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SCREENING NEW CYTOKINESIS GENES AND INVESTIGATION OF REGULATION OF HOF1 IN CYTOKINESIS

by

JUNG EUN PARK

A THESIS

Presented to the Faculty of the Graduate School of the

UNIVERSITY OF MISSOURI-ROLLA

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MASTER OF SCIENCE IN APPLIED AND ENVIRONMENTAL BIOLOGY

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Approved by

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Melanie Mormile

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ABSTRACT

In 1996, a complete sequence of the budding yeast genome was completed and numerous studies begun to discover the functions of genes. A primary concern of this study was to identify genes that may function in cytokinesis. Seven candidate genes were chosen, which were previously shown to localize to the bud neck, the site of cytokinesis, or interact with a protein that is involved in cytokinesis. Six of those uncharacterized genes, YHR149C, YLR187W, YOL070C, YMR124W, YPL158C, and YOR304C-A, were identified by data generated by GFP tagging of the yeast proteome. Data from a large-scale two-hybrid screen was used to identify YGR153W. The results from a PCR-mediated technique allowing single-step deletion of chromosomal genes revealed that those seven genes might not function in cytokinesis.

Another interest of this study was the role of phosphorylation of the PEST region of Hof1. Hof1p is a member of the *pombe* Cdc15p homology (PCH) family of proteins that localizes to actin-rich regions, such as cytokinetic actin ring. To determine if phosphorylation of the PEST region of Hof1 was important to regulate Hof1 dynamics and cytokinesis, *in vitro* mutagenesis on potential phosphorylation sites of the PEST domain was performed and further characterization is required.

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1. INTRODUCTION

Cytokinesis is the final stage of cell division that follows mitosis. During this process, cell constituents are separated between two daughter cells to ensure a complete genome and ample machinery to initiate the next cell cycle. In plant cells, cytokinesis is completed by a formation of a new cell wall constructed by vesicle fusion in the middle of a binucleated cell. In animal cells, which lack a cell wall, a contractile ring is formed to divide the plasma membrane. Our model organism, the budding yeast *Saccharomyces cerevisiae*, has both devices for cytokinesis. After the onset of anaphase, a contractile ring is formed containing non-muscle myosin II and actin filaments. By contraction of this ring, the cell membrane ingresses. After contraction, yeast construct primary and secondary septa to form a new cell wall between the daughter cells.

Although cytokinesis of budding yeast begins after chromosomes are pulled apart to each pole, the position of the division site is controlled in late G1 phase of cell cycle as the bud emerges. In fission yeast, the position of the division site is set in G2 by the position of the nucleus. In animal cells, the division plane is determined during anaphase by the position of the mitotic spindle (Balasubramanian et al., 2004). Despite differences in the spatial regulation of cytokinesis between yeasts and animal cells, many components of the actomyosin contractile ring are highly conserved (Field et al., 1999). Budding yeast are an excellent model system in which to study the assembly and contraction of the actomyosin ring. In budding yeast, the ring and the septum must coordinate temporally and spatially to perform cytokinesis because ring contraction may regulate the direction for efficient septum formation and the centripetal growth of the

septum follows the contraction of the actomyosin ring (Vallen et al., 2000; Bi 2001; Schmidt et al., 2002).

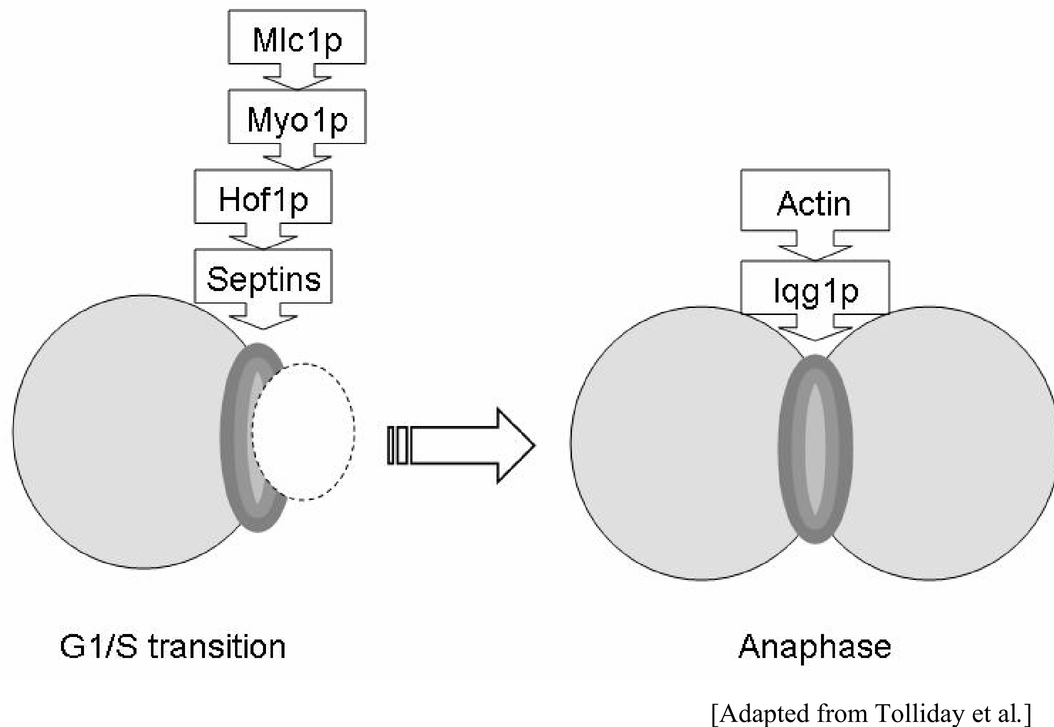
1.1. THE ACTOMYOSIN RING ASSEMBLY PATHWAY IN BUDDING YEAST

Many proteins are needed to form the actomyosin-based contractile ring and these proteins are recruited throughout the cell cycle, from G1 phase when the location of division plane is determined to late anaphase when the cytokinesis begins (Figure 1.1). Septins, Myo1p, Mlc2p, and formin Bnr1p are assembled in late G1 phase (Longtine et al., 1996; Lippincott and Li, 1998a; Luo et al., 2004; Kamei et al., 1998). Mlc1p and Hof1p are localized to the bud neck in S phase and S/G2 phase, respectively (Shannon and Li, 2000; Lippincott and Li, 2000b). In M phase, Iqg1/Cyk1p, formin Bni1p, and Cyk3p become localized to the bud neck. (Lippincott and Li, 1998a; Korinek et al., 2000; Luo et al., 2004). In anaphase, assembly of the contractile ring is completed by recruitment of actin filament in an Iqg1p-dependent manner (Lippincott and Li, 1998a).

