INVESTIGATING SEPTIN-DEPENDENT, ACTOMYOSIN-RING-INDEPENDENT CYTOKINESIS IN *SACCHAROMYCES CEREVISIAE*

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology.

Chapel Hill
2007

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ABSTRACT

NIEN-HSI KO: Investigating Septin-dependent, Actomyosin-ring-independent Cytokinesis in *Saccharomyces cerevisiae*
(Under the direction of John R. Pringle)

In the budding yeast *Saccharomyces cerevisiae*, a ring of myosin II forms in a septin-dependent manner at the presumptive budding site in late G1. This ring remains at the bud neck until the onset of cytokinesis, when actin is recruited to it and the resulting actomyosin ring contracts. Concurrently, a septum of cell wall (including a chitinous primary septum synthesized by Chs2p) is formed to complete cytokinesis. Mutants lacking *MYO1* (the only myosin II gene) have varyingly severe phenotypes that can be suppressed either by deletion of a nonessential subunit of the anaphase-promoting complex or cyclosome (APC/C) or by overexpression of *IQG1* or *CYK3*. Iqg1p is the only *S. cerevisiae* IQGAP and is required for both actomyosin-ring formation and primary-septum formation, and hence for normal cytokinesis. Because the actomyosin ring itself is not essential for primary-septum formation, Iqg1p must have another cytokinetic function(s), which may involve stimulating Cyk3p (a septin-dependent protein involved in cytokinesis) and Hof1p (a possible linker between the actomyosin ring and the primary-septum-synthesis machinery) to function in a septin-dependent, actomyosin-ring-independent pathway. Subsequent analyses showed that Iqg1p is increased in abundance and persists after cytokinesis in APC/C mutants, that Iqg1p is ubiquitinated directly by APC/C *in vitro*, and that a nondegradable Iqg1p (missing a novel recognition motif) can suppress *myo1Δ* phenotypes. These data suggest that Iqg1p is a direct target of the APC/C. To identify additional proteins involved in septin-dependent,

actomyosin-ring-independent cytokinesis, dosage-suppressor screens have been conducted, using *myo1Δ cyk3Δ* and *myo1Δ hof1Δ* synthetic-sick mutants. Some of the genes identified include *EGT2* (encoding a cell-wall endoglucanase), *ECM33* (encoding a GPI-anchored protein that may regulate cell-wall organization), and a truncated *CDC24* (encoding the GEF for the Rho-type GTPase Cdc42p) that lacks the C-terminal PB1 domain. Overexpressing Ecm33p or the truncated Cdc24p can also suppress the *iqg1Δ* and *chs2Δ* growth defects and restore primary-septum formation in *iqg1Δ* cells. Furthermore, the suppression by truncated Cdc24p appears to be Cdc42p-independent and may involve another Rho-type GTPase(s). Finally, the dosage-suppressor screen also uncovered a gene, *YJL055W*, that when overexpressed directly suppresses the inviability of $URA3^{+}$ cells in the presence of 5-fluoroorotic acid.

ACKNOWLEDGEMENTS

To the casual observer, a doctoral dissertation may appear to be solitary work. However, to accomplish a work of this magnitude requires a strong network of support, and thus I am indebted to many people. I want to first thank my thesis advisor, John Pringle, for his guidance and support. I also want to thank the many colleagues of mine in his laboratory, especially Ryuichi Nishihama for his mentoring. More importantly, I want to thank my parents, Yu-Kien and Yi Nan Ko, and my sisters, Lucy Chou and Pearl Chu for their unconditional love and extraordinary courage. Finally, deep from my loving heart, I want to thank my BABY for making my existence meaningful.

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CHAPTER 1

INTRODUCTION

Cell division is an essential process that partitions the contents of one cell into two cells. This process includes a nuclear segregation by mitosis followed by a division of cell surface and cytoplasm by cytokinesis. Of these two stages, mitosis has been shown to be highly conserved in plants, animals, and fungi, whereas cytokinesis mechanisms have appeared to be variable. In plant cells, cytokinesis starts when a transient preprophase band marks the division site on the plasma membrane in late G2 (Staehelin and Hepler 1996; Assaad, 2001; Verma, 2001; Jürgens, 2005a; Jürgens 2005b). Near the end of mitosis, the phragmoplast, an array made of cytoskeletal components, then transports materials via membrane trafficking and targeted vesicle fusion to form a cell plate from the center to the periphery of the division plane. The cell plate leads to the formation of plasma membranes that underlie the dividing cell wall, which is formed by secretion of cell-wall materials into the lumen of the expanding cell plate. In contrast, cytokinesis in animal cells involves a cleavage furrow containing a contractile actomyosin ring, which includes nonmuscle myosin II, F-actin, and other proteins (Satterwhite and Pollard, 1992; Rappaport, 1996; Glotzer, 1997; Field *et al*., 1999; Hales *et al*., 1999; Uyeda *et al*., 2004). In anaphase, the mitotic spindle determines the site at which the contractile ring and cleavage furrow will form. A transient structure, the midbody, forms as an intercellular bridge when the furrow reaches the remnants of the mitotic spindle. Finally, the common membrane that surrounds the two nascent cells is divided so that the

cells can separate.

Although plant and animal cytokinesis appear, at least superficially, quite different, recent studies have shown that the two processes actually share some similarities. For example, as in plants, animal cytokinesis also requires membrane trafficking and targeted vesicle fusion (Albertson *et al*., 2005; Matheson *et al*., 2005). Specifically, membrane delivery is required both for the increased surface area during cleavage furrow ingression and for the sealing of divided cells during vesicular abscission of the midbody. Important goals at present are to understand the process of targeted membrane addition during both animal and plant cytokinesis, the synthesis of the dividing cell wall via cell plate formation in plant cytokinesis, and more broadly the similarities and differences between cytokinesis in various cell types.

To answer these important questions, cytokinesis in various fungi seem like excellent study models, because fungal cytokinesis appears to be similar to both animal and plant cytokinesis. For example, in the fission yeast *Schizosaccharomyces pombe*, cells divide by utilizing both a contractile actomyosin ring and formation of a septum made of cell-wall materials. Specifically, the actomyosin ring assembles in the middle of the cell overlying the nucleus during early M phase. At the end of mitosis, the ring contracts as the septum forms at the site marked by the ring. The center layer of the septum is then digested away, leading to the separation of the two daughter cells (Chang and Nurse, 1996; Gould and Simanis, 1997; Field *et al*., 1999; Hales *et al*., 1999; Mulvihill and Hyams, 2003).

Similarly, cytokinesis in the budding yeast *Saccharomyces cerevisiae* also involves an actomyosin ring and a cell-wall septum. In *S. cerevisiae*, a myosin ring (made of Myo1p, the only type II myosin in this organism) is first formed, in a septin-dependent manner, at the

presumptive budding site in late G1 phase (Bi *et al*., 1998; Lippincott and Li, 1998). Septins are GTP-binding proteins that have been found at the division site in all fungal and animal cells examined (Longtine *et al*., 1996; Hall and Russell, 2004; Gladfelter, 2006) and may function as a diffusion barrier and a scaffold for the organization and localization of other proteins (Gladfelter *et al*., 2001; Longtine and Bi, 2003; Dobbelaere and Barral, 2004; Versele and Thorner, 2005; Spiliotis and Nelson, 2006). Remarkably, although the septins are required for cytokinesis in some cell types, including *S. cerevisiae*, they are not required in others, including *S. pombe* (Longtine *et al*., 1996; Adam *et al*., 2000; Nguyen *et al*., 2000; Kinoshita and Noda, 2001; An *et al*., 2004).

In *S. cerevisiae*, after its initial septin-dependent formation, the myosin ring then remains at the mother-bud neck until the onset of cytokinesis, when actin and other proteins are recruited to it to form the mature actomyosin ring, which soon contracts. Simultaneous with or soon after this contraction, the plasma membrane invaginates and the primary cell-wall septum is synthesized, principally by the chitin synthase Chs2p (Cabib *et al*., 2001; Schmidt *et al*., 2002). Secondary septa are then deposited on both sides of the primary septum to form the mature trilaminar septum, and the mother and daughter cells are separated by the action of a chitinase that partially hydrolyzes the primary septum (Kuranda and Robbins, 1991; Colman-Lerner *et al*., 2001).

Although the contractile actomyosin ring is involved in cytokinesis of animal cells, *S. pombe*, and *S. cerevisiae*, one major difference is the essentiality of the ring. The ring appears to be essential for cytokinesis in *S. pombe* and most animal cells (Rappaport, 1996; Gould and Simanis, 1997; Field *et al*., 1999; Hales *et al*., 1999; Mulvihill and Hyams, 2003), but it is not essential in *S. cerevisiae* (Rodriguez and Paterson, 1990; Bi *et al*., 1998;

Lippincott and Li, 1998; Schmidt *et al*., 2002). The non-essentiality of the actomyosin ring is certainly not unique to *S. cerevisiae*. For examples, amoebae cells of the slime mold *Dictyostelium discoideum* can carry out cytokinesis on substrates in the absence of the actomyosin ring (De Lozanne and Spudich, 1987; Uyeda *et al*., 2000; Nagasaki *et al*., 2002), and some animal cells, such as chicken embryo, epitheloid kidney, and normal rat kidney cells, appear to be able to carry out cytokinesis, albeit less efficiently, even when myosin II or actomyosin-ring contraction is inhibited or completely absent (Nunnally *et al*., 1980; Zurek *et al*., 1990; Uyeda *et al*., 2004; Kanada *et al*., 2005).

In *S. cerevisiae*, mutant cells lacking Myo1p do not form the actomyosin ring but are still viable in most genetic backgrounds, although their septa are typically thick and disorganized and often lack well defined primary-septum structures (Schmidt *et al*., 2002; Tolliday *et al*., 2003; Nishihama *et al*., 2007). However, some *myo1Δ* cells can form disorganized primary-septum-like structures made of chitin within the thick septa (Nishihama *et al*., 2007). The aberrant septa are presumably the reason that *myo1Δ* cells grow more slowly than wild type and fail to separate efficiently, but they also represent a means by which *S. cerevisiae* cells can carry out cytokinesis through a septin-dependent, actomyosin-ring-independent mechanism(s). This mechanism(s) may involve pathways and proteins functioning in membrane invagination, septum construction, and other related processes. Thus, elucidating this mechanism in *S. cerevisiae* may also shed new light on similar pathways in other organisms, including those that may be involved in cell-wall synthesis and targeted membrane addition during plant and animal cytokinesis. In other words, *S. cerevisiae* can serve as an informative model system to study the contractile-ring-independent process in cytokinesis.

