of proteins (Ubersax et al., 2003, Holt et al., 2009) and can either directly affect their activities, or stimulate or inhibit protein degradation. The main targets of Cdks include cell-cycle regulators and transcription factors that drive expression of cell cycle-regulated genes.

Cell cycle-regulated transcription, in yeast and mammals, is controlled by cyclin/Cdk complexes (Dynlacht, 1997). Of the waves of gene expression that are cell cycle-regulated, the G1/S cluster is the best characterized and is responsible for the progression into S-phase and the initiation of events for a new cell cycle. It is also the largest, comprising more than 200 genes (Haase and Wittenberg, 2014). The expression of the genes in this cluster in yeast is stimulated by Cln3/Cdk1, which phosphorylates and inactivates the transcriptional repressor, Whi5. This then leads to the activation of the transcriptional activators, SCB-binding factor (SBF) and MCB-

binding factor (MBF) (van den Heuval & Dyson, 2008; Costanzo et al., 2004; de Bruin et al., 2004). These activators are then phosphorylated by Cdk late in S-phase, which inhibits their functions (Bertoli et al., 2013). The G2/M gene cluster is also relatively well-characterized and is comprised of about 35 genes. The Fkh2 transcription factor when bound to its co-activator Ndd1 and the MADS protein Mcm1 are the primary regulators of the G2/M gene cluster and facilitate the G2/M transition (Haase and Wittenberg, 2014). Fkh2 and Ndd1 are also phosphorylated by Cdk, which fosters their interaction and recruits them to their target promoters (Pic-Taylor et al., 2004; Reynolds, 2003). Unlike the G1/S and G2/M waves of gene expression, little is known about how expression of S-phase genes may be regulated by Cdk1.

S-phase genes are regulated by the transcription factor Hcm1, which is one of four forkhead transcription factors in yeast that contain a winged helix DNA-binding domain and are important for the regulation of a variety of cellular processes (Pramilla et al., 2006). Hcm1 is a transcriptional activator that is expressed in late G1, but its targets peak in both early and late S-phase. It is estimated to regulate approximately 185 target genes that are involved in chromosome segregation, spindle fiber formation, and budding. Additionally, its targets include downstream transcription factors that drive expression of other cell cycle-regulated gene clusters (Haase and Wittenberg, 2014). For example, Hcm1 binds to and activates the transcriptional activators, Fkh1, Fkh2 and Ndd1, which are necessary for G2/M-phase gene expression. Cells lacking Hcm1 (*hcm1Δ*) are viable, but show chromosome loss and require the spindle checkpoint for viability (Pramila et al., 2006).

Importantly, the human homologue of Hcm1, FoxM1, is overexpressed in a number of human cancers such as liver, prostate, brain, breast and colon cancer. It is one of the most commonly upregulated genes in human tumors and its deregulation leads to cancer progression

and the development of cancer drug resistance (Koo et al., 2012). Like Hcm1, FoxM1 activates genes in G2-phase to help drive the G2/M transition and induce gene expression necessary for M-phase (Murakami et al., 2010). Interestingly, Hcm1 activity is low during G1/S, due to an auto-inhibitory interaction between its amino and carboxy-terminal domains. Cdk phosphorylation of the C-terminus of FoxM1 is required for its activation and reversing the auto-inhibition. FoxM1 contains 15 Cdk consensus sites, 12 of which are in its C-terminus and in its transactivation domain (TAD) (Anders et al., 2011). Ultimately, a better understanding of FoxM1 will aid in the treatment of a large number of cancers, and studying the regulation of its homolog in yeast, Hcm1, provides a way to achieve this goal.

1.1. Preliminary data

Similar to FoxM1, the Benanti lab has shown that Hcm1 is regulated by Cdk1 phosphorylation (Landry et al., 2014). Hcm1 contains 15 Cdk consensus sites: 3 in its N-terminus, 4 in its interior, and 8 in its C-terminus, as shown in the diagram in Figure 2A. When all 15 Cdk consensus sites (S/T-P motifs) of Hcm1 are mutated by changing the serine/threonines to non-phosphorylatable alanines, the mutant (Hcm1-15A) is more stable than the wild-type protein and is expressed at higher levels throughout the cell cycle (Figure 2B). However, Hcm1 target genes are also downregulated in cells expressing the Hcm1-15A (Figure 2C) (Landry et al., 2014). Cdk-regulated degradation and activation of Hcm1 can be uncoupled by separately mutating the clusters of Cdk sites that are found in its N-terminus (Hcm1-3N mutant) and its C-terminus (Hcm1-8C mutant) (Figure 2). Hcm1-3N shows increased protein expression over the cell cycle, whereas the Hcm1-8C expressed at levels similar to wild-type Hcm1 (Figure 2D). This shows that the N-terminal Cdk sites are necessary for the degradation of Hcm1 (Landry et

al., 2014). Additionally, these data point to coordinated positive (activation) and negative (degradation) regulation of Hcm1 by Cdk1 phosphorylation.