1.2. ROLE OF CONSERVED PROTEINS IN THE ACTOMYOSIN RING

Septins are a family of GTP binding and filament-forming proteins that are conserved from yeast to human. There are a total of seven septins in budding yeast, five of which are expressed during the vegetative cycle – Cdc3p, Cdc10p, Cdc11p, Cdc12p and Sep7p – and two that are expressed exclusively during sporulation – Spr3p and Spr28p (DeVirgilio et al.,1996; Fares et al. 1996, Longtine et al., 1996). Throughout the cell cycle, all five vegetative septins localize to the bud neck. Septins play a role in assembly of the actomyosin ring because they are required for the recruitment and

maintenance of Myo1p at the bud neck (Bi et al., 1998; Lippincott and Li, 1998). Septins also have a role in septum formation because they are essential for the anchoring of chitin synthases (CS), including CSII and SCIII, to the bud neck (DeMarini et al., 1997; Shaw et al., 1991). Therefore, septins play a role as a scaffold for holding actomyosin ring and septum-forming apparatus. (Bi et al., 1998; Lippincott et al., 1998a; Roh et al., 2002)



[Adapted from Tolliday et al.]

Figure1.1. Actomyosin ring assembly. At the G1/S transition, septins and Hof1p are localized to the incipient bud site (outer dark oval), then Myo1p and Mlc1p localize to the narrow ring (inner gray oval). Dashed lined indicates emerging bud. In late anaphase, Mlc1p recruits Iqg1p to the bud neck and then actin is recruited to the ring by Iqg1.

Myo1p, as the only type II myosin heavy chain in budding yeast, is required for actomyosin ring contraction (Lippincott and Li, 1998a; Bi et al., 1998). Deletion of Myo1p does not cause lethality but results in abnormal septum formation, inefficient cytokinesis and cell separation (Bi et al., 1998). Like other type II myosins, Myo1 binds

to essential and regulatory light chains. Mlc1p is the essential light chain, but its interaction with Iqg1 rather than Myo1 is required for actomyosin ring assembly (Shannon and Li, 2000; Luo et al., 2004). The regulatory light chain for Myo1p, Mlc2p, plays a role in Myo1p ring disassembly during and/or at the end of actomyosin ring contraction (Luo et al., 2004).

Iqg1 is an essential component for actin ring formation in budding yeast (Shannon and Li et al, 1999). Iqg1p is a member of the IQGAP family of proteins, which are implicated in the regulation of actin filaments from yeasts to humans. IQGAP family members have a conserved calponin-homology domain (CHD) at the N-terminus, internal IQ repeats, and a C-terminal GAP-related domain (GRD) (Machesky, 1998). The CHD of Iqg1 binds to F-actin *in vitro* and is required for the assembly of actin filaments in the ring (Shannon and Li, 1999). The IQ repeats of Iqg1 bind to Mlc1 and are required to localize Iqg1to the bud neck (Shannon and Li, 1999). The GRD of Iqg1 binds to the small GTPase Tem1 and is required for actomyosin ring contraction (Shannon and Li, 1999). Because the formins Bni1p and Bnr1p can nucleate actin filaments they are also required for actin ring formation. (Vallen et al., 2000; Pruyne et al., 2002; Sagot et al., 2002; Tolliday et al., 2002). Bni1p and Bnr1p interact with profilin, an actin binding protein, to control the reorganization of actin cytoskeleton as targets of Rho1p and Rho4p, respectively (Imamura et al., 1997).

1.3. REGULATION OF ACTOMYOSIN RING CONTRACTION

Exit from mitosis is regulated by a signaling pathway called the mitotic exit network (MEN) in budding yeast. The MEN is homologous to the septation initiation

network (SIN) in fission yeast. The SIN signaling pathway consists of three protein kinases Cdc7p (Fankhauser and Simanis., 1993), Sid1p (Guertin et al., 2000), and Sid2p (Sparks et al., 1999) and one small GTPase, Spg1p (Schmidt et al., 1997). Cdc14p (Fankhauser and Simanis, 1993) and Mob1p (Hou et al., 2000; Kuroda et al., 1996) proteins play a role as subunits of the Sid1p and Sid2p kinases, respectively. Sid 4p and Cdc11p form a complex that localizes to the SPB and may function as a scaffold essential for localization of all SIN components to the SPB (Chang and Gould, 2000; Guertin et al., 2000; Hou et al., 2000; Krapp et al., 2001; Sparks et al., 1999). SIN initiates contraction of the actin ring and synthesis of the division septum.

The MEN is required for exit from mitosis in budding yeast and monitors orientation of the mitotic spindle during anaphase as a part of checkpoint (Simanis, 2003a). The MEN enhances protein phosphatase Cdc14 activity to initiate mitotic cyclin-dependent kinase inactivation. The inactivation of cyclin-dependent kinase triggered by MEN activation results in disassembly of the mitotic spindle and the decondensation of chromosomes, and thus the cell exits from mitosis (Bardin et al, 2001). Cdc14 is controlled by Cfi1/Net1 (Shou et al., 1999; Straight et al., 1999; Vistintin et al., 1999; Traverso et al., 2001). Nucleolar protein Cfi1/Net1 inhibits Cdc14 during G1, S phase, and early mitosis but releases Cdc1 during nuclear division (Shou et al., 1999; Visintin et al., 1999). A regulator of mitotic exit Tem1, a GTP binding protein, and Tem1's exchange factor Lte1 are present in the bud only after the nucleus enters the during nuclear division (Bardin et al., 2000). During mitotic exit, MEN protein kinases Cdc5 (Song et al., 2001), Cdc15 (Xu et al., 2000), Dbf2 (Frenz et al., 2000) and Dbf20 (Toyn et al., 1991; Toyn and Johnston, 1994) and the Dbf2-associated factor Mob1 (Yoshida and

Toh-e., 2001; Luca et al., 2001) localize to the spindle pole body and those kinases localize at the bud neck during cytokinesis. The homology of the MEN to the SIN suggested that the MEN may regulate actomyosin ring contraction and septation in budding yeast as well exit from mitosis. In fact, the MEN protein Tem1 is required for actomyosin ring contraction (Lippincott et al., 2001).

A goal of this research is the identification of new genes and potential MEN targets in cytokinesis.

1.4. IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL CYTOKINESIS GENES

A genomic approach was used to identify the role of candidate genes of budding yeast that may function in cytokinesis. A complete sequence of yeast genome was identified in 1996 (Goffeau et al., 1996). Numerous studies have performed to discover the functions of genes by analyzing messenger RNA abundance and stability (Velculescu et al., 1997; Wang et al., 2002), biochemical activity (Martzen et al., 1999; Zhu et al., 2001), protein-protein interactions (Uetz et al., 2000), transcriptional regulation (Lee et al., 2002), gene disruption phenotypes (Ross-Macdonald et al., 1999; Winzeler et al., 1999; Tong et al., 2001; Giaever et al., 2002), protein localization (Huh et al., 2003) and protein abundance (Ghaemmaghami et al., 2003). Data generated by GFP tagging of the yeast proteome was used to identify six uncharacterized genes that localized to the bud neck (Huh et al., 2003) and data from large-scale two-hybrid screening was used to identify YGR153W (Uetz et al., 2000). Because these genes are in the location of cytokinesis, they may have a role in this process. In this study, a PCR-mediated

technique allowing single-step deletion of chromosomal genes was used to study our candidate genes.

1.5. HOF1 AS A POTENTIAL MEN TARGET

One candidate target of the MEN could be Hof1p which is phosphorylated MEN dependently in telophase and may function to coordinate actomyosin ring function and septum formation. (Vallen et al., 2000). During G2/M phase, Hof1p rings appear at the bud neck: first on the mother side, then on the daughter side. In telophase the two Hof1 rings are combined in a single ring. After the single ring has decreased in size, the ring is split into two rings and finally disappear (Lippincott and Li, 1998b; Vallen, 2000). Hof1p is not critical for assembly of the actomyosin ring but important to regulate the stability of the actomyosin ring during contraction (Lippincott and Li, 1998b). HOF1 deletion mutants are temperature-sensitive and defective in septum formation at 37°C (Lippincott and Li, 1998b; Vallen et al., 2000).