To date, some of the septin-dependent proteins that may function independently of the actomyosin ring in *S. cerevisiae* cytokinesis include Iqg1p, Cyk3p, and Hof1p. Iqg1p is the only known IQGAP (Brown and Sacks, 2006) in *S. cerevisiae* and has been shown to be indispensable for actomyosin-ring formation, primary-septum formation, and normal cytokinesis (Epp and Chant, 1997; Shannon and Li, 1999; Luo *et al*., 2004; Nishihama *et al*., 2007; see Chapters 2 and 3). Because the actomyosin ring itself is not essential for cytokinesis, Iqg1p must have at least one other cytokinetic function(s) that is independent of the actomyosin ring. To date, the exact function of Cyk3p is unclear, but its overexpression suppresses the *iqg1Δ* growth defect without restoring the actomyosin ring, and the *myo1Δ cyk3Δ* double mutant is synthetically sick, suggesting that Cyk3p also promotes cytokinesis through an actomyosin-ring-independent pathway (Korinek *et al*., 2000; Nishihama *et al*., 2007). Although the exact cellular function of Hof1p remains as a mystery, this protein has been shown to be a member of the Pombe-Cdc15-Homology (PCH) family, which also includes *S. pombe* Cdc15p and mammalian proteins PSTPIP1 and PSTPIP2 (Chitu and Stanley, 2007). PCH proteins have been suggested to function at the membrane-cytoskeleton interface by coordinating changes in the actin cytoskeleton and membrane morphology. Thus, Hof1p may function in cytokinesis as an adapter linking the primary-septum-synthesis machinery to the actomyosin ring (Vallen *et al*., 2000). Because the *myo1Δ hof1Δ* double mutant is synthetically inviable, Hof1p may also participate in an actomyosin-ring-independent pathway of cytokinesis.

In addition to the cytokinetic machineries described above, *S. cerevisiae* cells also have sophisticated regulatory pathways to coordinate cytokinesis with other late-cell-cycle events, such as the completion of chromosome segregation and the exit from mitosis. One

such regulatory pathway involves the anaphase-promoting complex or cyclosome (APC/C), which is an essential, conserved, and multi-subunit ubiquitin ligase that targets specific cell-cycle-related proteins for degradation (Peters, 2006). The APC/C initiates anaphase by triggering sister-chromatid separation via degradation of securin (Nasmyth, 2005), which unleashes the protease separase and activates the mitotic-exit network (MEN). The MEN further activates Cdc14p, which then activates the APC/C to promote exit from mitosis via degradation of mitotic cyclins. In addition to these major roles, the APC/C also regulates other cell-cycle proteins, such as the spindle-associated kinesins Kip1p and Cin8p (Harper *et al*., 2002).

To investigate the mechanisms of septin-dependent, actomyosin-ring-independent cytokinesis in *S. cerevisiae*, I have conducted synthetic-lethal and dosage-suppressor screens (based on the colony-sectoring assay described by Bender and Pringle, 1991) starting with septin and *myo1Δ* mutants. From these studies, described in Chapter 2, mutations in the nonessential subunits of the APC/C and overexpression of Iqg1p or Cyk3p were found to suppress the growth defects of *myo1Δ* cells. Furthermore, Iqg1p was found to be a direct substrate of the APC/C, and the elevated levels of Iqg1p in APC/C mutants accounted for the suppression of the *myo1Δ* growth defects. In addition, overexpression of Iqg1p or Cyk3p was also found to promote the formation of primary-septum-like structures in the absence of the actomyosin ring (Nishihama *et al*., 2007).

To identify additional components of the septin-dependent, actomysin-ring-independent mechanism of cytokinesis in *S. cerevisiae*, I have conducted dosage-suppressor screens [using the *URA3*/5-FOA-based lethality described by Boeke *et al*. (1984), Rothstein (1991), and Sikorski and Boeke (1991)] starting with *myo1Δ cyk3Δ* and *myo1Δ hof1Δ* double

mutants. In these studies, described in Chapter 3, several known and novel proteins that may function in cytokinesis have been identified. Specifically, overexpression of either Ecm33p (which is a GPI-anchored protein that may regulate cell-wall organization) or a truncated Cdc24p (which is the GEF for the Rho-type GTPase Cdc42p) lacking its PB1 domain was found to promote the formation of primary-septum-like structures and possibly more organized thick septa in the absence of the actomyosin ring. Furthermore, *ecm33Δ* cells displayed minor defects in cytokinesis at 37˚C. The suppression by the truncated Cdc24p appears to be Cdc42p-independent and may involve the action of other Rho-type GTPases, such as Rho1p, Rho2p, and/or Rho5p, and/or an unknown factor(s).

Taken together, these results suggest that Iqg1p, a novel APC/C target, may stimulate Cyk3p and other proteins (such as those involved in targeted membrane addition and/or cell-wall synthesis/integrity) in a cell-cycle-regulated manner to promote the construction of a more normal septum through the contractile-ring-independent mechanism of cytokinesis.

A by-product of the dosage-suppressor screens, unrelated to cytokinesis, and described in Chapter 4, was the discovery that overexpression of the little-characterized yeast gene *YJL055W* could directly suppress the inviability of $URA3^{+}$ cells in the presence of 5-FOA. This observation appear related to a previous report that the *yjl055wΔ* mutant has increased sensitivity to purine-analog mutagens, and suggests that Yjl055Wp may function in the detoxification of nucleotide analogs. However, the precise mechanism of Yjl055Wp action remains unclear.

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CHAPTER 2

IDENTIFICATION OF YEAST IQGAP (Iqg1p) AS AN ANAPHASE-PROMOTING-COMPLEX SUBSTRATE AND ITS ROLE IN ACTOMYOSIN-RING-INDEPENDENT CYTOKINESIS

ABSTRACT

In the yeast *Saccharomyces cerevisiae*, a ring of myosin II forms in a septin-dependent manner at the budding site in late G1. This ring remains at the bud neck until the onset of cytokinesis, when actin is recruited to it. The actomyosin ring then contracts, septum formation occurs concurrently, and cytokinesis is soon completed. Deletion of *MYO1* (the only myosin II gene) is lethal on rich medium in the W303 strain background and causes slow-growth and delayed-cell-separation phenotypes in the S288C strain background. These phenotypes can be suppressed by deletions of genes encoding nonessential components of the anaphase-promoting complex (APC/C). This suppression does not appear to result simply from a delay in mitotic exit, because overexpression of a nondegradable mitotic cyclin does not suppress the same phenotypes. Overexpression of either *IQG1* or *CYK3* also suppresses the *myo1Δ* phenotypes, and Iqg1p (an IQGAP protein) is increased in abundance and abnormally persistent after cytokinesis in APC/C mutants. *In vitro* assays showed that Iqg1p is ubiquitinated directly by APC/C^{Cdh1} via a novel recognition sequence. A nondegradable Iqg1p (lacking this recognition sequence) can suppress the *myo1Δ* phenotypes even when expressed at relatively low levels. Taken together, the data suggest that compromise of APC/C function allows the accumulation of Iqg1p, which then promotes actomyosin-ring-independent cytokinesis by activation of Cyk3p.

INTRODUCTION

Cytokinesis is the process that divides the cell surface and cytoplasm of one cell into two cells. Although the list of proteins known to be involved in cytokinesis has expanded significantly in recent years, a complete molecular understanding of this process remains elusive. Among the mysteries are the roles of the septins and of the actomyosin contractile ring. The septins are a family of GTP-binding proteins that have been found at the division site in all fungal and animal cells examined (Longtine *et al*., 1996; Hall and Russell, 2004; Gladfelter, 2006). Although their roles are still imperfectly understood, the septins appear to function as both a scaffold and a diffusion barrier for the localization and organization of other proteins (Gladfelter *et al*., 2001; Longtine and Bi, 2003; Dobbelaere and Barral, 2004; Versele and Thorner, 2005; Spiliotis and Nelson, 2006). Surprisingly, although the septins are indispensable for cytokinesis in some cell types, they are dispensable in others (Longtine *et al*., 1996; Adam *et al*., 2000; Nguyen *et al*., 2000; Kinoshita and Noda, 2001; An *et al*., 2004). Similarly, it has recently become clear that the actomyosin ring at the division site is also dispensable for cytokinesis in a variety of cell types (Bi *et al*., 1998; Nagasaki *et al*., 2002; Kanada *et al*., 2005). In the budding yeast *Saccharomyces cerevisiae*, the septins are essential for formation of the actomyosin ring. However, this cannot be their only role, because they are essential for cytokinesis in this organism, whereas the actomyosin ring is not essential in most strain backgrounds (Bi *et al*., 1998; Schmidt *et al*., 2002; Nishihama *et al*., 2007). A major challenge at present is to elucidate the actomyosin-ring-independent role(s) of the septins in cytokinesis in yeast and (presumably) in other cell types.

In *S. cerevisiae*, Myo1p (the only type II myosin in this organism) forms a ring at the presumptive budding site in late G1 (Bi *et al*., 1998; Lippincott and Li, 1998). This ring

remains at the mother-bud neck until the onset of cytokinesis, when actin and other proteins are recruited to it to form the mature actomyosin ring, which soon contracts. Concurrent with this contraction, the plasma membrane invaginates and the primary cell-wall septum is synthesized, principally by the chitin synthase Chs2p (Cabib *et al*., 2001; Schmidt *et al*., 2002). Secondary septa are then deposited on both sides of the primary septum to form the mature trilaminar septum, and the mother and daughter cells are separated by the action of a chitinase that partially hydrolyzes the primary septum (Kuranda and Robbins, 1991; Colman-Lerner *et al*., 2001). In viable *myo1Δ* cells, no actomyosin ring forms, and the septa that form are typically disorganized and often lack well defined primary-septum-like structures (Schmidt *et al*., 2002; Tolliday *et al*., 2003; Nishihama *et al*., 2007). These disorganized septa are presumably the reason that *myo1Δ* cells grow more slowly than wild type and fail to separate efficiently, resulting in the formation of multi-cell clusters.

Among the proteins recruited to the division site in a septin-dependent manner just before cytokinesis are Iqg1p and Cyk3p. Iqg1p is the only member of the IQGAP family (Brown and Sacks, 2006) in *S. cerevisiae*, and it has been reported to be essential both for formation of the actomyosin ring and for cytokinesis (Epp and Chant, 1997; Shannon and Li, 1999; Luo *et al*., 2004). Because the actomyosin ring itself is not essential for cytokinesis, Iqg1p must have at least one cytokinetic function that is actomyosin-ring independent. The function of Cyk3p is not known, but its overexpression suppresses the *iqg1Δ* lethality without restoring the actomyosin ring, suggesting that Cyk3p also promotes cytokinesis through an actomyosin-ring-independent pathway (Korinek *et al*., 2000; Nishihama *et al*., 2007).

For successful cellular reproduction, cytokinesis must be coordinated with other late-cell-cycle events such as the completion of chromosome segregation and the exit from

mitosis. Not surprisingly, cells have sophisticated regulatory pathways to ensure this coordination. Regulation of late mitotic events depends largely on the anaphase-promoting complex or cyclosome (APC/C), an essential multi-subunit ubiquitin ligase that targets specific cell-cycle-related proteins for degradation (Peters, 2006). The APC/C, with its activating subunit Cdc20p, initiates anaphase by triggering sister-chromatid separation via degradation of the securin Pds1p (Nasmyth, 2005), which unleashes the protease separase. In *S. cerevisiae,* separase activation also initiates release of the protein phosphatase Cdc14p from its nucleolar inhibitor Net1p. Activation of the mitotic-exit network (MEN) further activates Cdc14p, which then activates Cdh1p, a second APC/C-activating subunit that targets Clb2p and other mitotic cyclins for degradation in late mitosis. In addition to these major roles, the APC/C also regulates other cell-cycle proteins, such as the spindle-associated kinesins Kip1p and Cin8p (Harper *et al*., 2002).