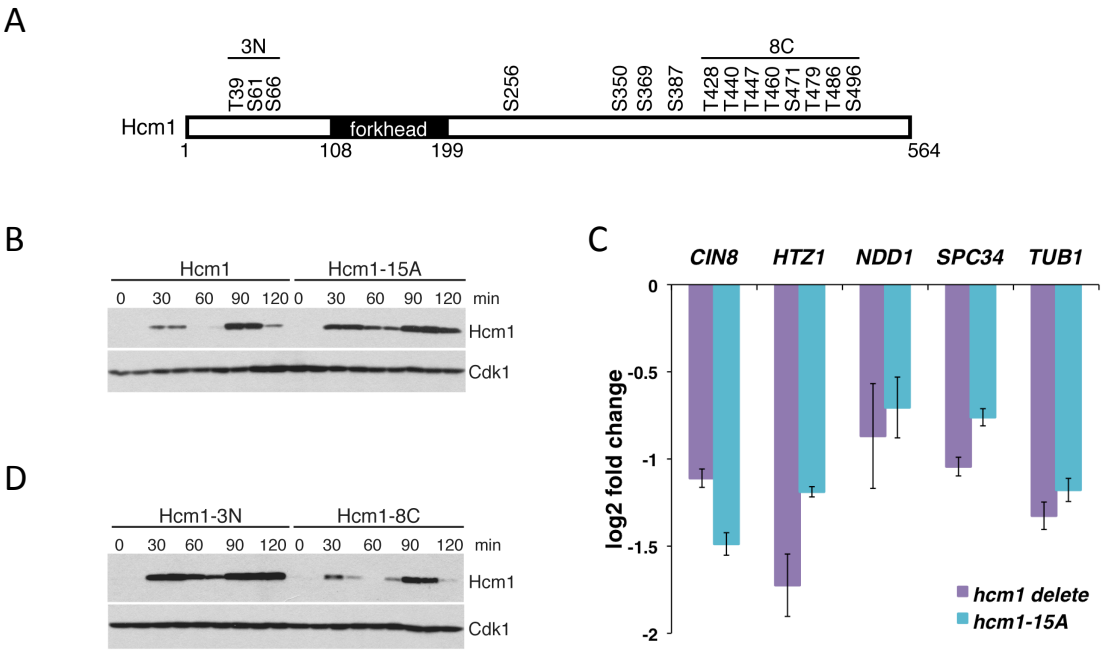

Figure 2: Cdk1 regulates Hcm1 activation and degradation.
(A) Diagram of the transcription factor Hcm1, detailing the Cdk consensus sites (S/T-P sites). **(B)** Expression of wild-type Hcm1 and Hcm1-15A over the cell cycle. Cells were arrested in G1, released into the cell cycle and samples were collected at 15-minute intervals. Protein levels were visualized by Western blotting. Cdk1 levels are shown as a loading control. **(C)** RT-qPCR data showing the expression of Hcm1 target genes in *hcm1Δ* and *hcm1-15A* cells relative to expression in wild-type cells. Cells were arrested in G1 and collected 45 minutes after release when cells were in late S-phase. **(D)** Phosphorylation of the N-terminus of Hcm1 promotes its degradation. Expression of Hcm1-3N and Hcm1-8C over the cell cycle. Cells were arrested in G1, released into the cell cycle and samples were collected at 15-minute intervals. Protein levels were visualized by Western blotting. Cdk1 levels are shown as a loading control Data are reproduced from Landry et al., 2014.

To determine which phosphorylation sites are required for Hcm1 function, mutants were then grown on plates containing the microtubule poison, benomyl, as the targets of Hcm1 are involved in regulating the mitotic spindle and cells lacking Hcm1 function are benomyl-sensitive (Horak et al., 2002; Daniel et al., 2006). As shown in Figure 3, the Hcm1-8C mutant is sensitive to the poison and mimicked the growth of cells expressing the Cdk-deficient allele of Hcm1,

Hcm1-15A, and *hcm1Δ* cells. Therefore, the 8 C-terminal Cdk consensus sites of Hcm1 are important for stimulating its activity (Landry et al., 2014). This data is bolstered by the fact that cells expressing the Hcm1-8C mutant show decreased association with target gene promoters compared to wild-type Hcm1 (Landry et al., 2014). From these data, we conclude that Cdk1 phosphorylation of some or all of these C-terminal sites are required for Hcm1 activation.

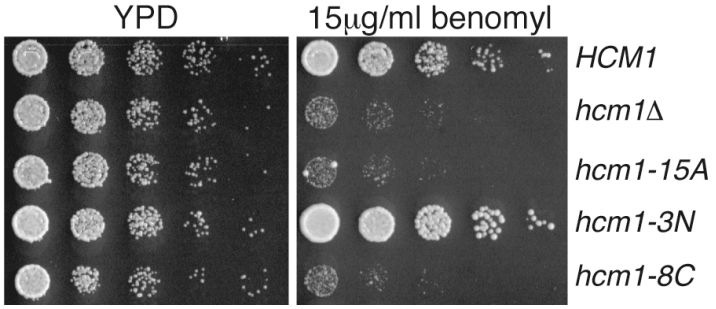

Figure 3: Phosphorylation of the C-terminus of Hcm1 stimulates its activity.
5-fold dilutions of strains with the indicated genotypes on a rich medium plate (YPD) or a plate containing 15μg/mL of the spindle poison benomyl (reproduced from Landry et al., 2014).

1.2. Hypothesis and Goals

The consequences of Hcm1 phosphorylation at the molecular level are not well understood. The goal of this project is to understand how phosphorylation of Hcm1 stimulates its activity. I set out to test the hypothesis that Cdk1 phosphorylation of Hcm1 at one or more specific sites in its C-terminus is important for its recruitment to its target promoters, thus allowing for the correct and timely gene expression of Hcm1 target genes.

2. Results

2.1. Mutagenesis

In order to investigate how Cdk1 phosphorylation of Hcm1 stimulates its activity, I first wanted to determine if specific Cdk consensus sites in its C-terminus are required for activation. However, since there are 11 Cdk consensus sites in the C-terminal half of Hcm1, it is not practical to generate every possible combination of mutations in these sites. For this reason, I took a randomized approach, utilizing the property of homologous recombination in *S. cerevisiae* (Ma et al., 1987). Homologous recombination enables the reconstruction of a plasmid in yeast, upon transformation with several fragments of DNA with overlapping sequences. We developed a strategy in which we could use this approach to randomly recombine fragments of DNA containing either wild-type Hcm1 sequences and sequences with Hcm1 phosphosite mutations, which could then be screened to determine which of the resulting mutants were functional (see Figure 4).