Hof1p is a member of the *pombe* Cdc15p homology (PCH) family of proteins that localize to actin-rich regions, such as the cytokinetic actin ring (Lippincott and Li, 2000b). There are eleven PCH family members: Hof1 and YHR114W in budding yeast, Cdc15, Imp2 and YB65 in fission yeast, PSTPIP, PSTPIP2, PACSIN, and PACSIN2 in mouse, CIP4 in human, and FAP52 in avian. The Hof1 homolog in fission yeast, Cdc15, is a critical gene and its mutants are defects in cytokinesis (Balasubramanian et al., 1998) and produce long filamentous cells having multiple nuclei (Fankhauser et al., 1995). Among mouse PCH family proteins, the Hof1 homologous protein is PSTPIP. A hyperphosphorylated mutant of PSTPIP delays in the completion of cytokinesis after

cleavage furrow formation (Angers-Loustau et al., 1999). Most PCH family proteins contain a potential coiled coil domain at the N-terminus (Lupas et al., 1991), PEST sequences that are often characteristic of proteins that are rapidly turned over (Rechsteiner, 1990), and a carboxy-terminal Src homology 3 (SH3) domain (Fankhauser et al., 1995). The goal of this study was to determine if phosphorylation of the PEST region of Hof1 was important to regulate Hof1 dynamics and cytokinesis. To do this *in vitro* mutagenesis was used and examined the effects on Hof1.

2. IDENTIFICATION AND CHARACTERIZATION OF POTENTIAL CYTOKINESIS GENES

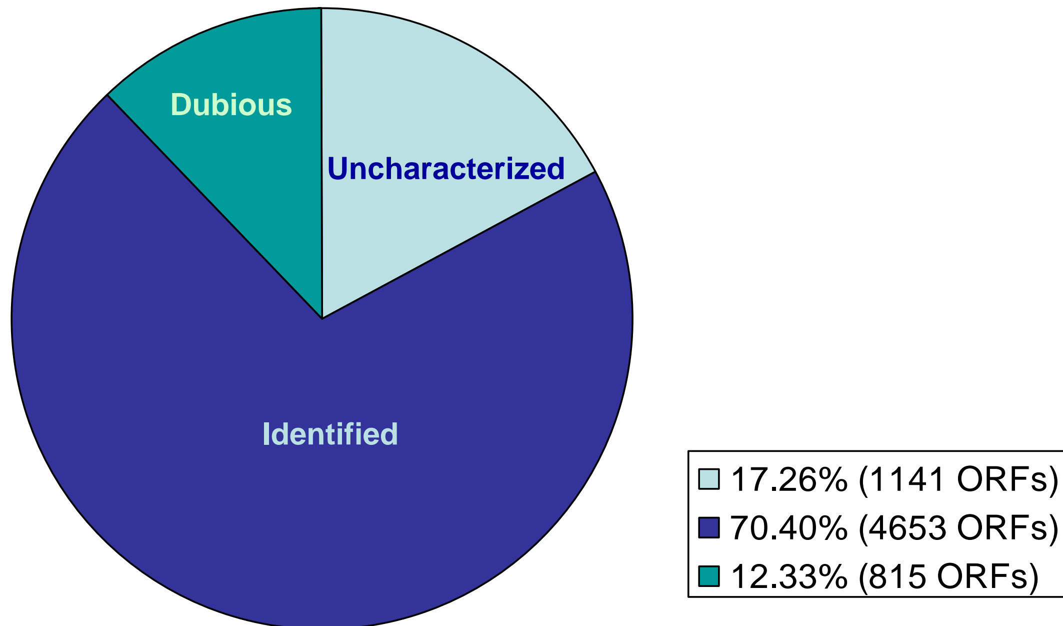
2.1. INTRODUCTION

Due to its biological properties, the budding yeast is used industrially and as a model organism for scientific studies. One of the common yeast species is a baker's yeast *Saccharomyces cerevisiae: Saccharomyces* means 'sugar mold' and *cerevisiae* means 'beer', derived from Greek and Latin respectively. As its name suggests, yeast is used mostly in fermentation of sugars. Yeast plays a huge role as a model organism in genetics and cell biology because it has great similarities to human cells and thus other mechanisms are comparable.

The yeast genome sequencing began in 1989 and was fully sequenced in 1996 (Goffeau et al., 1996). This effort took the work of many labs: 55% of genome was sequenced by 70 laboratories in the European Union, 17% at the Sanger Centre in England, 15% at Washington University, 7% at Stanford University, 4% at McGill University in Canada and 2% at The Institute of Physical and Chemical Research (RIKEN) in Japan. Genome-wide screens provide information about its proteome. Among 6,609 total open reading frames (ORFs) of yeast, 4653 ORFs are verified but there are still 1,142 ORFs yet to be characterized (Figure 2.1).

There have been many approaches to analyze the localization of proteins in *S. cerevisiae*. Those were mostly transposon-mediated random epitope-tagging and overexpression of epitope-tagged proteins. Recently, genomic approaches have been developed to avoid potential problems from using previous method such as interruption of localization signals or abnormal subcellular localization of overexpressed proteins.

Through this approach, a yeast strain collection expressing GFP-tagged full-length proteins was generated through homologous recombination (Huh et al., 2003).



[Adapted from the web site www.yeastgenome.org]

Figure 2.1. Protein coding genes of *S. cerevisiae*

In a living organism, genetic recombination occurs naturally during reproduction and results in a rearrangement of genetic material. If there are two strands of DNA having homology that align closely together, crossover will occur to produce an exchange of components between two strands. The homologous recombination technique is extremely efficient in budding yeast and requires only small amounts of homology (about 40 base pairs) and is used to modify, by deleting, tagging or introducing new genes, gene sequences (Baudin et al., 1993).

The goal of this study is to discover some uncharacterized yeast genes with a role in cytokinesis. In this study, there are seven candidate ORFs: YHR149C, YLR187W, YOL070C, YMR124W, YOR304C-A, and YPL158C, which are uncharacterized but prior GFP screening localized them to the bud neck (Huh et al., 2003) and YGR153W, which is screened as bub2 interactor (Uetz et al., 2000). As cytokinesis occurs at bud neck, these uncharacterized genes are positioned in a subcellular region that suggests that they may function in cell division. To determine the function of these genes, the single copy of the gene in haploid cells was deleted by taking advantage of the one-step gene replacement and then, the morphology of the deletion mutants were examined to determine if cytokinesis was affected. (Figure 2.2)