13 subunits of the APC/C have been identified in *S. cerevisiae*. Many of the core subunits are essential for APC/C function and thus for viability. However, some subunits, including Cdc26p, Apc9p, Doc1p, Swm1p, and Mnd2p, are not essential. Cdc26p and Apc9p appear to be involved in the assembly of the APC/C (Passmore, 2004), Doc1p is involved in promoting the association between the APC/C and its substrates (Carroll and Morgan, 2002; Passmore *et al*., 2003; Carroll *et al*., 2005), Swm1p is required for full catalytic activity of the APC/C (Schwickart *et al.,* 2004; Page *et al*., 2005), and Mnd2p appears to have little role in mitotic control but is important in regulating the APC/C during meiosis (Oelschlaegel *et al.,* 2005; Page *et al*., 2005)

To investigate the mechanisms of septin-dependent, actomyosin-ring-independent cytokinesis in *S. cerevisiae*, we have been conducting synthetic-lethal and dosage-

suppression screens starting with septin and *myo1Δ* mutants. During these studies, we unexpectedly observed that mutations in nonessential subunits of the APC/C could suppress the phenotypes of *myo1Δ* mutants. We present evidence that Iqg1p is a direct substrate of the APC/C and that the elevation of Iqg1p levels in APC/C mutants accounts for the suppression of *myo1Δ* phenotypes.

MATERIALS AND METHODS

Strains, Plasmids, Growth Conditions, and Genetic Methods

The strains and plasmids used in this study are listed in Tables 2.1 and 2.2; their construction is described below or in the tables. Yeast were grown on liquid or solid synthetic complete (SC) medium lacking specific nutrients as needed to select plasmids or transformants, YP rich liquid or solid medium, or YM-P rich, buffered liquid medium (Lillie and Pringle, 1980; Guthrie and Fink, 1991). 2% glucose was used as carbon source except for experiments involving induction of gene expression under *GAL* promoter control, for which 1% or 2% raffinose plus 0.5% or 2% galactose was used, as indicated. Yeast strains were grown at 23˚C except as noted. The antibiotic geneticin (G418; Cambrex, Walkersville, MD) was used to select for cells containing the kan^R marker, and 5-fluoroorotic acid (5-FOA; Research Products International, Mt. Prospect, IL) was used to select for *ura3*-mutant cells. To arrest cells in G1, α-factor (Sigma-Aldrich, St. Louis, MO) was used at the concentration indicated. The microtubule-depolymerizing drug benomyl (DuPont, Wilmington, DE) was used at 50 μg/ml to arrest cells in M phase. To block translation, cycloheximide [MP Biomedicals, Solon, OH (Figure 2.6) or Sigma-Aldrich (Figure 2.9)] was added to the growth medium at the concentration indicated. Standard procedures were used for growth of *Escherichia coli*, genetic manipulations, polymerase chain reaction (PCR), and other molecular biological procedures (Sambrook *et al*., 1989; Guthrie and Fink, 1991; Ausubel *et al*., 1995).

Strain and Plasmid Constructions

Genes were deleted using the PCR method (Baudin *et al*., 1993; Longtine *et al*., 1998) and the primers indicated in Table 2.3; in each case, the entire coding region was deleted. The success of each deletion was confirmed by two PCR tests that used check primers that were

upstream and downstream, respectively, of the deleted region together with primers internal to the selectable markers (Longtine *et al*., 1998; Table 2.3). C-terminal tagging with sequences encoding the 3HA epitope or GFP(F64L,S65T,V163A) was also done using the PCR method with plasmid pFA6a-3HA-His3MX6 (Longtine *et al*., 1998) or pFA6a-GFP(F64L,S65T,V163A)-His3MX6 (see below) as template. The success of the tagging was confirmed by two PCR tests, essentially as described above. To construct strains DOY138-141, an *IQG1-TAP:His3MX6* C-terminal fragment was PCR-amplified using genomic DNA from strain IQG1-TAP as template and the primers indicated in Table 2.3. This cassette was then transformed into appropriate parental strains as indicated in Table 2.1.

Plasmid pFA6a-GFP(F64L,S65T,V163A)-His3MX6 was constructed by subcloning an *Msc*I-*Bst*BI fragment containing the three mutations from YEpGFP*-BUD8F (Schenkman *et al*., 2002) into *Msc*I/*Bst*BI-cut pFA6a-GFP(S65T)-HIS3MX6 (Wach *et al*., 1997). Plasmid pTSV31A-MYO1 was constructed by subcloning the 7.0-kb *Sal*I-*Bam*HI *MYO1* fragment from pBS-MYO1 (a gift from E. Bi, University of Pennsylvania, Philadelphia, PA) into *Sal*I/*Bam*HI-cut pTSV31A (a 2μ *URA3 ADE3* plasmid; M. Tibbetts and J.R. Pringle, unpublished results). Plasmid pGT04 was constructed using two steps of PCR. In the first step, a fragment of *IQG1* (nucleotides -262 to +3 relative to the A of the start codon) was amplified from genomic DNA with a *Bam*HI site incorporated into the 5' primer and a 3' primer that included nucleotides corresponding to positions +127 to +141 of *IQG1*. A second fragment (nucleotides +127 to +277) was also amplified from genomic DNA using a 5' primer that included nucleotides corresponding to positions -15 to +3 of *IQG1* and a 3' primer that included an *Xba*I site. In the second step, the PCR products from the first step were purified and used as template with the *Bam*HI-site-containing 5' primer and the *Xba*I-

site-containing 3' primer. The resulting product, which contained 262 nucleotides of the *IQG1* promoter, a start codon, and 151 nucleotides (from +127 to +277) of open-reading-frame sequence, was cut with *Bam*HI and *Xba*I, gel-purified, and inserted into *Bam*HI/*Xba*I-cut pRS305 (Sikorski and Hieter, 1989). Plasmids pGAL-IQG1-TAP and pGAL-iqg1Δ42-TAP were constructed by transforming yeast cells with *Bam*HI/*Hin*dIII-cut pRSAB1234 (see Supplemental Materials and Methods of Gelperin *et al*., 2005) with PCR-amplified full-length or truncated (lacking codons 2-42) *IQG1*; the amplified fragment contained 22 (5') and 21 (3') base pairs of flanking vector sequences to allow the *in vivo* recombination.

Growth Rates, Cell-Cluster Indices, Colony-Sectoring Assays, and FACS Analysis

To determine growth rates, exponential-phase cultures ($OD_{600} \approx 0.4$) in YM-P medium were diluted two-fold with fresh YM-P, and incubation was continued. The times needed to return to the original OD_{600} were recorded as the doubling times.

To determine cell-cluster indices, strains were streaked onto the indicated media, grown overnight, scraped from the plates, washed once with water by centrifugation, resuspended, sonicated briefly, and observed by DIC microscopy. Each unbudded (one cell body) or budded (two cell bodies) cell was scored as one nonclustered unit, and entities with three, four, five, or six-or-more cell bodies were scored as one, two, three, or four clusters, respectively. Each count was continued until the number of clusters plus nonclustered units was 100, and the number of clusters was recorded as the cluster index. To minimize the possibility that the cell-cluster index determined for the *myo1Δ* single mutant would be influenced by spontaneously arising suppressors, we examined nine different *myo1Δ* strains that were obtained as segregants from RNY112 and its double-mutant derivatives.

The colony-sectoring assay was based on that described by Bender and Pringle

(1991). *ade2-1 ade3Δ ura3-3 myo1Δ* strains carrying a *URA3 ADE3 MYO1* plasmid were grown on SC-Ura plates, then streaked onto YP or YPGalRaf plates to observe sectoring or nonsectoring single colonies. In some experiments, a *LEU2*-marked plasmid (empty or carrying *IQG1* or *CYK3*) was also present. In these cases, the strains were first grown on SC-Ura-Leu plates, then streaked onto SC-Leu plates to observe sectoring or nonsectoring single colonies.

To assess the extent of G2 delay using the fluorescence-activated cell sorter (FACS), cells were grown to exponential phase ($OD_{600} \approx 0.4$) in YM-P medium, then collected, fixed, stained with SYTOX Green (Invitrogen, Carlsbad, CA), and examined using standard procedures (Haase and Reed, 2002).

Screen for Dosage Suppressors of myo1Δ

A W303-background *myo1Δ* haploid strain, RNY798, which contained an *ADE3 MYO1* plasmid and could only form nonsectored viable colonies (see above), was transformed with a genomic-DNA library in the low-copy plasmid YCp50-LEU2 (Bi and Pringle, 1996; the library was constructed using DNA from an S288C-background strain and was kindly provided by F. Spencer and P. Hieter, Johns Hopkins University, Baltimore, MD). From ~20,000 transformants screened on SC-Leu plates, 85 reproducibly sectoring transformants were identified. Isolation of plasmids and retransformation of strain RNY798 yielded 14 plasmids that rescued the lethality of the *myo1Δ* strain. Sequencing and subcloning of the inserts revealed that nine plasmids contained full-length *MYO1* or C-terminal *MYO1* fragments, and that in three other plasmids, the gene responsible for suppression was either *IQG1* (two cases) or *CYK3* (one case). The suppressing genes in the final two plasmids have not yet been identified.

Protein Analysis and Ubiquitination Assays

Iqg1p-3HA levels were determined by Western blotting in both asynchronous and synchronous cultures. For synchronous cultures, the supersensitive *bar1Δ* (Sprague, 1991) strains were grown to exponential phase in SC-His medium, treated for 3 h with 70 ng/ml α-factor to arrest cells in G1, released from arrest by centrifugation and resuspension in SC-His medium, and sampled at 20-min intervals. At each time point, one sample was taken for protein extraction, and a second sample was fixed for 10 min in 70% ethanol at 0˚C, resuspended in PBS, stained with DAPI (Sigma-Aldrich), and used to evaluate cell-cycle progression by scoring the percentages of large-budded cells with two well separated chromosome sets. To extract proteins, cells were collected by centrifugation, resuspended in 1.85 M NaOH containing 2% β-mercaptoethanol, and incubated for 10 min at 0˚C. Trichloroacetic acid was then added to 50%, and incubation was continued for 15-25 min at 0˚C. Insoluble material was collected by centrifugation, mixed with SDS-sample buffer, boiled for 5 min, and analyzed on 7% SDS-polyacrylamide gels. After blotting proteins electrophoretically to nitrocellulose transfer membrane (GE Healthcare, Piscataway, NJ), Iqg1p-3HA was detected using the rat monoclonal anti-HA antibody 3F10 (Roche Molecular Biochemicals, Indianapolis, IN) as primary antibody. Protein bands were then visualized using either mouse anti-rat-IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA), alkaline-phosphatase-conjugated goat anti-mouse-IgG tertiary antibody (Sigma-Aldrich), and the AttoPhos AP fluorescent-substrate system (Promega, Madison, WI) (Figure 2.6D), or HRP-conjugated goat anti-rat-IgG secondary antibody (GE Healthcare), and the ECL chemiluminescence system (GE Healthcare) (Figure 2.7). Actin was detected using either a goat polyclonal anti-actin antibody (Karpova *et al*., 1993) (Figures 2.6D and 2.7B) or

the mouse monoclonal anti-actin antibody MAB1501 (Chemicon, Temecula, CA) (Figure 2.7A) as primary antibody. Protein bands were then visualized using alkaline-phosphatase-conjugated rabbit anti-goat-IgG secondary antibody (Sigma-Aldrich) and the AttoPhos AP system (Figure 2.6D), HRP-conjugated donkey anti-goat-IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the ECL system (Figure 2.7B), or alkaline-phosphatase-conjugated goat anti-mouse-IgG secondary antibody and the AttoPhos AP system (Figure 2.7A). In Figure 6D, the intensities of the protein bands were measured using the Storm Scanner Model 840 (GE Healthcare), and the values for Iqg1p-3HA were normalized using the actin bands from the same samples.