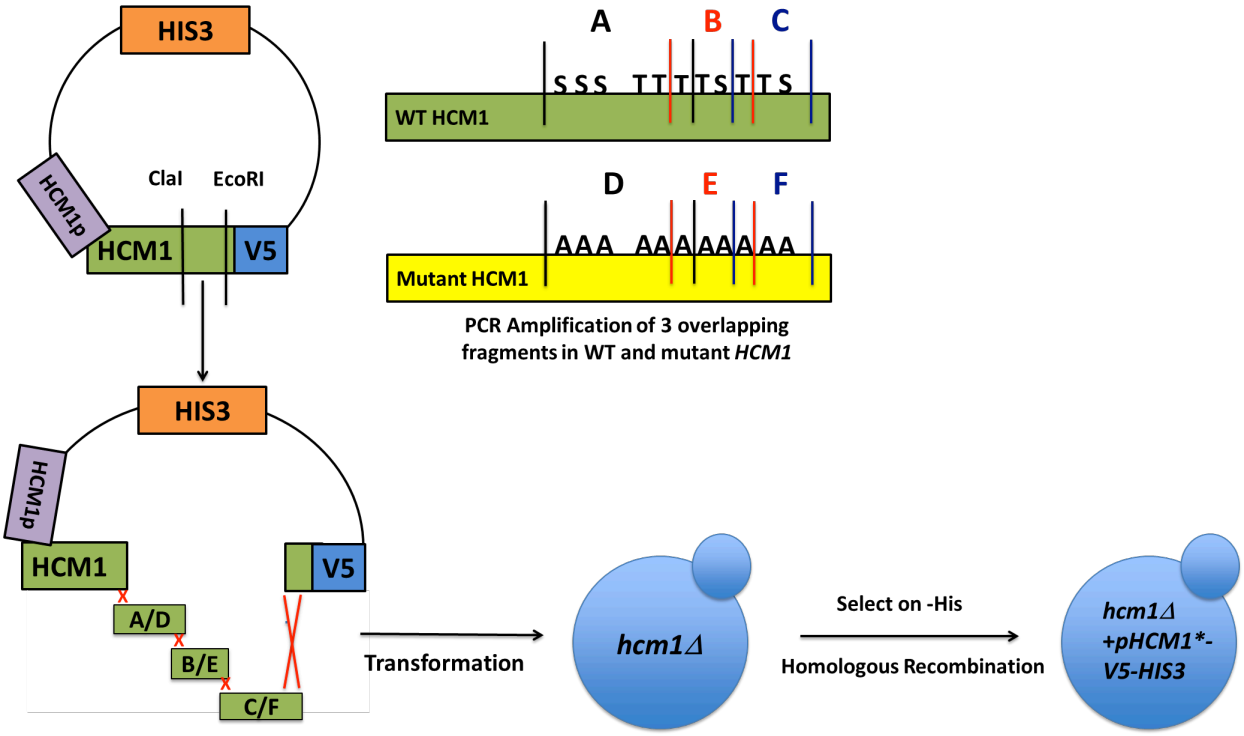

Figure 4: Randomized mutagenesis strategy.
Model detailing the mutagenesis conducted to randomly recombine 11 C-terminal phosphosites in Hcm1 using the principle of homologous recombination in yeast. Asterisk indicates mutated *HCM1* generated by recombination.

First I constructed a plasmid in which the wild-type *HCM1* gene with a C-terminal epitope tag was expressed from its endogenous promoter, as seen at the top of Figure 4. I digested the plasmid and purified the linear fragment that lacked the sequence encoding the C-terminus of Hcm1. Next, I used PCR to amplify 6 overlapping sequences that contained either wild-type *HCM1* sequence or *HCM1* sequence with mutations changing the serine/threonine residues to non-phosphorylatable alanines (Figure 4; A-C are wild-type sequences, D-F are phosphomutant sequences). All 6 fragments and the digested vector backbone were then co-transformed into an *hcm1Δ* strain and cells containing the plasmid were selected for on plates lacking histidine (bottom of Figure 4). An *hcm1Δ* background was used to determine if the different combinations of phosphosites received could rescue the benomyl-sensitivity of this

strain. Since all 6 PCR products contained overlapping sequences, it was expected that they would recombine with the vector backbone in random combinations and generate a large number of unique *HCM1* mutants. These mutants could them be assayed to gain insight into which sites in the C-terminus of Hcm1 are required for its activation by Cdk1.

2.2. Primary Screen of Mutants

Hcm1 activates transcription of a number of genes that regulate the mitotic spindle. As a result, cells without functional Hcm1 are sensitive to drugs that interfere with microtubule polymerization and perturb spindle function (Pramila et al., 2002; Daniel et al., 2006). I took advantage of this sensitivity to develop a screen that was used to determine which Cdk consensus sites are important for Hcm1 function. First, colonies from the transformation that grew on the selection plates were picked, as they were predicted to carry an intact plasmid with some combination of the 11 phosphosite mutations. Of those selected, 94 colonies were screened on plates containing the spindle poison benomyl, and assayed for their ability to rescue the *hcm1Δ* phenotype. Isolates were compared to strains expressing wild-type Hcm1 from a plasmid, an empty vector control plasmid, or the Hcm1-8C mutant that we previously found conferred benomyl-sensitivity (Figure 3, Figure 5). I then sequenced 18 plasmids that rescued benomyl-sensitivity similar to wild-type Hcm1, and 18 plasmids that failed to rescue the benomyl-sensitivity phenotype, so that I could draw conclusions as to which sites must be phosphorylated for the activation of Hcm1. For example, if a collection of sites was mutated in a particular isolate and it was as sensitive as the *hcm1Δ* strain, this would suggest that these sites are necessary for Hcm1 function. Conversely, if the phenotype of the mutant mimicked wild-type Hcm1-expressing cells, then this would indicate that phosphorylation of this cluster of sites is not required for Hcm1 activation.