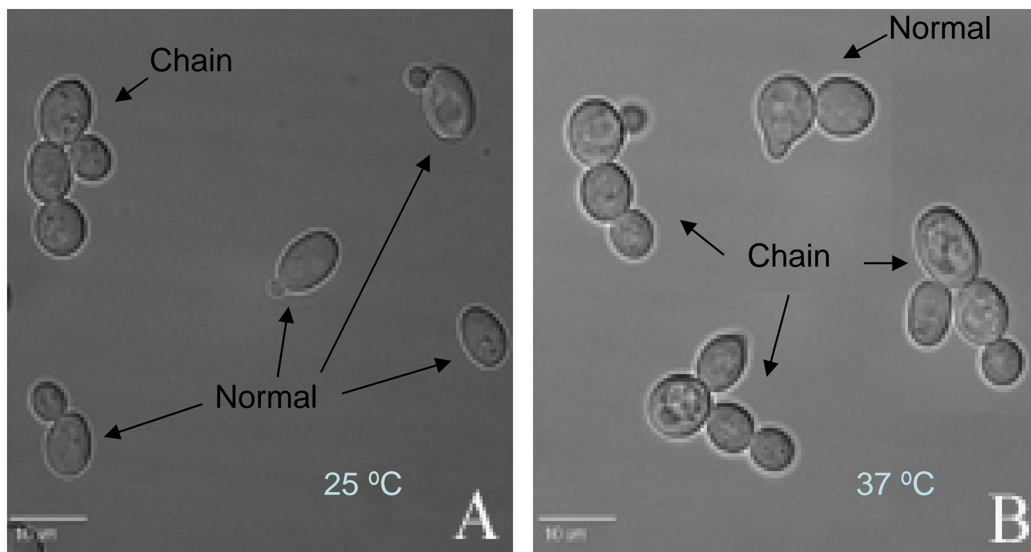


Figure 2.2. Morphology of yeast cells: normal vs chain phenotype. Example of temperature sensitive cytokinesis mutant. A: Low temperature, mostly normal cells. B: mostly chains of cell bodies, indicating a cytokinesis defect.

2.2. MATERIALS AND METHODS

2.2.1. Strains, Growth Conditions, and DNA Methods. All *S. cerevisiae* strains were derived from KSY3 (a ura3 leu2 his3 trp1 ade2 Δbar1) (Li). Yeast were grown in YPD (yeast extract peptone dextrose) liquid medium, or on – HIS or – LEU drop-out solid medium at 30 ºC, or at 37 ºC.

HIS3 gene for deletion of uncharacterized open reading frame was amplified by PCR in 50 µl reactions containing 0.5 µl of template DNA (pRS303) (100 pmol/1 µl), 1 µl of forward primer (100 pmol/1 µl) (sequences shown in Table 2.1), 1 µl of reverse primer (100 pmol/1 µl), 5 µl of dNTP (25 mM/1 µl), 5 µl of 10X buffer provided with Taq polymerase, 1 µl of Taq polymerase, and 36.5 µl of dH_2O. PCR reactions were run for 30 cycles of 2 minutes at 95 ºC, 30 seconds at 95 ºC, 30 seconds at 50 ºC, and 5 minutes at 72 ºC; the 30 cycles were followed by a 12 minutes extension at 72 ºC. For yeast transformation, five PCR reactions were pooled and concentrated. The reactions were combined in a single 1.5 ml eppendorf tube and 500 µl of phenol:chloroform:IAA was added. After vortexing and spinning down in microcentrifuge at maximum speed for 5 minutes, supernatant was harvested and incubated in 1 ml 100% EtOH containing 40 µl of 4 M AmOAc on ice for 10 minutes. The mixture was pelleted by microcentrifugation for 10 minutes and air-dried pellet were resuspended in 20 µl of sterile water.

PCR screening of transformants for insertion by homologous recombination was performed using primers that annealed to the target gene locus outside the region modified (primer sequences for insertion screening shown in Table 2.1).

2.2.2. Yeast Transformation. Yeast transformations were performed by modified method from the lithium acetate methods (Gietz et al., 1992). After 30 minutes incubation

Table 2.1. Primers used in this study

NAME	SEQUENCE
PCR-mediated deletion	
YLR187W Forward	CTGCAAGAACACCAAGAAGCTACAGTAAGAA
YLR187W Reverse	CTCGGCTCGATGCGCCTAAACGAAACGCGACGCC
YOL070C Forward	GGCTGGCACCATCAAGAAGAGAAAGAACGCTGATA
YOL070C Reverse	GCCGGACTTGTCCAAGTATCAATGAATACAAGCCA
YMR124W Forward	GAGCAGAGAGGTACAGCCTGATCCGATAAAGCAGTG GG
YMR124W Reverse	CCCATTATACACTTTCACCTGTTGCTCAGTGTG
YHR149C Forward	GTGGGGAGCAGGCCTTGTCTTTATCCATATCGAAT
YHR149C Reverse	CGAGGCAGCAAATACAAATGATGTGAGGCGCCTGAG C
YPL158C Forward	CTGGGATAACCAGATTGCTGGGCTTTCAGCAGGTCAT CAA
YPL158C Reverse	CGGTAGACAGAATAGGCGTTTGTTATCGTCGATGGTG
YOR304C-A Forward	GCGGCTTGGCTTACAGGTACATTTTACCTTAGCGC
YOR304C-A Reverse	GGCACGATCTGTAGTTAATTGCAACCTAGTTACGCG
YNL335W Forward	CTCCACCGCACAATAGTTTGTCGGGAAGTCATCAATC
YNL335W Reverse	GGAGAAAGGAGATCATCACTGTTCATTCTGAACTCGC CTC
YGR153W Forward	GCCTTTCTCTATTGAACTGTATGACTGAGATACGCCT GC
YGR153W Reverse	GTGCCATTAGGCCCAAAGCCTGACATTTCTAGATT
Insertion screening	
YLR187W Forward	ACATTAGTGAAGCAAGACCTG
YLR187W Reverse	AGATCAAATCACCGACACGA
YOL070C Forward	TTCCCTCGGATAACTCTTCCA
YOL070C Reverse	TTCCCACAGTTACAACTGTGT
YMR124W Forward	GGCGTTTTTTGCTTGACTGA
YMR124W Reverse	GCCCAGTTTCATTTGACGTA
YHR149C Forward	AAAGTGCGCTGAACACTTGA
YHR149C Reverse	TCGCAACAAAAATTCAATGGG
YPL158C Forward	GGAAAGTTTCTGCTTAGAGAC
YPL158C Reverse	GCACTTACATGAATAAGCTCA
YOR304C-A Forward	CCGAATTAAAGTCACTATTTTTG
YOR304C-A Reverse	GCTGTTAACAAGTACGGACTTCGT
YNL335W Forward	CATGCTATAACGTAAAACAATGTAG
YNL335W Reverse	TTCTGATCTTTCCTTGCCACAAC
YGR153W Forward	CCTTCTTCCACACTTATTACC
YGR153W Reverse	TTAGAACCTGTTCTAGGGCT

at 30 ºC, 50 µl of DMSO (dimethyl sulfoxide) was added and mixed. Heat shock was given at 42 ºC for 15 minutes. Cells were pelleted briefly in microcentrifuge and resuspended in 500 µl of 1X TE (10 mM Tris-HCl, 1mM Na_2EDTA, pH8.0). The resuspension was plated on –HIS drop-out media and incubated at 30 ºC for two-three days.

2.2.3. Genomic DNA Purification. Transformants were grown in 10 ml YPD for overnight and genomic DNA of the transformants was prepared as described (Hoffman and Winston, 1987).