Iqg1p-TAP levels were determined by Western blotting using the peroxidase anti-peroxidase soluble complex produced in rabbit (Sigma-Aldrich, Cat. No. P1291) and the SuperSignal West Pico chemiluminescence system (Pierce Biotechnology, Rockford, IL). For the experiments of Figure 2.6B and C, cell lysates were prepared as described previously (Ostapenko and Solomon, 2005), and Iqg1p-TAP was precipitated from equal amounts of lysate by incubation for 90 min at 4°C with IgG-Sepharose (GE Healthcare) in 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, containing 1% NP-40 and protease inhibitors (10 mg/ml each of leupeptin, chymostatin, and pepstatin; all from Chemicon). Precipitated proteins were separated on 10% SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Billerica, MA), and detected as described above using an overnight incubation at 4°C in 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, containing 0.1% Tween-20 and 5% dry milk. For the experiments of Figure 2.9C and D, protein extracts were prepared and analyzed by 10% SDS-PAGE and Western blotting as described previously (Ubersax *et al.*, 2003). Iqg1p-TAP and Iqg1Δ42p-TAP were detected as described above, and Clb2p was detected using a rabbit polyclonal

anti-Clb2p primary antibody (Kellogg and Murray, 1995), an HRP-conjugated donkey anti-rabbit-IgG secondary antibody (GE Healthcare), and the SuperSignal West Pico system.

To perform ubiquitination assays, reaction components were expressed and purified as described previously (Charles *et al*., 1998; Carroll and Morgan, 2002, 2005). Substrates were produced in rabbit-reticulocyte lysates by coupled transcription and translation in the presence of ^{35}S-methionine, following the manufacturer's instructions (Promega). Truncated *IQG1* constructs with a T7 promoter sequence and Kozak site added upstream were made using two steps of PCR. In the first step, the desired *IQG1* fragment was PCR-amplified using a 5' primer containing a sequence of 23 nucleotides that overlapped the 5' primer used in the second step plus 30-37 nucleotides of *IQG1* coding sequence beginning with a start codon. The 3' primer contained ~20 nucleotides of coding sequence ending with a stop codon. In the second step, the product from the first step was used as template with the same 3' primer and a 5' primer of 119 nucleotides that included a T7 promoter and a Kozak site. Point mutations were introduced by incorporation into the 5' primer used in the first step. Similarly, full-length *PDS1* and *CYK3* constructs were generated with the T7 promoter sequence and Kozak site upstream of the start codons. Ubiquitination reactions were performed and monitored using a Molecular Dynamics PhosphorImager (GE Healthcare) as described previously (Carroll and Morgan, 2002, 2005; Carroll *et al*., 2005).

Microscopy and Quantitation of GFP Fluorescence

DIC and fluorescence microscopy were performed using a Nikon (Tokyo, Japan) Eclipse E600-FN microscope and an ORCA-2 cooled CCD camera (Hamamatsu Photonic Systems, Bridgewater, NJ). To quantitate Iqg1p-GFP signal intensities, cells were grown to exponential phase in SC-His medium, collected by centrifugation, and resuspended in water

before observation by DIC and fluorescence microscopy. The average intensities of the GFP signals at the mother-bud neck were determined for each strain using MetaMorph version 5.0 (Universal Imaging Corporation, Downington, PA); a box of fixed size was drawn to contain the neck of each large-budded cell with a detectable signal, and the total fluorescence of the boxed area was measured and recorded using the regional-measurement function of MetaMorph.

RESULTS

Suppression of myo1Δ *Phenotypes by APC/C Mutations*

We performed a screen for mutations synthetically lethal with *myo1Δ* in the S288C strain background at 23˚C. The first several mutants analyzed proved to be temperature sensitive for growth (*i.e.*, unable to grow at 37˚C even when the *MYO1* plasmid was present), which seemed to offer an easy method to clone the genes harboring the synthetic-lethal mutations. Using a genomic-DNA library to rescue the temperature sensitivity of one mutant, we recovered *CDC26*, which encodes a subunit of the APC/C that is nonessential at 23˚C but essential at 37˚C. Further investigation revealed that the parental *myo1Δ* strain used in the screen was itself temperature sensitive and harbored a *cdc26* mutation [a *YJRWdelta11* or *YJRWdelta13* sequence (338 nucleotides; *Saccharomyces* Genome Database) inserted at nucleotide -7 relative to the start site of the *CDC26* ORF] that accounted for the temperature-sensitive phenotype. Because *myo1Δ* strains grow less well than wild type (Rodriguez and Paterson, 1990; Bi *et al*., 1998; Lippincott and Li, 1998) and can accumulate spontaneous suppressor mutations (Tolliday *et al*., 2003), this suggested that the *cdc26* mutation might have been selected because it alleviated the phenotype of the original *myo1* mutant. Indeed, when we intentionally introduced mutations of *CDC26* or other nonessential APC/C subunits into freshly prepared *myo1Δ* strains, we found that these mutations could suppress both the clustering and slow-growth phenotypes in the S288C background (Figure 2.1) and the lethal phenotype (Tolliday *et al*., 2003; Nishihama *et al*., 2007) in the W303 background (Figure 2.2). Similar results were obtained with mutations of *CDH1*, the APC/C activator for exit from mitosis (Figures 2.1B and C; Figure 2A). In contrast, mutation of *PDS1*, which encodes securin, the APC/C target whose degradation triggers anaphase onset, did not alter

the phenotypes in either background (Figures 2.1B and C; Figure 2.2A). Taken together, these results suggested that APC/C mutations do not suppress *myo1Δ* phenotypes by affecting the timing of anaphase.

Lack of* myo1Δ *Suppression by Delayed Mitotic Exit

It also seemed possible that APC/C mutations might suppress *myo1Δ* phenotypes by delaying mitotic exit and thus allowing more time for an inefficient process of cytokinesis to be completed successfully. To explore this possibility, we first introduced a deletion of *NET1* into an S288C-background strain that was heterozygous for a *myo1Δ* mutation. Because *NET1* encodes an inhibitor of Cdc14p, which activates the APC/C for mitotic exit, it seemed possible that a *net1* mutation might exacerbate the *myo1Δ* phenotypes by accelerating mitotic exit. Indeed, the doubly heterozygous diploid yielded only synthetic-lethal or synthetic-sick *myo1Δ net1Δ* double-mutant segregants (Figure 2.3A), and the poor viability was suppressed by deletion of a nonessential APC/C subunit (Figure 2.3B). These data were consistent with the hypothesis that APC/C mutations might suppress the *myo1Δ* phenotypes by delaying mitotic exit. However, this hypothesis was difficult to reconcile with the observation that mutation of *MND2*, which appears to have little effect on mitotic exit (Oelschlaegel *et al.,* 2005; Page *et al*., 2005), suppressed the *myo1Δ* phenotypes as effectively as did mutations of other APC/C subunits (Figure 2.1B and C; Figure 2.2A).

To explore this matter further, we wanted to delay mitotic exit by means independent of effects on the APC/C. To this end, we constructed strains in both backgrounds in which the nondegradable mitotic cyclin Clb2pΔDB (Amon *et al*., 1994) is expressed from the inducible *GAL1* promoter. FACS analysis on asynchronous cultures showed that in glucose medium, APC/C-mutant strains showed a higher ratio of G2/M cells to G1 cells than did

wild-type strains (Figure 2.4A and B, panels 1 and 4), indicating a delay in mitotic exit in the APC/C mutants. A shift to galactose medium produced a modest decrease in the G2/M-to-G1 ratio in these strains (Figure 2.4A and B, panels 2, 3, 5, and 6), presumably reflecting a delay in cell-cycle initiation on the poorer carbon source (Pringle and Hartwell, 1981). In contrast, the P_{GAL}-*clb2ΔDB* strains showed no accumulation of G2/M cells on glucose medium (Figure 2.4A and B, panels 7) but, as expected, showed a significant accumulation of such cells when production of Clb2pΔDB was induced by growth in galactose medium (Figure 2.4A and B, panels 8 and 9). The apparent delay in mitotic exit was similar to (W303 background) or greater than (S288C background) the delay produced by APC/C mutations. However, in contrast to APC/C mutations, induction of Clb2pΔDB produced little or no suppression of either the *myo1Δ* clustering phenotype in the S288C background (Figure 2.4C) or the *myo1Δ* lethal phenotype in the W303 background (Figure 2.4D and E). Thus, neither reduced degradation of the APC/C^{Cdh1} target Clb2p nor the resulting delay in mitotic exit appears to suppress *myo1Δ* phenotypes. These results suggested that some other APC/C target(s) and/or pathway(s) might be involved in the suppression.

Suppression of* myo1Δ *Phenotypes by Overexpression of Iqg1p or Cyk3p

In parallel with the synthetic-lethal screen, we also screened for dosage suppressors of *myo1Δ* lethality in the W303 background (see Materials and Methods). From this screen, low-copy plasmids carrying *IQG1* and *CYK3* were isolated. Further investigation showed that both low-copy and high-copy *IQG1* and *CYK3* plasmids could indeed suppress *myo1Δ* inviability in the W303 background, as judged by tests either of spore viability (Figure 2.5A) or of the ability of vegetative *myo1Δ* cells to lose a *MYO1* plasmid (Figure 2.5B). Sequencing of both genes in the W303 background revealed no mutations (Nishihama *et al*.,

2007), suggesting that the suppression of *myo1Δ* lethality was due simply to increased amounts of one or the other wild-type protein. In further support of this hypothesis, either low-copy or high-copy plasmids containing either *IQG1* or *CYK3* could also suppress the clustering phenotype of the *myo1Δ* mutant in the S288C background (Figure 2.5C). These results suggested that Iqg1p, Cyk3p, or both might be a previously unrecognized target(s) of the APC/C, so that APC/C mutations would suppress *myo1Δ* phenotypes by increasing the amount of that protein(s).

Role of APC/C in the Post-Mitotic Degradation of Iqg1p

To ask if Iqg1p or Cyk3p might be a target of the APC/C, we first searched their sequences for the known APC/C-recognition motifs called the destruction box (DB) (King *et al*., 1995; Burton and Solomon, 2001; Burton *et al*., 2005), KEN box (Pfleger and Kirschner, 2000), A box (Littlepage and Ruderman, 2002), and GxEN motif (Castro *et al*., 2003). No such sequences were found in Cyk3p, but two possible DBs, a possible KEN box, and a GKEN sequence (a possible KEN box or GxEN motif) were found in Iqg1p (Figure 2.6A). Thus, we focused on this protein. Wild-type cells arrested in G1 phase had significantly lower levels of Iqg1p and a significantly higher rate of Iqg1p turnover than cells arrested in M phase (Figure 2.6B; Figure 2.6C, top two panels), suggesting that the APC/C might target Iqg1p for degradation at the end of mitosis, as it does other proteins. Consistent with this hypothesis, we found that Iqg1p levels were significantly elevated in asynchronous populations of APC/C-mutant cells (Figure 2.6D) and that the rapid rate of Iqg1p degradation in G1 cells was eliminated in APC/C mutants (Figure 2.6C, bottom two panels). Moreover, in synchronous cultures, Iqg1p levels were found to increase before mitosis and drop precipitously after mitosis in wild-type cells (Figure 2.7A and B, left panels), and the post-

mitotic decrease was largely eliminated in an APC/C mutant (Figure 2.7A and B, right panels).