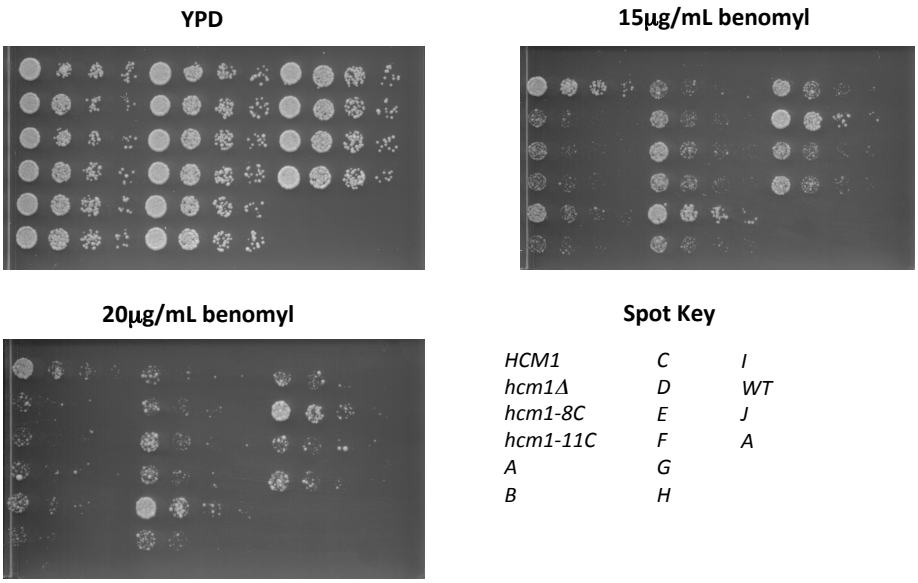

Figure 5: Growth of the Hcm1 mutants expressed from plasmids on benomyl plates. 5-fold dilutions of strains indicated in the key were spotted in three columns on a rich media plate (YPD) or plates containing 15μg/mL or 20μg/mL of the spindle poison benomyl.

Among the 36 colonies that I sequenced, 10 different combinations of the 11 C-terminal phosphosite mutations were obtained (Table 1). Of these 10, 5 combinations were sensitive to benomyl, 4 were not, and 1 combination had mixed results (Figure 5). Also, several combinations were isolated more than once.

Table 1: Combinations of phosphosite mutations isolated during mutagenesis and benomyl-sensitivity screen. The mutants were named with a letter and "X" denotes whether the site was mutated for that particular isolate. The sensitivity of the mutant to benomyl was compared to controls (WT, wild-type Hcm1; 11C and 8C, previously characterized, non-functional mutants) and the number of times a combination was received is denoted.

Name	Cdk-Consensus Sites											Benomyl Sensitivity	Number of Isolates
	S350	S369	S387	T428	T440	T447	T460	S471	T479	T486	S496		
WT												No	12
11C	X	X	X	X	X	X	X	X	X	X	X	Yes	7
8C				X	X	X	X	X	X	X	X	Yes	1
A							X	X	X	X	X	Yes/No	4
B	X	X	X	X	X	X			X	X	X	Yes	2
C	X	X	X	X	X				X	X	X	Yes	1
D	X	X	X	X	X	X						Yes	3
E							X	X	X	X		Yes	1
F	X	X	X	X								Yes	1
G											X	No	1
H	X	X	X								X	No	1
I	X	X	X									No	1
J	X	X	X				X	X				No	1

In order to confirm that the mutations of the Cdk consensus sites of Hcm1 were not affecting the expression of the protein, levels were compared by Western blot (Figure 6). Consistent with our previous findings that mutation of the C-terminal sites did not affect Hcm1 stability, all mutants were expressed at levels similar to wild-type Hcm1. There was no Hcm1 protein expressed by the *hcm1Δ* cells, as expected.

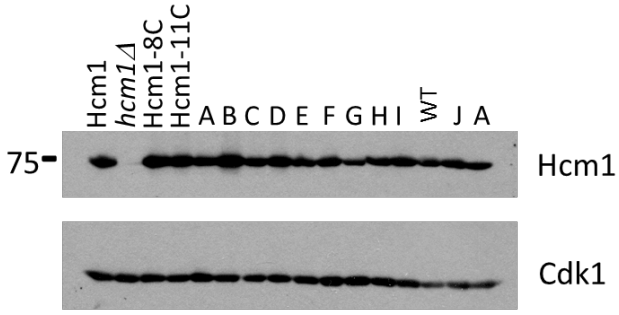

Figure 6: Expression of Hcm1 mutants from plasmids.
Western blot showing V5-tagged Hcm1 proteins, expressed from the plasmids described in Table 1. Cdk1 levels are shown as a loading control.

Although some differences were evident in the primary screen, biological replicates of some mutants showed inconsistent results using this screening technique. One possible explanation for this variability is that strains could not be grown under conditions to select for the plasmid, since I found that benomyl was ineffective in synthetic drop out medium plates. For this reason, the assay was carried out under non-selective conditions. This could possibly influence the plasmid copy number per cell, altering expression of Hcm1 (see more discussion below). Therefore, in order to strengthen my conclusions as to which Cdk consensus sites in the C-terminus of Hcm1 are important for its function, I proceeded to integrate a few representative mutations isolated in the screen into the yeast genome.

2.3. Integration of Mutants into the Genome

Combinations of the 11 Cdk consensus site mutations that I predicted would be informative were chosen to integrate in the genome (Table 2). For the integration, I decided to focus on the cluster of 8 sites found at the C-terminus of the Hcm1 protein, since we previously found that mutation of just these sites conferred benomyl-sensitivity (Figure 2, Figure 3). Combinations A, D, E, J and G were selected from Table 1. Plasmids A and D were selected because A contains mutations in the last 5 of the 8 phosphosites (T460, S471, T479, T486, S496), and D is the converse, containing mutations in only the first 3 of the 8 phosphosites (T428, T440, and T447). Thus I predicted that looking at the growth and sensitivity of these mutants would indicate if those particular clusters of sites are important for the stimulation of Hcm1 activity. Additionally, plasmid A showed mixed results in the plasmid-based screen. Plasmids E and J were selected because they differ in two phosphosites, T479 and T486, and thus screening these combinations could indicate the roles of those particular sites. Finally, plasmid G was selected in that it contains a mutation in the last phosphosite, S496, only. This group of combinations

also provides clusters of phosphosite mutations ranging from 5 to 1, which may suggest that an overall charge difference is necessary for the stimulation of Hcm1 activity, rather than specific sites.