2.2.4. Morphological Observations. All deletion mutants were treated with zymolyase before observing phenotypic changes. 670 µl of 37% formaldehyde was added to 5 ml overnight culture of each deletion mutant strain and then, cells were incubated at room temperature on the roller for one hour. Cells were washed twice with 1 ml 1X PBS and then, washed one time with 1 ml Sorbitol Buffer (1 M Sorbitol in 50 mM KPO_4, pH7.5). Cells were resuspended in 1 ml Sorbitol Buffer with 0.2 mg/ml zymolyase 20T (Seikagaku Kogyo Co, Tokyo,. Japan) and 2 mM DTT (dithiothreitol). Cells were incubated at 37 ºC for ten minutes and then, immediately put on ice. Morphology was observed and cells were counted under Olympus CH2, objective EA40 NA0.65.

2.2.5. T-TEST Microsoft Exel T-test program was used to calculate P value.

2.3. RESULTS

One of the candidate genes, YGR153W, is a bub2 interactor. Six other candidate genes, YHR149C, YLR187W, YOL070C, YMR124W, YPL158C, and YOR304C-A, were chosen from genes screened as coding a protein which localizes to the bud neck

because that is the place where the cytokinesis occurs and thus, the genes might have role in cytokinesis.

PCR-mediated gene disruptions were performed to generate deletion mutants of candidate genes (Table 2). First PCR was done for amplifying HIS3 gene with 40 base pairs of homology to the target ORF at each end and second PCR was performed after transformed the DNA into the wild type strain KSY3 to check if the ORFs were replaced with HIS3 marker gene successfully. (Figure 2.3.)

Table 2.2 Yeast strains

Strain	Genotype	Background	Source
KSY3	a ura3 leu2 his3 trp1 ade2 Δbar1	W303	R.Li
KSY48	a ura3 leu2 his3 trp1 ade2 Δbar1 ΔYHR149C:HIS3	W303	This study
KSY49	a ura3 leu2 his3 trp1 ade2 Δbar1 ΔYOL070C:HIS3	W303	This study
KSY50	a ura3 leu2 his3 trp1 ade2 Δbar1 ΔYMR124W:HIS3	W303	This study
KSY65	a ura3 leu2 his3 trp1 ade2 Δbar1 ΔYLR187W:HIS3	W303	This study
KSY75	a ura3 leu2 his3 trp1 ade2 Δbar1 ΔYGR153W:HIS3	W303	This study
KSY124	a ura3 leu2 his3 trp1 ade2 Δbar1 ΔYOR304C-A:HIS3	W303	This study
KSY125	a ura3 leu2 his3 trp1 ade2 Δbar1 ΔYPL158C:HIS3	W303	This study

Cytokinesis defects can be detected by the morphology of the cell. If cells have cytokinetic problems, they cannot divide properly. Therefore, they can have three or more cell bodies attached. However, it is hard to tell if the chain shaped cell bodies are caused by defects in cytokinesis or septation. To determine whether they have cytokinesis defects, cell walls of overnight cultures were digested through zymolyase treatment and

then cells were counted. Zymolyase is the enzyme that digests cell wall and thus the possibility of septation problem would be excluded.

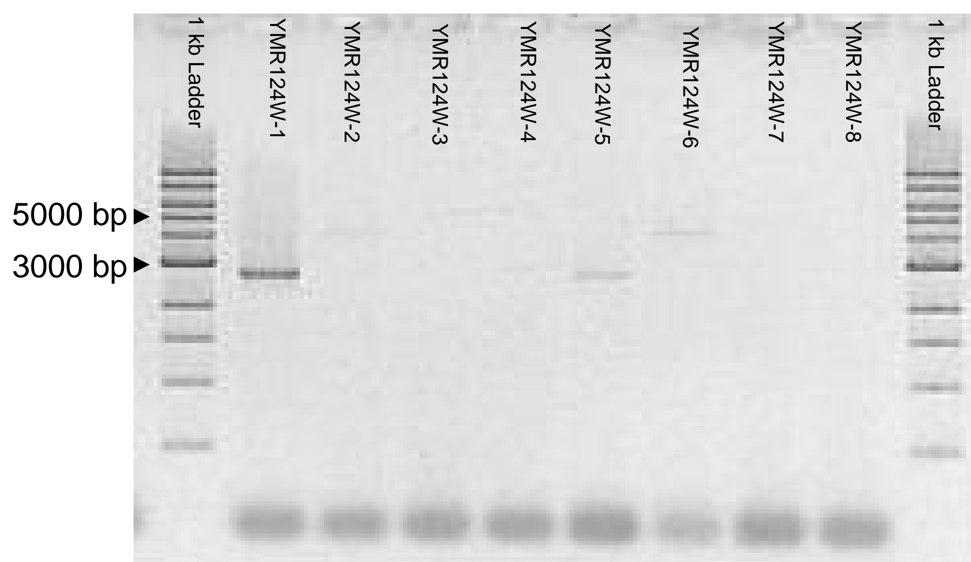


Figure 2.3. Gel electrophoresis for YMR124W. For checking insertion of HIS3 gene, check PCR was done with genomic DNA from transformants and check primers. In wild type cells, check primers for YMR124W should give 4830bp product; however, in deletion mutant, those give 2699bp product. YMR124W-1 and YMR124W-5 are the examples of deletion mutants. YMR124W-2 and YMR124W-6 are not deleted.

A total of one hundred cells were counted and sorted into two groups: cells with one or two cell bodies as a normal and cells with three or more cell bodies which show cytokinetic defect. T-test was used to calculate P value. If P value is lesser than .05, it is significant. All of the P values calculated from the samples were greater than .05; therefore, there were no significant differences between wild strain and deletion mutant strains (Figure 2.4).

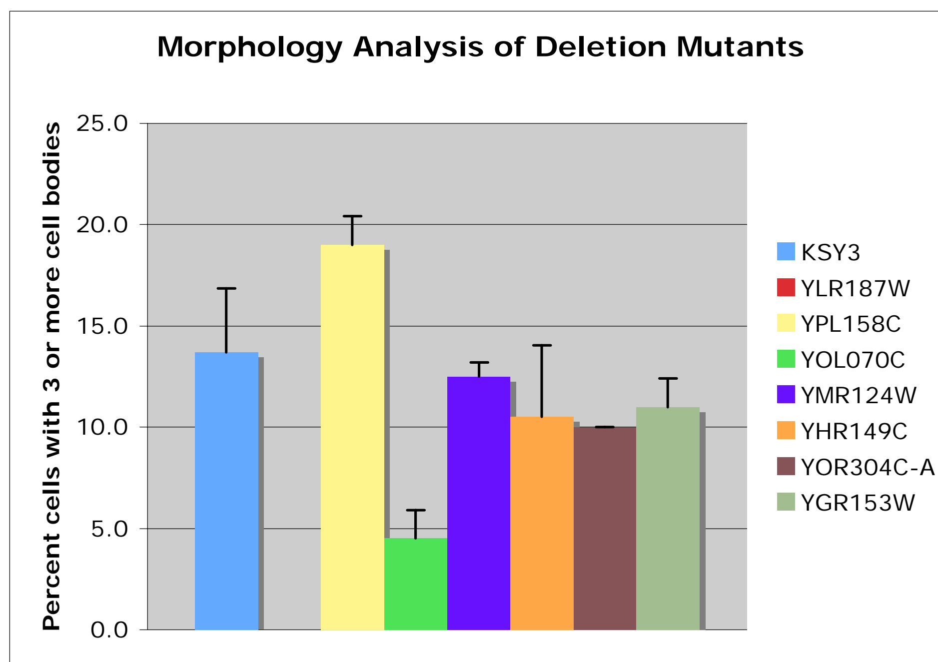


Figure 2.4. Cell counting assay. T-test results show that there was no significant difference between wild type strain and mutants.