Finally, we also examined cells expressing Iqg1p-GFP from the chromosomal *IQG1* promoter. As reported previously (Epp and Chant, 1997; Shannon and Li, 1999), Iqg1p localized to a ring at the mother-bud neck in medium-budded and large-budded wild-type cells. However, the neck signal was rather weak (Figure 2.8A) and was observed in only ~50% of the medium-budded and large-budded cells. Moreover, the Iqg1p-GFP did not appear to persist through cell division, because signal was never observed in unbudded cells (Figure 2.8B, left panel). In contrast, in APC/C mutants, Iqg1p-GFP signal was observed at the neck in a higher percentage (~80%) of medium-budded and large-budded cells and was significantly brighter there (Figure 2.8A). Moreover, Iqg1p-GFP appeared to persist through cell division in at least some cells, because patches of signal were sometimes observed in unbudded cells (Figure 2.8B, right panel).

Taken together, the results strongly suggest that Iqg1p is degraded in a cell-cycle-dependent manner that depends, directly or indirectly, on the APC/C.

Ubiquitination of Iqg1p by the APC/C and Identification of a Novel APC/C-Recognition Motif

To ask if Iqg1p is a direct target of the APC/C, *in vitro* ubiquitination assays were performed using various fragments of Iqg1p and wild-type APC/C with its activator Cdh1p. Under conditions in which the well characterized APC/C substrate Pds1p was ubiquitinated effectively (Figure 2.9A, lanes 1 and 2), Iqg1p(1-750) and Iqg1p(33-250) were ubiquitinated in an APC/C-dependent manner (Figure 2.9A, lanes 3 and 4; Figure 2.9B, lanes 1 and 2), whereas Iqg1p(43-750), Iqg1p(400-1100), and Iqg1p(741-1495) were not (Figure 2.9A, lanes 5-10). These data indicated that Iqg1p is indeed a direct target of the APC/C and that a

recognition sequence between amino acids 1 and 42 is required for this ubiquitination. Although this region contains no clear DB or other known APC/C-recognition sequence (see above), amino acids 34-42 have some resemblance (RxxxxxxxN) to previously characterized DBs (RxxLxxxxN/D/E), and mutating residues within this sequence reduced (single substitutions: Figure 2.9B, lanes 3-10) or eliminated (multiple substitutions: Figure 2.9B, lanes 11-14) APC/C^{Cdh1}-dependent ubiquitination.

We also performed similar experiments using full-length Cyk3p. However, consistent with the lack of recognizable APC/C-recognition sequences in this protein, no ubiquitination was detected (data not shown).

To ask if the apparent APC/C^{Cdh1}-recognition sequence in Iqg1p is also important for its turnover *in vivo*, we compared the stabilities of full-length Iqg1p and Iqg1p(43-1495) in both the W303 and S288C strain backgrounds. As expected, the truncated protein was substantially more stable both during prolonged arrest in G1 (Figure 2.9C) and at the end of mitosis in a synchronized population (Figure 2.9D).

Suppression of* myo1Δ *Phenotypes by Nondegradable Iqg1p

The data presented above suggest that the elevated levels of Iqg1p resulting from APC/C mutations might explain the suppression of *myo1Δ* phenotypes by such mutations. In this case, synthesis at normal levels of a nondegradable Iqg1p should also be able to suppress *myo1Δ* phenotypes. In agreement with this prediction, we found that expression of Iqg1p(43-1495) from the chromosomal *IQG1* promoter could suppress both the *myo1Δ* lethality in the W303 background (Figure 2.10A) and the *myo1Δ* clustering phenotype in the S288C background (Figure 2.10B). Somewhat surprisingly, segregants expressing the nondegradable Iqg1p in an otherwise wild-type background showed little or no defect in

growth rate (Figure 2.10A). However, overexpression of the nondegradable Iqg1p from a *GAL* promoter did cause severe growth defects (data not shown).

DISCUSSION

Suppression of Actomyosin-ring Defects by APC/C Mutations

The actomyosin contractile ring is involved in cytokinesis in most if not all animal and fungal cells. Nonetheless, it has recently become clear that this structure is dispensable for cytokinesis in a variety of cell types (see Introduction), indicating that actomyosin-ring-independent mechanisms are critical, and in some cases sufficient, for cytokinesis. In an attempt to elucidate these mechanisms, we are performing genetic studies in *S. cerevisiae.* In this organism, the phenotypes associated with loss of the actomyosin ring (due to deletion of the myosin II gene *MYO1*) vary greatly in severity depending on the strain background, presumably due to differences among the strains in the efficiency of the actomyosin-ring-independent mechanisms, as discussed in more detail elsewhere (Nishihama *et al.,* 2007).

During our studies, we made the surprising observation that mutations in genes encoding nonessential subunits of the APC/C could suppress both the lethal phenotype of *myo1Δ* in the W303 strain background and the slow-growth and delayed-cell-separation phenotypes of *myo1Δ* in the S288C strain background. Initially it seemed likely that this suppression might result simply from changes in the timing of late-cell-cycle events; for example, a delay in mitotic exit due to APC/C malfunction might allow more time for an inefficient process of cytokinesis to be completed successfully. However, several lines of evidence (the suppression by *cdh1* and *mnd2* mutations; the lack of effect of a *pds1* mutation or of expression of a nondegradable Clb2p; see Results for details) suggested strongly that the suppression is not due to changes in the timing of either anaphase or mitotic exit, and hence that it is likely to involve some novel APC/C target(s) and/or pathway(s). Indeed, there is now good evidence that suppression occurs because the APC/C defects result in an

increased abundance of Iqg1p, a novel APC/C^{Cdh1} target that is important in actomyosin-ring-independent cytokinesis, as discussed further below.

This model provides a plausible explanation for why a *net1* mutation exacerbates the phenotype of *myo1Δ* in the S288C strain background. We initially interpreted this observation in terms of effects on the timing of late-cell-cycle events, reasoning that an acceleration of mitotic exit could provide insufficient time for an inefficient cytokinesis process to be completed. However, it now seems more likely that premature activation of Cdc14p resulting from the absence of Net1p leads to premature activation of Cdh1p, and thus targeting of Iqg1p for destruction by APC/C^{Cdh1} before its role in cytokinesis can be completed. Of course, the effects of *net1* mutations could also be more complex.

Identification of Iqg1p as an APC/C target

In a dosage-suppressor screen, we observed that even low-copy plasmids containing *IQG1* or *CYK3* could suppress the *myo1Δ* phenotypes in both the S288C and W303 genetic backgrounds. These observations suggested that Iqg1p, Cyk3p, or both might be previously unidentified targets of APC/C. Thus, mutations that compromised APC/C function might allow the intracellular concentrations of Iqg1p, Cyk3p, or both to rise to levels that could suppress the *myo1Δ* phenotypes [by mechanisms that we consider in more detail elsewhere (Nishihama *et al*. (2007)]. Although it remains possible that Cyk3p is an APC/C target, there is as yet no evidence to support this possibility: Cyk3p contains no recognizable APC/C recognition sequences, and we failed to detect ubiquitination *in vitro* by APC/C^{Cdh1}. In contrast, a variety of *in vivo* and *in vitro* experiments indicate that Iqg1p is a target of APC/C^{Cdh1}. First, in wild-type cells, both the levels of Iqg1p and the rates of Iqg1p degradation fluctuate during the cell cycle in a manner consistent with APC/C-triggered

degradation at the end of mitosis. Second, in APC/C (including *cdh1*) mutants, the rates of Iqg1p degradation are drastically reduced; correspondingly, Iqg1p is present at higher levels and persists abnormally through cell division. Finally, *in vitro* assays showed that Iqg1p can be ubiquitinated directly by APC/C^{Cdh1}. The observation that the *myo1Δ* phenotypes can be suppressed by expression of a stabilized Iqg1p (lacking the APC/C^{Cdh1} recognition site) from the chromosomal *IQG1* promoter provides strong support for the hypothesis that the suppression by APC/C mutations indeed results from the elevated and more persistent levels of Iqg1p in the mutant strains.

Interestingly, recognition of Iqg1p by APC/C^{Cdh1} does not appear to depend on any of the sequences that correspond to previously characterized APC/C-recognition motifs. Instead, the ubiquitination of Iqg1p depends on a novel sequence (33-LRPQSSSKIN-42) near the N-terminus that is similar but not identical to the consensus DB [RxxLxxxxN/D/E: Burton and Solomon, 2001] and KEN-box (KENxxxN/D: Pfleger and Kirschner, 2000) motifs. Further dissection of the amino acids important for recognition of this site by the APC/C should shed light on the mechanisms of APC/C-target interaction. In the meantime, the results highlight the dangers of attempting to identify APC/C targets solely on the basis of the known recognition motifs.

Another interesting conclusion from this study concerns the role of Mnd2p. Although this APC/C subunit appears to have little or no role in mitotic control (Oelschlaegel *et al.,* 2005; Page *et al*., 2005), we found that an *mnd2* mutation suppressed the *myo1Δ* phenotypes as strongly as did other APC/C mutations. This apparent discrepancy might be explained if there are specific roles of particular APC/C subunits in the interactions with different specific APC/C targets. However, it also seems possible that the explanation simply involves

differential strengths of the interactions of the APC/C with different targets. In particular, because the APC/C roles in anaphase promotion and mitotic exit are essential for cell survival, the interactions with the relevant targets might be stronger than the interactions with a target like Iqg1p whose APC/C-mediated degradation is not essential for cell survival. Thus, the loss of an accessory subunit like Mnd2p might have little effect on the strong interactions but a major effect on the weak interactions. An attraction of this model is that it may also explain why mutating any of the nonessential APC/C subunits has essentially the same effect on suppression of the *myo1Δ* phenotypes, even though each subunit appears to have a distinct role in APC/C function (Carroll and Morgan, 2002; Passmore *et al*., 2003; Schwickart *et al.,* 2004; Carroll *et al*., 2005; Page *et al*., 2005).

Significance of Timely APC/C-mediated Degradation of Iqg1p

An interesting and unresolved question is the role of the APC/C-dependent proteolysis of Iqg1p, a question that is highlighted by the observation that expression at endogenous levels of a nondegradable Iqg1p has little effect on cell growth. One possible answer involves the multiple roles of the myosin light chain Mlc1p. Recent studies have shown that *S. cerevisiae* cells are highly sensitive to the cellular levels of this protein, which is a light chain for Iqg1p, Myo1p, and the type V myosin Myo2p, and that the Mlc1p-Myo2p interaction is critical for septum formation, cell separation, and new bud formation (Luo *et al*., 2004). Thus, heterozygous *mlc1Δ* diploid cells display haploinsufficiency that can be suppressed by reduced the copy number of *MYO2* (Stevens and Davis, 1998), and overexpression of a Myo1p C-terminal tail (containing the Mlc1p-binding IQ motifs) leads to cytokinetic defects that can be alleviated by overexpression of Mlc1p (Tolliday *et al*., 2003). In addition, our own recent studies have shown that overexpression of Iqg1p from a high-copy plasmid with

its normal promoter in wild-type cells can cause multi-cellular clustering (Nishihama *et al*., 2007). Finally, high levels of overexpression of the nondegradable Iqg1p from a high-copy plasmid with the *GAL* promoter appear to cause severe cytokinetic defects. Taken together, these results suggest that the association of Mlc1p with each binding partner may need to be fine-tuned temporally and that the timely APC/C-mediated degradation of Iqg1p may be important to release Mlc1p particularly for interaction with Myo2p.