Five strains were constructed with these genotypes. In order to do this, the desired sites were PCR amplified from the plasmids and then transformed into either wild-type *HCM1* cells or *hcm1-8C* cells depending on the combination of C-terminal phosphosites desired (see Methods for more details). The strains created are referred to as Hcm1C-5A, Hcm1C-3A, Hcm1C-4A, Hcm1C-2A, and Hcm1C-1A, respectively (Table 2).

Table 2: Strains expressing Hcm1 mutants from the genomic locus. The strain names are indicated and an "X" denotes whether the site was mutated for that particular strain. The behavior of the mutant on benomyl was characterized in comparison to the growth of controls.

			8C Sites							
Hcm1 Mutant	**Plamid Name**	**Benomyl Sensitivity**	**T428**	**T440**	**T447**	**T460**	**S471**	**T479**	**T486**	**S496**
Hcm1C-5A	A	Yes				X	X	X	X	X
Hcm1C-3A	D	Yes	X	X	X					
Hcm1C-4A	E	Yes				X	X	X	X	
Hcm1C-2A	J	No				X	X			
Hcm1C-1A	G	No								X

In order to ensure that the integration of the Cdk consensus site mutations did not affect the expression of the Hcm1 protein, levels were compared by Western blot (Figure 7). The five strains were tested alongside wild-type Hcm1, *hcm1Δ,* Hcm1-15A, and Hcm1-8C cells as controls. As expected, no protein was observed in *hcm1Δ* cells and increased expression of Hcm1-15A was observed, as the 3 N-terminal phosphosites that are required for its degradation are changed in this mutant (Landry et al., 2014). Importantly, all five unique Hcm1 mutants that I generated were expressed at similar levels to the wild-type Hcm1 and Hcm1-8C, confirming that mutation of these sites does not affect protein expression.

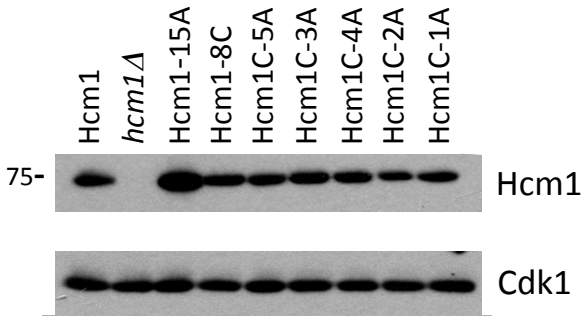

Figure 7: Expression of Hcm1 mutants integrated into the genome.
Western blot showing Hcm1 and Cdk1 protein levels for the strains from Table 2.

Strains expressing phosphosite mutations from the genomic locus were then screened using the benomyl assay described above (Figure 8). They were tested alongside wild-type *HCM1*, *hcm1Δ, hcm1-15A*, and *hcm1-8C* strains and their sensitivities compared (Table 2). I found that Hcm1C-5A, 3A- and 4A-expressing cells grew in a manner similar to the *hcm1Δ* mutants, whereas the Hcm1C-1A and 2A strains were substantially healthier and grew similar to wild-type *HCM1* cells. Biological replicates confirmed these results. While this data does not yet point to specific sites as necessary for Hcm1 activation, it does demonstrate that only a subset of these 8 C-terminal sites are required for Hcm1 activation, and it suggests several possible models for how this phosphoregulation may work.

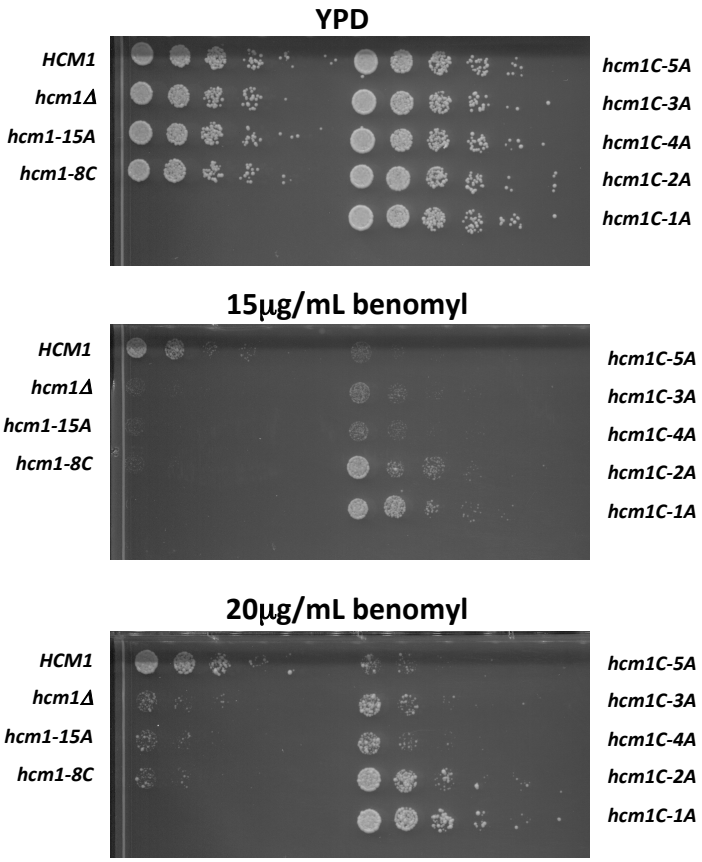

Figure 8: Benomyl-sensitivity of strains expressing Hcm1 phosphomutants from the genomic locus.
5-fold dilutions of strains with the indicated genotypes were spotted in two columns on a rich media plate (YPD) or plates containing 15μg/mL or 20μg/mL of the spindle poison benomyl, with genotypes labeled to indicate the identity of the spots.

3. Discussion

3.1 Mutagenesis Approach and Benomyl Sensitivity Screen

Here, I used a novel approach to randomly recombine 11 phosphosite mutations in the C-terminus of Hcm1, in order to generate a collection of unique combinations of mutations. This randomized strategy proved to be extremely effective for the creation of Hcm1 phosphomutants with different combinations of these phosphosites. As seen in Table 1, 10 unique combinations were constructed and screening more colonies from the original transformation could have led to

the identification of additional combinations. This method was more efficient than a site-directed approach as different combinations of the phosphosite mutations could be generated rapidly.