2.4. CONCLUSION

Candidate genes were chosen through analysis of genomic screen data. To identify whether these genes have role in cytokinesis, deletion mutants were generated. If the genes function in cytokinesis, deletion mutants will show defects in cytokinesis such as the number of cells having 3 or more cell bodies would be significantly increased. However, the results show that all those seven ORFs are not involved in cytokinesis.

3. INVESTIGATION OF THE ROLE OF PHOSPHORYLATION ON HOF1 IN CYTOKINESIS

3.1. INTRODUCTION

In budding yeast, pathways for cytokinesis can be sorted by two groups. The first pathway is required for the assembly of a functional actomyosin-based contractile ring. The second pathway controls cell separation and septum formation and involves numerous proteins including the formin-related protein Bnr1 and Hof1 (Vallen et al., 2000). In Hof1 deletion mutants, the actomyosin ring forms normally but soon after contraction begins, the ring disassembles quickly and Hof1 seems to regulate septin distribution (Lippincott and Li, 1998b).

Hof1 is a member of the pombe Cdc15 homology (PCH) family proteins (Lippincott and Li, 2000b). PCH family proteins contain an N-terminal FER-CIP4 Homology (FCH) domain (Aspenström, 1997), a coiled coil domain, proline-glutamic acid-serine-threonine rich (PEST), and a Src homology domain 3 (SH3) at the carboxyl termini. (Figure 3.1)

Although Hof1 is essential for assembly of anctomyosin ring early in mitosis, it is specifically degraded late in mitosis and remains unstable during the entire G1 phase (Blondel et al., 2005). The Skp1-Cullin-F-box protein Grr1 is found both in the nucleus and the cytoplasm and it is also accumulates at the bud neck late in mitosis (Blondel et al., 2000). Grr1 is localized to the mother-bud neck region after activation of the mitiotic-exit network (Vallen et al., 1999), and interacts with PEST domain of Hof1 to degrade (Blondel et al., 2005). The PEST domain of Hof1 is found between amino acids 418 and 438 through the PEST-Find program (Rechsteiner and Rogers, 1996). (Figure 3.1)

Phosphorylation of Hof1 by MEN proteins such as Cdc15 and/or Dbf2 at the end of mitosis may trigger its degradation (Blondel et al., 2005). Actin ring formation and upstream MEN protein function is required to localize the downstream effector kinase, Dbf2p, to the bud neck and that it correlates with Hof1p phosphorylation (Corbett et al., 2006).

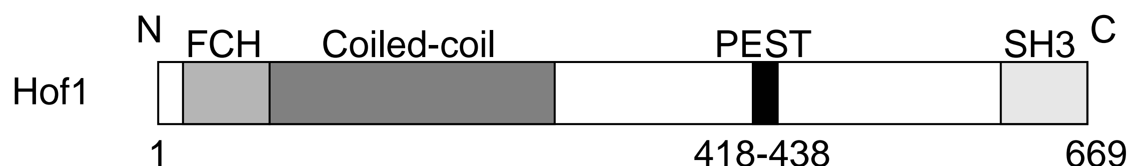


Figure 3.1. Schematic representation of Hof1p

In this study, Hof1 phosphorylation sites within the PEST diomain were mutated to test if non-phosphorylated Hof1 mutants have cytokinesis defects or defects in Hof1 localization.

3.2. MATERIALS AND METHODS

3.2.1. Strains, Plasmids, Growth Conditions, and DNA Methods. The yeast strains and plasmids used in this study are shown in Tables 1 and 2. The yeast strains were derived from KSY3, KSY7, and KSY9. Yeast were grown in YPD, –HIS or -LEU liquid medium, or on –HIS or –LEU drop-out solid medium at room temperature, or at 30ºC.

3.2.2. Yeast Transformation. Yeast transformation was performed by modified method from the lithium acetate methods (Gietz et al., 1992) as described in Section 2.

3.2.3. Mutagenesis. QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used for making mutant strains. Mutant strand synthesis was performed by PCR in 50 µl reactions containing 1 µl of template DNA (100 ng/µl), 1 µl of forward primer (200 ng/µl) (sequences shown in Table 3.1), 1 µl of reverse primer (200 ng/µl), 1 µl of dNTP mix from the kit, 5 µl of 10X reaction buffer from the kit, 3 µl of QuickSolution, 37 µl of dH_2O, and 1 µl of *PfuUltra* HF DNA polymerase. PCR reactions were run for 1 cycle of 6 minutes at 95 ºC, for 18 cycles of 60 seconds at 98 ºC, 60 seconds at 55 ºC, and 20 minutes at 65 ºC; the 18 cycles were followed by a 7 minutes extension at 72 ºC. Amplified PCR products were digested with1µl of *Dpn* I at 37 ºC for 1 hour to remove parental dsDNA.

For the reaction to be transformed, XL10-Gold ultracompetent cells were gently thawed on ice and 45 µl of the ultracompetent cells were aliquotted to a pre-chilled Falcon®2059 polypropylene tube. 2 µl of the β-ME mix provided with the kit were added to the cells and the cells were swirling gently every 2minutes while they were incubated on ice for 10 minutes. 2 µl of the *Dpn* I-treated DNA from the PCR reaction were transferred to the cells and then, the cells were incubated on ice for 30 minutes. Heat-pulse was given to the tube in a 42 ºC water bath for 30 seconds and then, cells were incubated on ice for 2 minutes. After 0.5 ml of pre-heated LB broth was added, the cells were incubated at 37 ºC for 1 hour with shaking at 225-250 rpm. 200 µl of cells were harvested and plated on LB-ampicillin agar plate. The transformation plate was incubated at 37 ºC for over 16 hours.

3.2.4. Time-Lapse Imaging Cells were grown overnight in selective medium and placed on agarose pads. Because YPD gives high background fluorescence, 0.2 g of agarose were melted in 1 ml -TRP media by boiling at 100 ºC. Lab tape was attached onto the glass slides to make it about 0.1 cm thicker. Two taped slides were placed parallel to each other and then, one empty glass slide was placed in between the two taped glass slides. 170 µl of melted agarose was placed on the empty glass slide and then, pressed by another clean glass slide. After 3 to 5 minutes drying, taped slides and covered slide were removed. 5 µl of cells, washed one time with distilled water, was placed on the agarose pad and the pad was covered with cover slip and then, sealed with valap (1:1:1 mixture of Vaseline, lanolin, and paraffin). Living cells were viewed using an Olympus IX51 inverted microscope at 1,000X total magnification using a UPLSAPO 100X NA 1.4 objective. FITC (EX 482/35 506DM EM 536/40) filter was used (Brightline). Images were captured with a Hamamatsu ORCA285 CCD camera. Shutters, filters, and camera were controlled using Slide Book software (Intelligent Imaging Innovations, Denver, CO). Images were collected with 0.2 s of exposure to fluorescent light every 2 minutes.