Possible Conserved Regulation of IQGAPs by the APC/C

Iqg1p is the only known member of the conserved IQGAP family in *S. cerevisiae* (Brown and Sacks, 2006), and because IQGAPs and the APC/C are both conserved, it is possible that other IQGAPs are also regulated by the APC/C. This hypothesis is particularly attractive for Rng2p in *S. pombe* and IQGAP1 in mammalian cells, because these proteins are also involved in cytokinesis (Eng *et al*., 1998; Wu *et al*., 2003; Skop *et al*., 2004). However, although each protein has a single DB consensus sequence, neither Rng2p nor IQGAP1 has a sequence corresponding to the novel APC/C-recognition motif identified in Iqg1p. Thus, the possible regulation of these proteins by the APC/C will require further investigation.

ACKNOWLEGEMENTS

We thank Erfei Bi for plasmid pBS-MYO1, Forrest Spencer and Phil Hieter for the YCp50-LEU2 genomic library, Steve Haase and his lab members for help with the FACS analysis, Erin O'Shea and Jonathan Weissman for strain IQG1-TAP from the TAP-tagged strain collection, Akanksha Gangar for the protein-extraction protocol, and John Cooper for the goat anti-actin antibody. We also thank Danny Lew, Pat Brennwald, Steve Haase, members of their laboratories, and members of the Pringle laboratory for helpful suggestions and support.

FIGURES (1 to 10)

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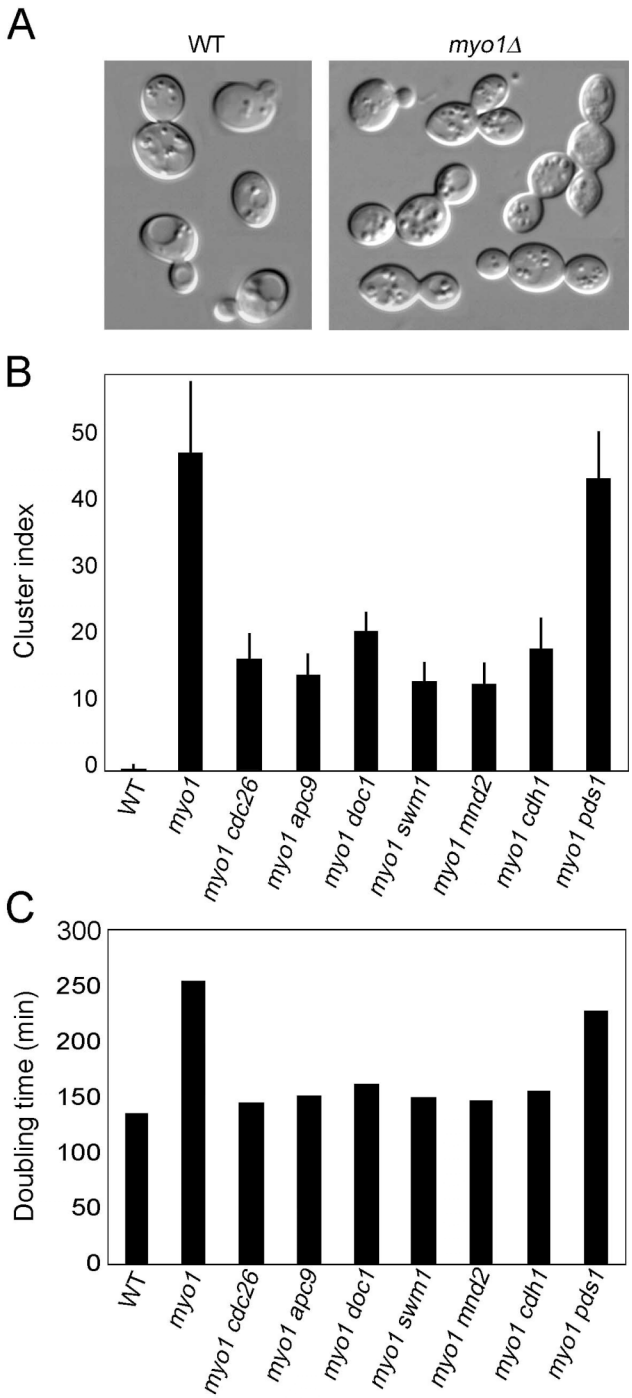

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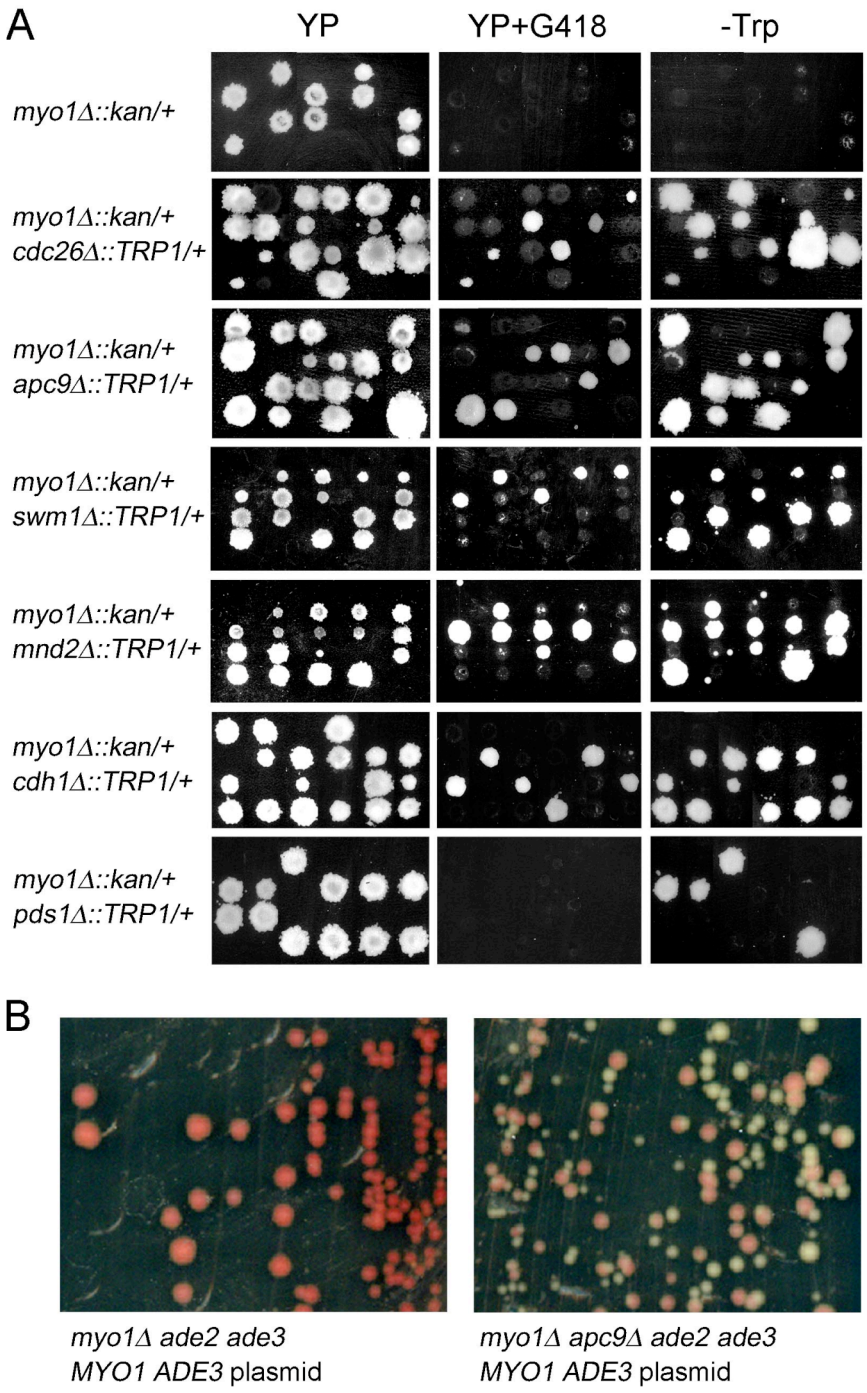

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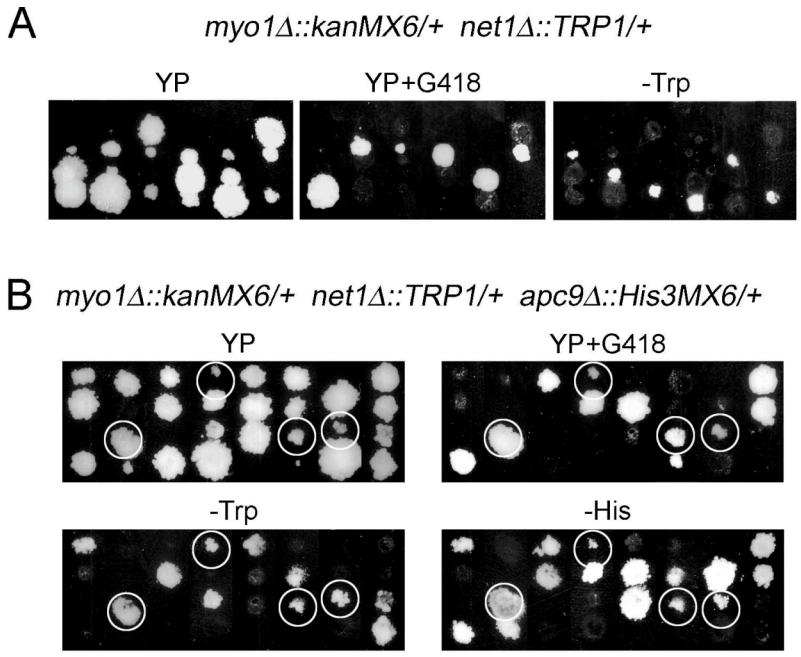