I then used a benomyl sensitivity screen to determine which Cdk consensus sites in the C-terminus of Hcm1 are important for its function. I performed a primary screen with the Hcm1 mutants expressed from plasmids and assayed for their ability to rescue the sensitivity of an *hcm1Δ* strain, as compared to strains expressing wild-type Hcm1 from a plasmid, an empty vector control plasmid, or the Hcm1-8C mutant that we previously found was nonfunctional (Landry et al, 2014). Although this screen led to some preliminary conclusions, biological replicates of some mutations gave inconsistent results using this screening approach (Table 1). One possible reason for this is that for cells to retain the plasmids, they must be grown in synthetic media lacking histidine. However, I found that benomyl was ineffective in synthetic media. Therefore, cells were assayed on rich media plates containing benomyl and it is possible that they lost the plasmid and were no longer expressing Hcm1. This expression pattern could vary each time I conducted the assay, which could account for the inconsistency. Protein levels were approximately equal by Western blot for all plasmid strains (Figure 6), however, in contrast to the benomyl assay, cells were grown in selective media lacking histidine in this experiment. In order to draw better conclusions about the Hcm1 phosphomutants, we decided to integrate the informative combinations identified from the randomized mutagenesis into the genome and repeat the benomyl sensitivity assay with strains expressing Hcm1 mutants from the *HCM1* locus.

When I performed the benomyl screen with the strains expressing the mutations from the genomic locus, the results were more conclusive. Biological replicates showed consistent results each time the assay was performed. Control strains grew as predicted on the benomyl plates further validating the screen. Therefore, the randomized mutagenesis strategy was successful in

generating different combinations of phosphosite mutations in the C-terminus of Hcm1, and while the plasmid-based screen provided preliminary conclusions, the benomyl screen conducted with the strains expressing the Hcm1 mutants from the genomic locus enabled me to effectively investigate which Cdk phosphorylation sites are necessary for Hcm1 activation.

3.2 Hcm1 Phosphoregulation

My data suggests two possible models for Hcm1 phosphoregulation: one possibility is the phosphorylation of two or more specific sites are required for Hcm1 activation, while the other implies an overall negative charge may be required (Figure 9).

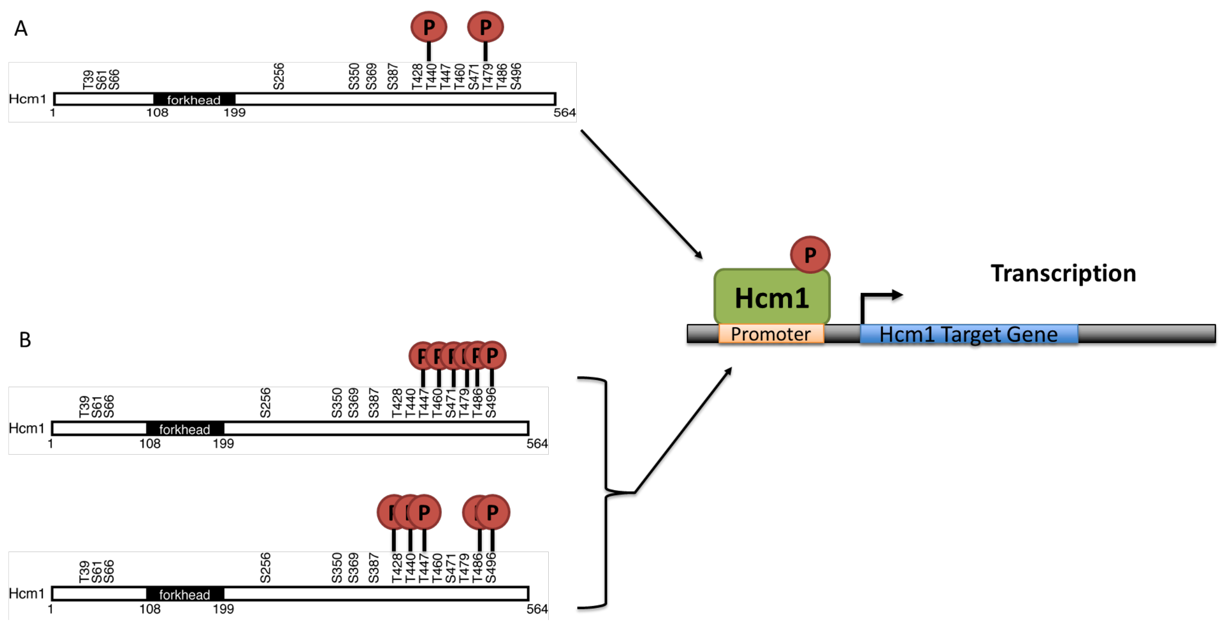

Figure 9: Possible Mechanisms of Hcm1 Phosphoregulation
(A) Phosphorylation of more than one specific site may be required for the activation of Hcm1, for example T440 and T479, whereas phosphorylation of only 1 of these sites would be insufficient. **(B)** An overall negative charge of the C-terminus of Hcm1 is required. For example, phosphorylation of any combination of 5 sites may be sufficient.

As seen in Table 2 and Figure 8, the Hcm1C-5A and Hcm1C-3A strains were both sensitive to benomyl. Hcm1C-5A contains mutations in the last 5 of the 8 phosphosites (T460, S471, T479, T486, S496), and Hcm1C-3A is the converse, containing mutations in only the first

3 of the 8 phosphosites (T428, T440, and T447). Thus, one possibility is that phosphorylation at one or more sites in each of these mutated clusters may be required for Hcm1 activation. For example, as suggested in Figure 9A, it is possible that both sites T440 and T479 are needed, but phosphorylation of only one of the two would be insufficient to recruit Hcm1 to its target promoters and promote timely gene expression. In the Hcm1C-5A and Hcm1C-3A strains, one of these two sites is mutated in each, which could be why they were sensitive to benomyl. Cdk phosphorylation of Ndd1, another transcriptional activator, provides an example of a transcription factor regulated by site-specific phosphorylation (Reynolds et al., 2003). Phosphorylation of Ndd1 on site T319 is required for its recruitment to target gene promoters and association with other binding factors. Mutation of this phosphosite to a non-phosphorylatable alanine significantly reduces the association of Ndd1 with its target promoters and impairs transcriptional regulation.