Table 3.1 Primers used in this study

Name	Sequence
Mutagenesis	
HOF1-P1-F	GGTGCATAAAAGAAATCAAGCTCTCGCCGCACCAGCA GAATCAAG
HOF1-P1-R	CTTGATTCTGCTGGTGCGGCGAGAGCTTGATTTCTTTTA TGCACC
HOF1-P2-F	CACCAGCAGAAGCAGCTGCTGCTAATCCAACGGATTTT AGCC
HOF1-P2-R	GGCTAAAATCCGTTGGATTAGCAGCAGCTGCTTCTGCT GGTG
HOF1-P3-F	GCTAATCCAGCGGATTTTGCCCACATCAAAAAGAGAC
HOF1-P3-R	GTCTCTTTTTGATGTGGGCAAAATCCGCTGGATTAGC
HOF1-SEQ2	GATACACGAAGTGGGCGCAATTATCC

3.3. RESULTS

To test if non-phosphorylated Hof1 mutants have defects in cytokinesis or defects in localization of Hof1, 13 nucleotides of PEST domain in Hof1 gene were mutated with QuikChange® II XL Site-Directed Mutagenesis Kit from Stratagene to generate pJP3 (Figure 3.2 and 3.3). Mutagenesis was confirmed by sequencing. Phosphorylation is observed on serine (S), threonine (T), and tyrosine residues. As a result of mutagenesis, total 10 of phosphorylation sites were mutated.

```
pJP3      AATCAAGCTCTCGCCGCACCAGCAGAAGCAGCTGCTGCTAATCCAGCGGATTTTGCCCAC
          |||||| |||||  | ||||| ||||| ||  | || |||||||| ||||||||  ||||
Hof1 wt   AATCAATCTCTCAGCTCACCATCAGAATCAAGTTCTTCTAATCCAACGGATTTTAGCCAC
```

Figure 3.2. Mutagenized Hof1 PEST domain nucleotide sequence

```
pJP3           N Q A L A A P A E A A A A N P A D F A H
               | |   |     |   |         | |   | |   |
Hof1 wt        N Q S L S S P S E S S S S N P T D F S H
```

Figure 3.3. Mutagenized Hof1 PEST domain amino acid sequence

pLP3 was transformed into KSY3, KSY7, and KSY9 to make Hof1-GFP controls and pJP3, mutagenized from pLP3 at PEST domain, into KSY3, KSY7, and KSY9. (Table 3.2 and 3.3)

To see the Hof1p localization pattern, Hof1-GFP images of KSY92, KSY93, and KSY94 were collected. As reported previously (Lippincott and Li, 1998), Hof1 ring was appeared as double ring and then, the double ring merges into one ring. Next, the combined ring decreases in width as it increases the thickness. Finally as cell separation occurs, Hof1 localizes to the new cells and becomes fainter as it is degraded (Figure 3.4.).

Table 3.2 Yeast strains

Name	Genotype	Background	Source
KSY3	a ura3 leu2 his3 trp1 ade2 Δbar1	W303	R Li
KSY7	a ura3 leu2 his3 trp1 ade2 cdc15-2	W303	R Li
KSY9	a cdc5-1 his trp1	W303	R Li
KSY92	ura3 leu2 his3 trp1 ade2 Δbar1 pLP3(Hof1-GFP, HIS3)	W303	This study
KSY93	a ura3 leu2 his3 trp1 ade2 cdc15-2 pLP3(Hof1-GFP, HIS3)	W303	This study
KSY94	a cdc5-1 his trp1 pLP3(Hof1-GFP, HIS3)	W303	This study
KSY126	a ura3 leu2 his3 trp1 ade2 Δbar1 pJP3(Hof1-GFP, HIS3)	W303	This study
KSY127	a ura3 leu2 his3 trp1 ade2 cdc15-2 pJP3(Hof1-GFP, HIS3)	W303	This study
KSY128	a cdc5-1 his trp1 pJP3(Hof1-GFP, HIS3)	W303	This study

Table 3.3 Plasmids used in this study

Name	Description
pLP3	CEN plasmid, HOF1-GFP HIS3
pJP1	HOF1 mutant P1 GFP HIS3
pJP2	HOF1 mutant P2 GFP HIS3
pJP3	HOF1 mutant P3 GFP HIS3