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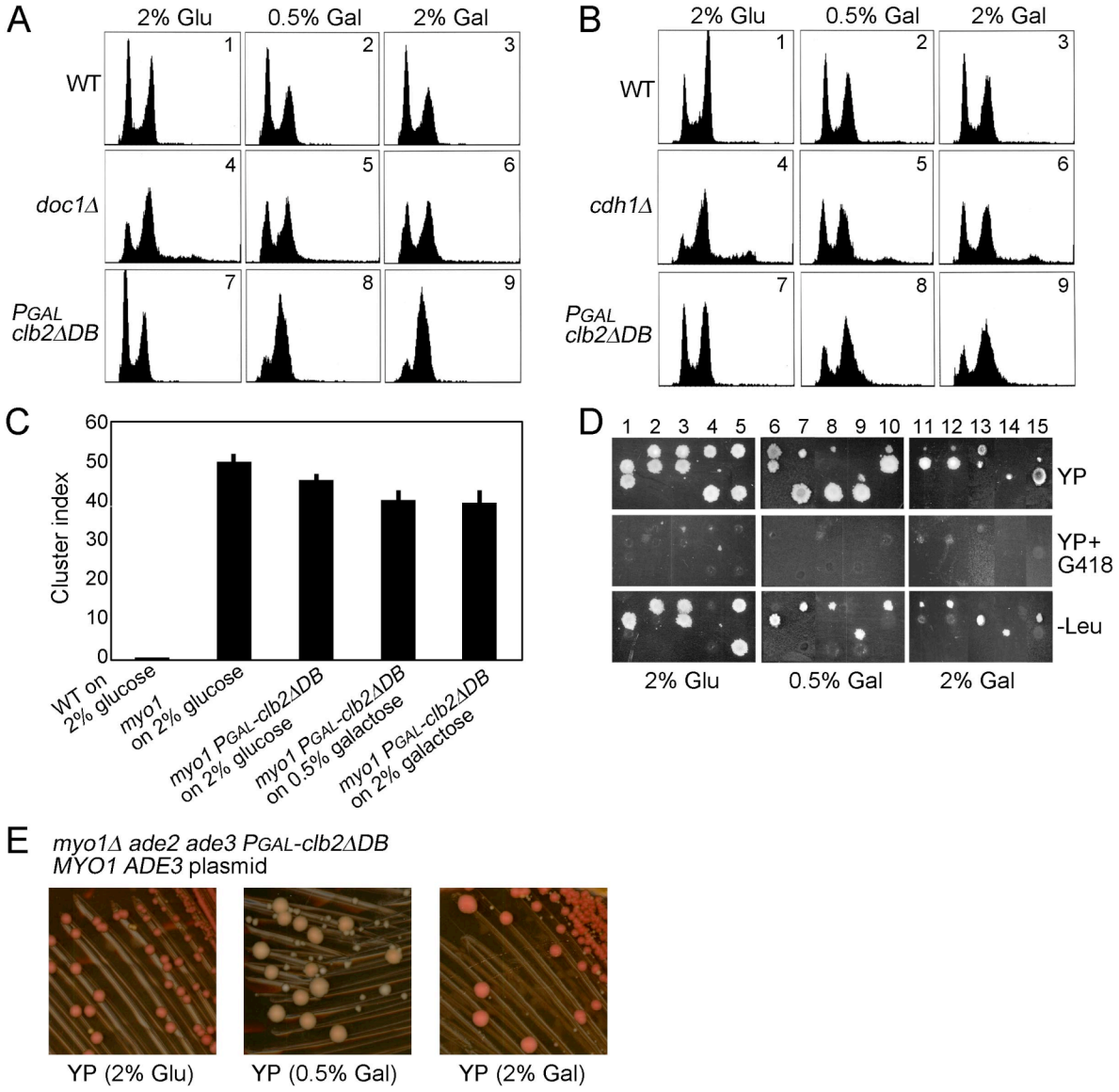

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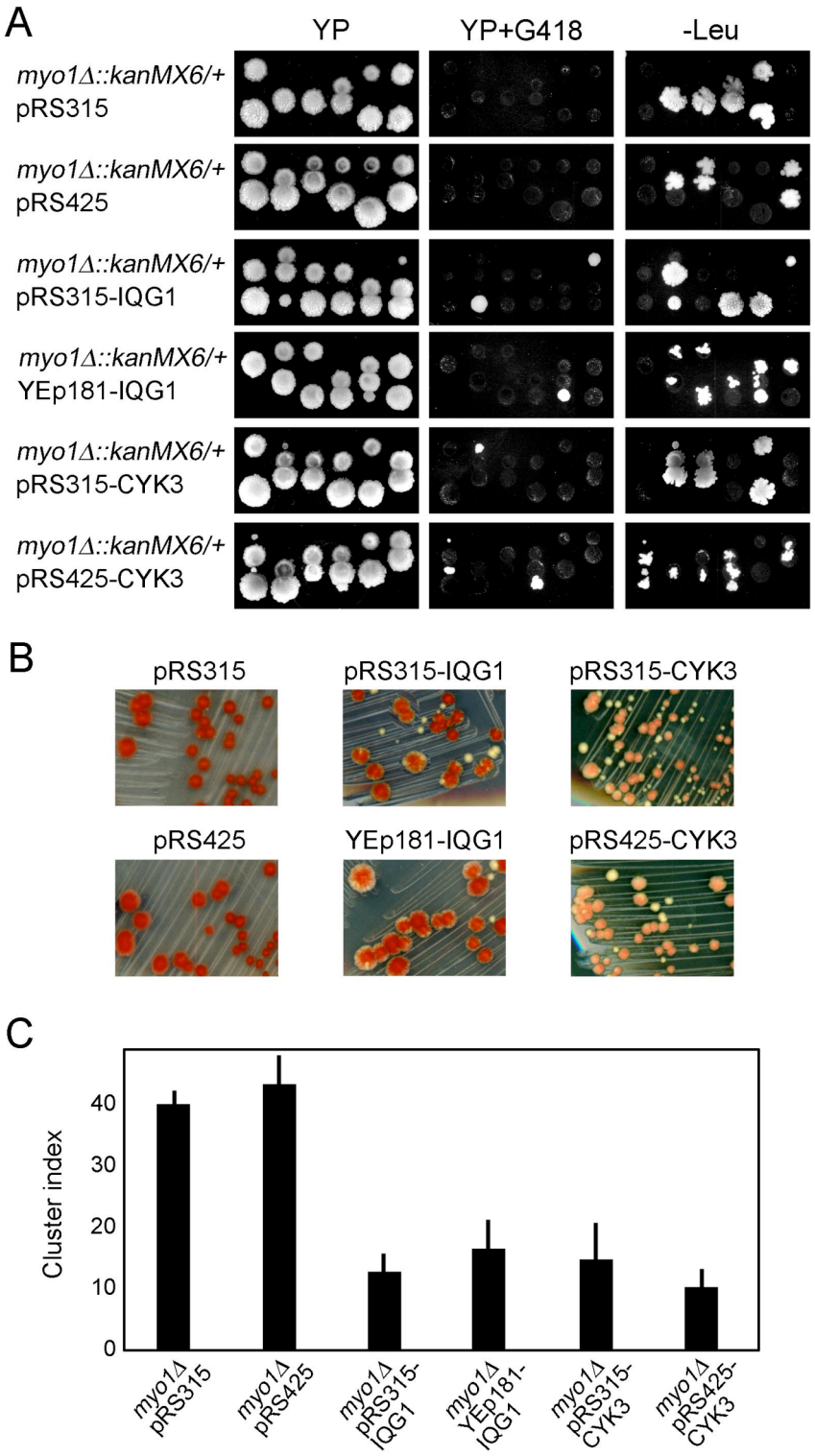

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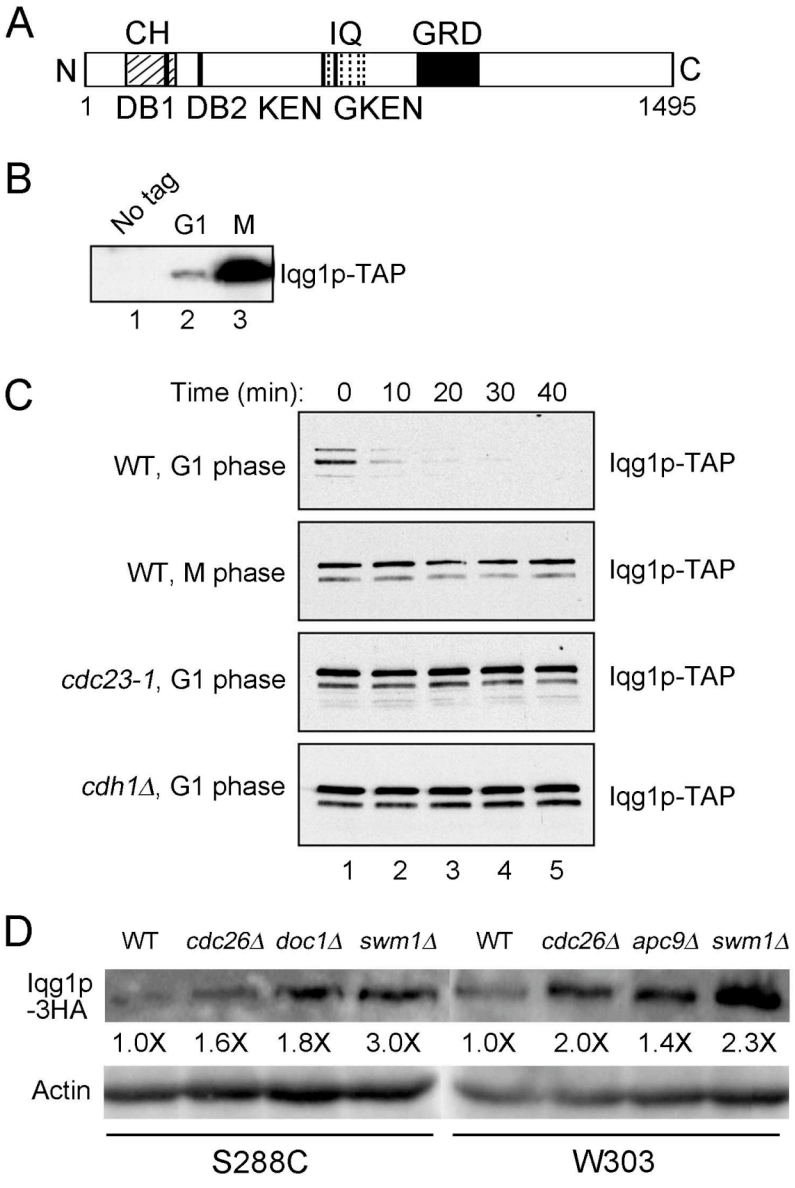

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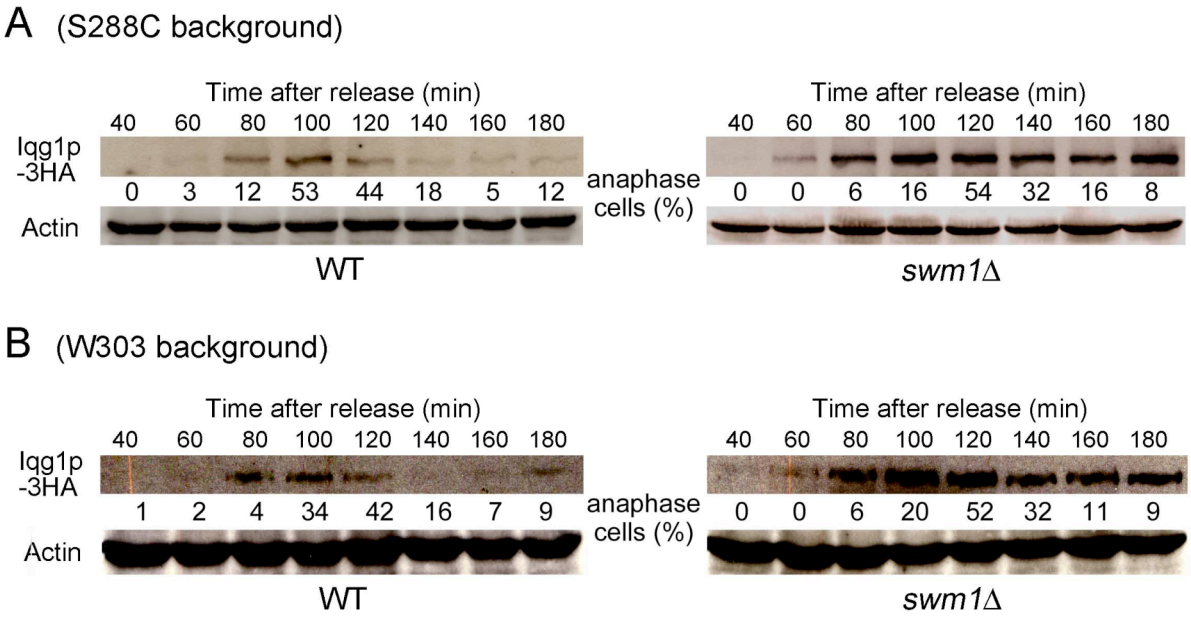