When I repeated the benomyl screen using strains that expressed the mutants from the genomic locus, the Hcm1C-5A, 3A and 4A expressing cells grew in a manner similar to the *hcm1Δ* mutants, as they were sensitive to benomyl. In contrast, the Hcm1C-1A and 2A strains grew similar to wild-type *HCM1* cells. These data suggest an alternative model in which an overall negative charge may be necessary for Hcm1 activation, rather than phosphorylation at specific sites. The Hcm1C-1A and Hcm1C-2A contain fewer phosphosite mutations and thus more sites can be phosphorylated by Cdk. This creates a greater overall negative charge on the C-terminus of Hcm1, which may be important for Hcm1 function and recruitment to target promoters. Figure 9B presents this model of Hcm1 phosphoregulation. In support of this model, multisite phosphorylation and thus overall charge have been found to be critical for regulation and degradation of other proteins. For example, multisite phosphorylation of Ste5 (a scaffold

protein in yeast involved in mating) during G1 phase is required for its inhibition and the promotion of a new division cycle (Strickfaden et al., 2007). Additionally, Sic1, which is a B-type cyclin/CDK inhibitor, must be phosphorylated on at least 6 phosphosites for its inhibition and subsequent cell-cycle progression (Nash et al., 2001).

It is also possible that these models are not mutually exclusive. Rather, the phosphorylation at two specific sites (such as T440 and T479) could be necessary for phosphorylation of additional sites in the C-terminus, creating an overall negative charge that stimulates Hcm1 activity. Support for this combined model comes from what is known about FoxM1, the human homologue of Hcm1. FoxM1 contains 15 Cdk consensus sites, 12 of which are in its C-terminus in its transactivation domain (TAD). Phosphorylation of this TAD domain promotes FoxM1 activation and 3 specific Cdk consensus sites within the domain, T600, T611 and S638, critically contribute to its function (Laoukili et al., 2008). However, phosphorylation of these three sites is not sufficient for FoxM1 activation, as an additional 2 to 4 sites in the TAD are also required (Anders et al., 2011). Therefore, these three sites could be required for the phosphorylation of the 2 to 4 other Cdk consensus sites, producing an overall negative charge in the TAD domain. Due to the fact that Hcm1 is structurally and functionally similar to FoxM1, this proposed mechanism of Hcm1 phosphoregulation involving both specific sites and an overall charge is highly plausible.

In the future, we hope to use these mutants that I have constructed to better understand the molecular consequences of Hcm1 phosphoregulation and how phosphorylation actually promotes binding to its target genes. One possibility is that Cdk phosphorylation may stimulate Hcm1 activity by promoting its interaction with another protein required for its binding to DNA. Cdk phosphorylation of the C-terminus of FoxM1 stimulates its binding to target promoters by

initiating interaction with the coactivator CREB-binding protein, whose role is to activate transcription (Chen et al., 2009). Due to the fact that FoxM1 is the human homologue of Hcm1, it is possible that a similar interaction exists in yeast and the phosphorylation promotes the recruitment of Hcm1 and this binding factor to target gene promoters to ensure correct and timely gene expression.

3.3 Future Directions

My data suggests several additional experiments that will lead to a better understanding of Hcm1 activation. First, it will be important to integrate other unique and informative combinations of phosphosite mutations at the *HCM1* genomic locus and repeat the benomyl sensitivity screen. This will lead to a better understanding of which Cdk consensus sites are needed for Hcm1 stimulation. To test the proposed model of overall charge being necessary for Hcm1 activation (Figure 9B), I will generate several phosphomutants that each has a different combination of 4 phosphosite mutations. If the phosphorylation of each cluster, regardless of the 4 specific sites it contains, results in activation of Hcm1, it would validate the overall charge model of phosphoregulation. To investigate the model of site-specific phosphorylation (Figure 9A), I will mutate individual sites within the 3A and 4A phosphomutants. If I find a single site among those mutated in the 3A phosphomutant, and a single site among those mutated in the 4A phosphomutant, that are required for activity, this would support the model that there are specific sites in each cluster that are required for Hcm1 phosphoregulation. Additionally, we will need to determine if the benomyl sensitivity of these Hcm1 phosphomutants correlates with the decreased binding of Hcm1 to target promoters and decreased expression of target genes, similar to what we observed in *hcm1Δ*, *hcm1-15A,* and *hcm1-8C* strains. Finally, in order to understand the molecular consequences of Hcm1 phosphoregulation, it will be important to identify other

proteins that bind to Hcm1 target gene promoters in both the wild type and phosphomutant-expressing cells. This could confirm our prediction that Cdk phosphorylation stimulates Hcm1 activity by promoting interaction with another protein required for its DNA binding.

In conclusion, the human homologue of Hcm1, FoxM1, is overexpressed in a number of human cancers. Understanding the function and control of Hcm1 in yeast could lead to a better understanding of the regulation of FoxM1 and other transcription factors with similar TAD domains, as the mechanisms of phosphoregulation by Cdk1 could be conserved in other systems. In fact, many similarities in regulation have already been found between Hcm1 and FoxM1. Understanding the mechanisms of cell cycle-regulation in yeast will provide valuable information about such regulation and breakdowns in control in humans. This will better aide the investigation of many types of cancers.

4. Methods

Cloning

To construct the *HCM1* expression plasmid, 3V5-tagged *HCM1* and 500 base pairs of its promoter were amplified by polymerase-chain reaction (PCR) using a proofreading polymerase and cloned into a pGEM-T Easy cloning vector. The correct sequence was confirmed by sequencing. The plasmid was then digested and the fragment containing *HCM1* and its promoter was gel-purified. This fragment was sub-cloned into the pRS313 vector and confirmed by sequencing. This plasmid expressing 3V5-tagged *HCM1* from its endogenous promoter was used for the randomized mutagenesis strategy described in the results section.