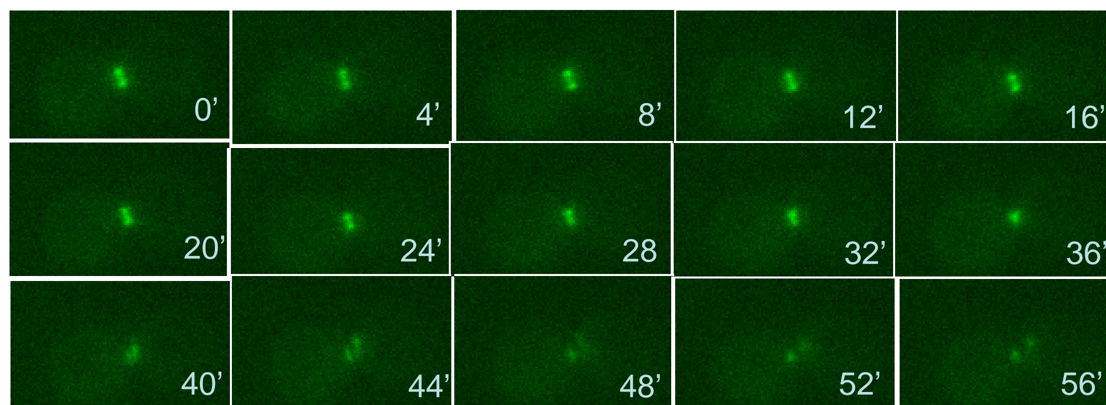


Figure 3.4. KSY92

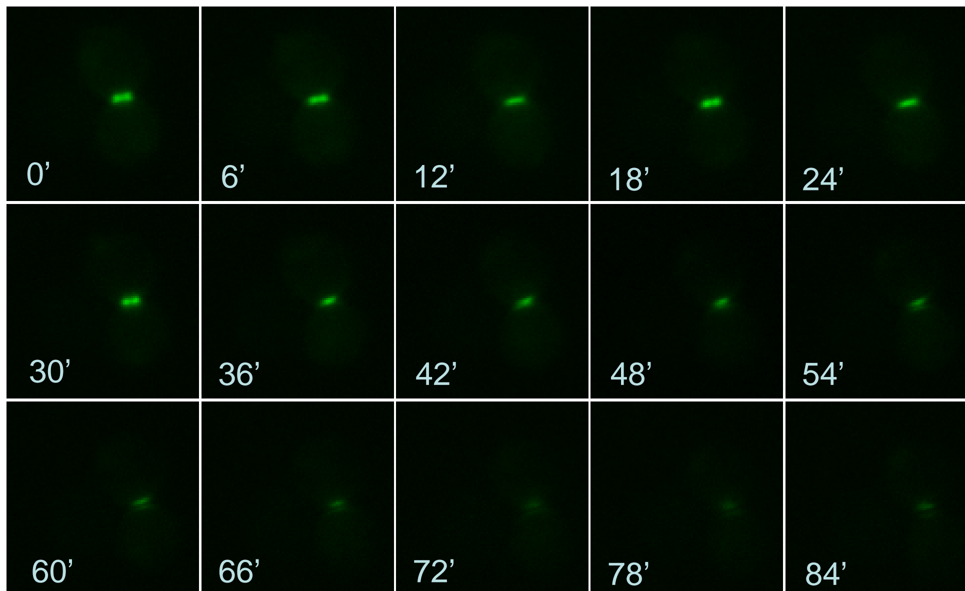


Figure 3.5. KSY93

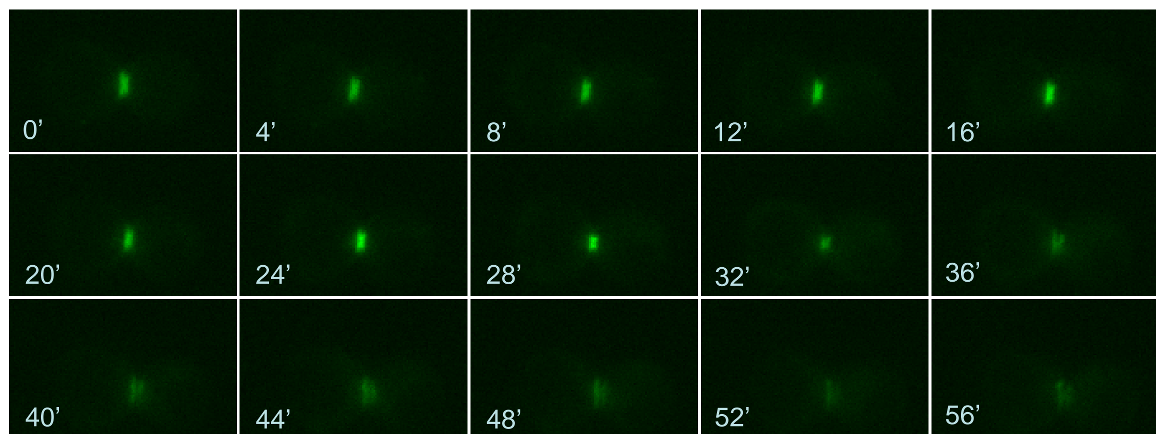


Figure 3.6. KSY94

Mutagenized Hof1-GFP localization pattern is similar to controls, but with wt no differences. First, contraction of Hof1 ring occurs faster in mutant than in controls (Figure 3.4). Secondly, the intensity of the Hof1 signal does not decrease in mutant cells as rapidly as in controls.

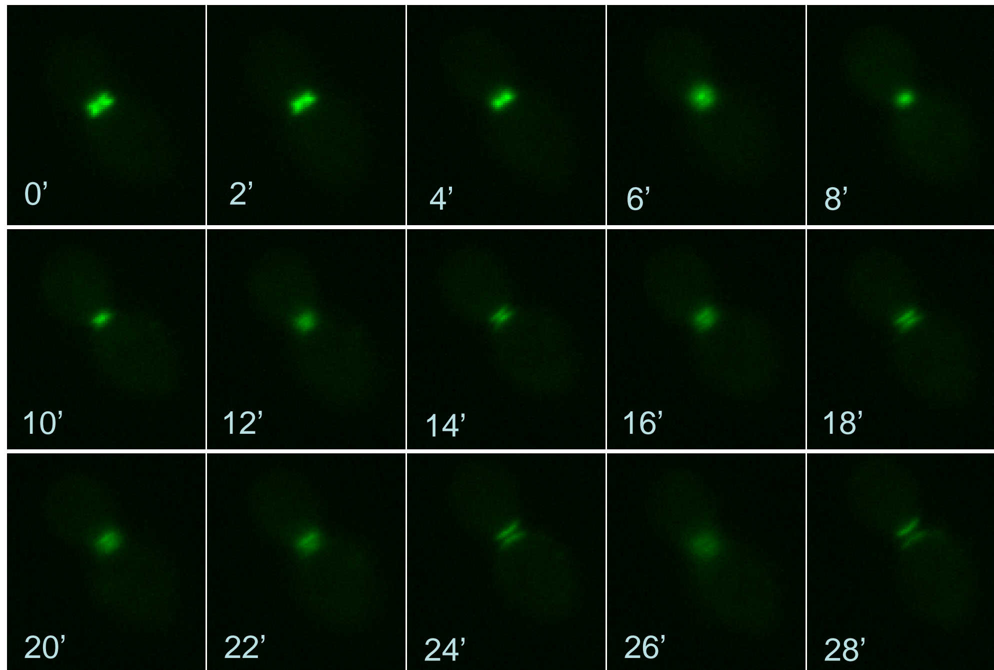


Figure 3.7. KSY126

3.4. CONCLUSIONS

It seems that mutation of 10 Hof1 phosphorylation sites causes changes in Hof1 localization. However, more images of KSY126, KSY127, and KSY128 need to be collected to compare with control group. Characterization of Hof1 mutant strains KSY126, KSY127, and KSY128 is also required to understand more about the role of phosphorylation of Hof1 in cytokinesis.

4. CONCLUSION

There are many methods to address the function of genes in cells such as analyzing messenger RNA abundance and stability, biochemical activity, protein-protein interactions, transcriptional regulation, gene disruption phenotypes and protein abundance.

To see the function of genes, candidate genes were deleted and chromosomal genes were tagged through a PCR-mediated technique. Morphological changes were observed to see if deletion mutants show cytokinesis defects. The results show that all seven ORFs, YHR149C, YLR187W, YOL070C, YMR124W, YOR304C-A, YPL158C and YGR153W, were successfully deleted but they were not involved in cytokinesis. Double deletion mutants can be made to see if there is redundancy through checking synthetic lethality with known cytokinesis genes.

There were many efforts to discover the role of Hof1p in cytokinesis and those works have revealed its functions. Hof1p is localized to the bud neck during cell cycle and interact with Bnr1p, a formin homology (FH) protein that binds to an actin-binding protein profillin (Evangelista et al., 1997). Hof1 may function as an adapter linking the primary septum synthesis machinery to the actomyosin system (Vallen at al., 2000) and regulates actomyosin ring dynamics and septin distribution (Lippincott and Li, 1998b). Hof1 double rings merge into a single ring that contracts slightly and may colocalize with the actomyosin structure (Vallen et al., 2000). Hof1p is phosphorylated, and this modification may regulate its dynamics or degradation (Blondel et. al., 2005).

This study was focused on the role of phosphorylation of HOF1 PEST domain. There may be other phosphorylation sites in HOF1 outside of this domain. To determine

if all *in vivo* phosphorylation sites in HOF1 mutant KSY126 have been deleted, further characterization experiment such as Western Blot should be performed.

Hof1 phosphorylation depends on MEN genes (Blondel et. al., 2005). Hof1-GFP was transformed into KSY 7 and KSY9, stains that have temperature sensitive mutations in Cdc5 and Cdc15, respectively. Control experiments were performed at the permissive temperature, and Hof1-GFP localization looked similar to wild type strains. Future experiments will examine Hof1-GFP localization at the semi-permissive temperature to determine effects on localization after inactivation of the Cdc5 and Cdc15 kinases.

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