Benomyl Screen Assay

In this assay, cells were grown in rich media with 2% dextrose until they reached mid-log phase. Five-fold dilutions were then plated on rich media plates (YPD) with 2% dextrose and 0μg/mL, 15μg/mL or 20μg/mL of the spindle poison benomyl. These plates were incubated at 30°C and were removed when colony sizes were approximately equal.

Western Blotting

First, equal optical densities of cells were collected and pelleted. Cells were lysed in pre-heated SDS sample buffer (50 mM Tris pH 7.5, 5 mM EDTA, 5% SDS, 10% glycerol, 0.5% β-mercaptoethanol, bromophenol blue, 1 mg/mL leupeptin, 1 mg/mL bestatin, 1 mM benzamidine, 1 mg/mL pepstatin A, 17 mg/mL PMSF, 5 mM sodium fluoride, 80 mM β-glycerophosphate and 1 mM sodium orthovanadate) and heated at 95°C for 5 minutes. Next, glass beads were added and samples were bead-beat with a Mini-BeadBeater-96 for 3 minutes. The lysates were then clarified by centrifugation. The extracts obtained were run on 10% SDS–polyacrylamide gels and were transferred to nitrocellulose membranes. The Western blots were incubated overnight at 4°C in primary antibody, followed by secondary antibody for 30min at room temperature the following day. Primary antibodies were diluted in 4% non-fat milk/PBS and were against V5 and Cdc28 (Cdk1). Blots were visualized using SuperSignal West Femto Chemiluminescent Substrate on film.

Strain Construction to integrate Hcm1 Phosphosite Mutations

Plasmids A, D, E, J and G were chosen from the primary screen as informative combinations. To integrate these mutants into the genome, I utilized strains that had been previously generated in the lab that contain either wild-type *HCM1* tagged with GFP and marked

with the KanMX selection cassette (YJB598), or *HCM1-8C* that is tagged with GFP and marked with KanMX (YJB600). I then used homologous recombination to replace the C-terminus of HCM1 in these strains with different wild-type or phosphomutant sequences, along with a 3V5 tag and the *HIS3* marker. Using this strategy I converted wild-type sites in YJB598 to phosphomutant sites (mutants A, E, J, G), or reverted the phosphomutant sites in YJB600 to wild-type sites (mutant D). The newly synthesized strains were named according to the number of phosphosite mutations the allele contained in its C-terminus. Table 3 summarizes this information and the genotypes for the strains are in Supplemental Table 1.

These strains were verified in a 4-step process. They were first selected for their ability to grow on plates lacking histidine and their inability to grow on plates containing KanMX (since the KanMX cassette was replaced by the *HIS3* cassette upon recombination). Next, I confirmed that the 3V5-tag and HIS3 cassette were correctly integrated at the HCM1 locus using PCR. Then, the C-terminal sites of each strain were PCR amplified with a proofreading polymerase and sequenced to confirm the integration of the desired phosphosite mutations. Finally, protein expression was verified by Western blot, as described above.

Table 3: Construction of Strains expressing Hcm1 mutants from the genomic locus. The strains were named according to the number of phosphosite mutations each contained. Indicated sequences were amplified and transformed into the strains indicated.

Strain Name	Specific Sequence Amplified	Strain Transformed with PCR Product
Hcm1C-5A	T460A, S471A, T479A, T486A, S496A	YJB598
Hcm1C-3A	T460, S471, T479, T486, S496	YJB600
Hcm1C-4A	T460A, S471A, T479A, T486A	YJB598
Hcm1C-2A	T460A, S471A	YJB598
Hcm1C-1A	S496A	YJB598

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6. Supplemental Table

Table S1: Yeast Strains

Name	Genotype	Figure
YBL176	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 HCM1-3HA-HIS3*	2B, 3
YBL177	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-15A-3HA-HIS3*	2B-C, 3
hcm1Δ	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx*	2C, 3, 7, 8
YHA38	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-3N-3HA-HIS3*	2D, 3
YHA65	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-8C-3HA-HIS3*	2D, 3
YKP8	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-3V5-HIS3*	5, 6
YKP10	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-HCM1-3V5-HIS3*	5, 6
YKP16	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313- hcm1-11C-3V5-HIS3*	5, 6
YKP17	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313- hcm1-8C-3V5-HIS3*	5, 6
YKPA	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313- hcm1-T460A S471A T479A T486A S496A-3V5-HIS3*	5, 6
YKPB	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1-S350A S369A S387A T428A T440A T447A T479A T486A S496-3V5-HIS3*	5, 6
YKPC	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1-S350A S369A S387A T428A T440A T479A T486A S496-3V5-HIS3*	5, 6
YKPD	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1-T428A T440A T447A-3V5-HIS3*	5, 6
YKPE	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1-T460A S471A T479A T486A-3V5-HIS3*	5, 6
YKPF	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1- S350A S369A S387A T428A-3V5-HIS3*	5, 6
YKPG	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1-S496A-3V5-HIS3*	5, 6
YKPH	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1- S350A S369A S387A S496A-3V5-HIS3*	5, 6
YKPI	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313-hcm1- S350A S369A S387A-3V5-HIS3*	5, 6
YKPJ	*MATa his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 hcm1Δ::KanMx + pRS313- hcm1- T460A S471A-3V5-HIS3*	5, 6
YBL192	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 HCM1-3V5-KanMx*	7, 8
YBL193	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-15A-3V5-KanMx*	7, 8
YBL398	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-8C-3V5-KanMX*	7, 8
YJB598	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 HCM1-GFP-KanMX*	7, 8
YJB600	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-8C-GFP-KanMX*	7, 8
YKP11	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-T460A S471A T479A T486A S496A-3V5-HIS3*	7, 8
YKP12	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-T428A T440A T447A-3V5-HIS3*	7, 8
YKP13	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1-T460A S471A T479A T486A-3V5-HIS3*	7, 8
YKP14	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ0 hcm1- T460A S471A-3V5-HIS3*	7, 8
YKP15	*MATa ura3Δ0 leu2Δ0 his3Δ0 met15Δ hcm1-S496A-3V5-HIS3*	7, 8