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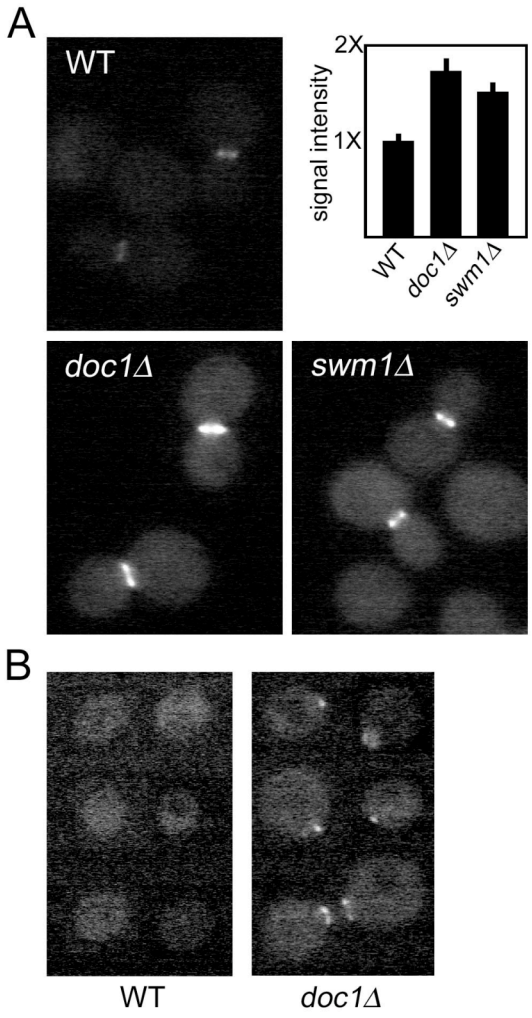

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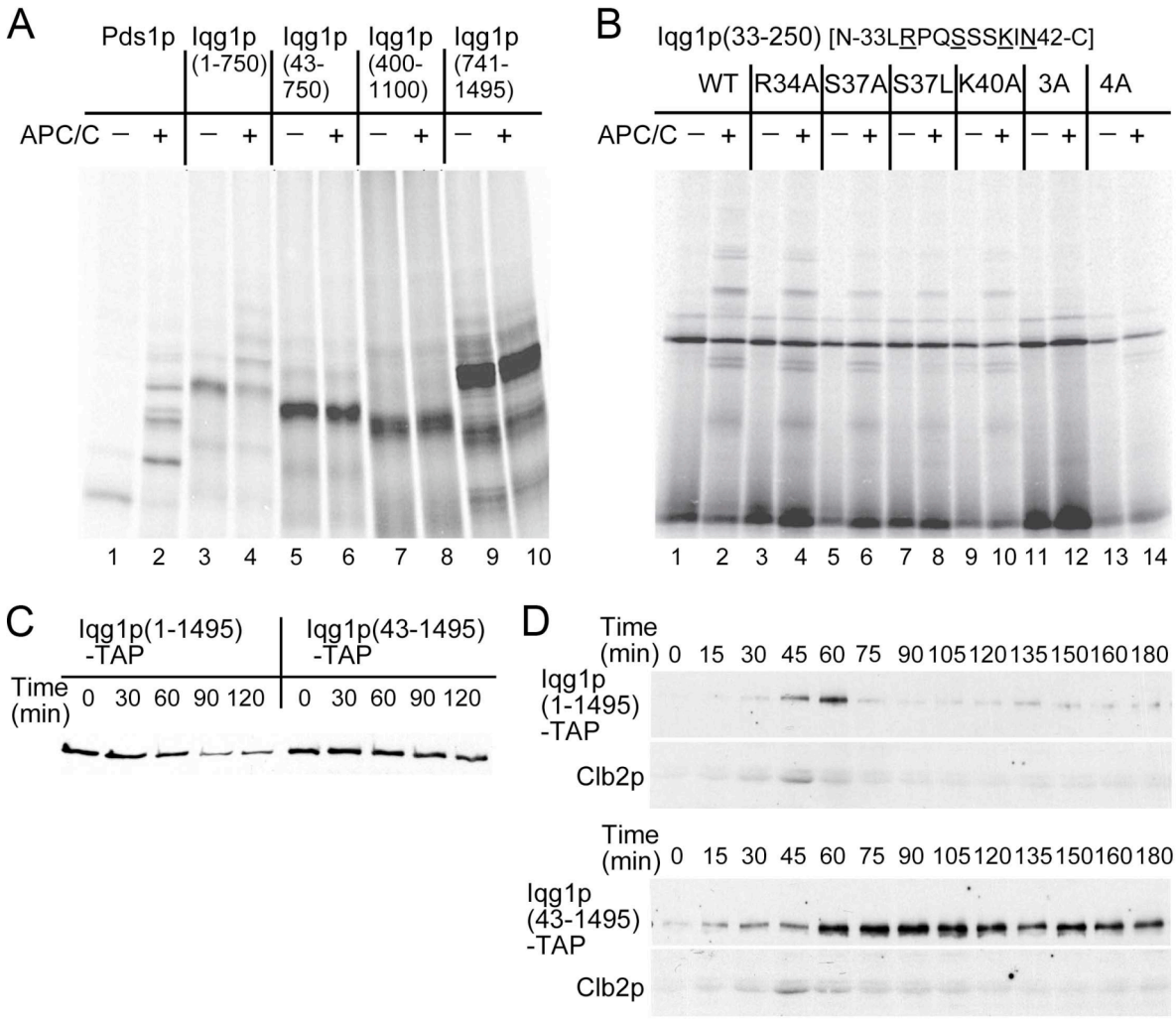

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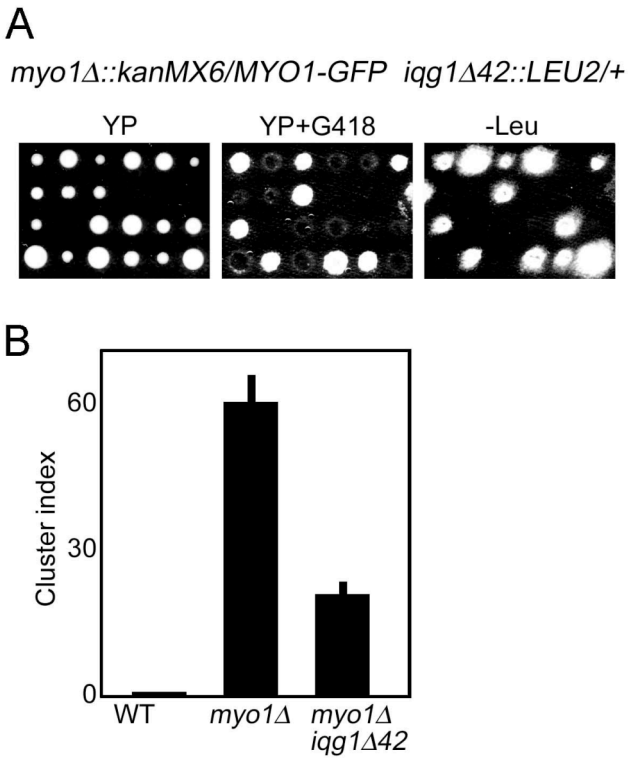

FIGURE LEGENDS (1 to 10)

Figure 2.1. Suppression of *myo1Δ* clustering and slow-growth phenotypes by APC/C mutations in the S288C strain background. (A) Presence of multi-cell clusters in a *myo1Δ* strain but not in a congenic wild type (WT). Strains YEF473A and KO608 were grown overnight on YP plates and observed by DIC microscopy as described in Materials and Methods. (B) Suppression of clustering phenotype. Strains were grown as in A, and clustering was scored as described in Materials and Methods. Strains were YEF473A, KO608 and related strains (for *myo1Δ*; see Materials and Methods), KO397, KO421, KO401, KO405, KO415, KO257, and KO139. Values shown are the means of three to nine counts except for *myo1Δ* (mean of 25 counts); standard deviations are also indicated. (C) Suppression of slow-growth phenotype. Strains were the same as in B. The doubling times shown are the averages of two measurements made as described in Materials and Methods.

Figure 2.2. Suppression of *myo1Δ* lethality by APC/C mutations in the W303 strain background. (A) Suppression of spore lethality. Tetrads were dissected from strains heterozygous for *myo1Δ::kanMX6* or for both *myo1Δ::kanMX6* and an APC/C or *pds1* mutation marked by *TRP1*. All viable *myo1Δ* segregants (scored by growth on YP+G418) also carried an APC/C mutation (scored by growth on SC-Trp). The strains analyzed were RNY509, KO213, KO216, KO346, KO351, KO354, and KO246. (B) Demonstration of suppression by a colony-sectoring assay. Strain RNY798 (*myo1Δ ade2 ade3*; *MYO1 ADE3* plasmid) and its *apc9Δ* derivative KO313 were streaked onto YP plates. Only strain KO313 could form viable subclones (sectors) that had lost the plasmid and thus were white due to the loss of Ade3p activity.

Figure 2.3. Synthetic lethality between *myo1Δ* and *net1Δ* in the S288C background and its suppression by an APC/C mutation. (A) Tetrads were dissected from a strain (KO199) heterozygous for the unlinked mutations *myo1Δ::kanMX6* and *net1Δ::TRP1*. Most tetrads, like the six shown here, yielded no viable double-mutant spores. In 18 additional tetrads, only two viable spores were recovered that carried both mutations and thus grew, albeit very slowly, on both YP+G418 and SC-Trp plates. (B) Tetrads were dissected from a strain (KO275) heterozygous for *myo1Δ::kanMX6*, *net1Δ::TRP1*, and *apc9Δ::His3MX6*. In 54 tetrads dissected, 24 viable segregants were recovered that carried both *myo1Δ* and *net1Δ* (see examples in circles); 20 of these also carried *apc9Δ* (as shown by growth on SC-His plates), and the other four grew very slowly.

Figure 2.4. Lack of *myo1Δ* suppression by expression of a nondegradable mitotic cyclin. All galactose media also contained 1% raffinose. (A and B) Delayed mitotic exit produced by APC/C mutations or by expression of a nondegradable cyclin. (A) S288C-background strains KO492, KO399, and KO494 were grown to exponential phase in YM-P medium containing glucose or galactose, as indicated, and FACS analysis was performed as described in Materials and Methods. (B) W303-background strains KO372, KO529, and KO369 were grown and analyzed as described for panel A. (C-E) Lack of suppression of *myo1Δ* phenotypes. (C) Clustering phenotype in the S288C background. Strains YEF473A, KO608, and KO570 were grown overnight on YP plates containing the indicated sugars, and

clustering was scored as described in Materials and Methods. Values shown are the means of three to five counts; standard deviations are also indicated. (D and E) Lethal phenotype in the W303 background. (D) Three sets of tetrads from strain KO336 [*myo1Δ::kanMX6/+* and heterozygous for P_{GAL}-*clb2ΔDB:LEU2* (see Table 2.1)] were dissected (top panel) on YP + 2% glucose (tetrads 1-5), YP + 0.5% galactose (tetrads 6-10), or YP + 2.0% galactose (tetrads 11-15). Tetrads were then replica-plated to YP plates containing the indicated sugars plus G418 (to identify any segregants carrying *myo1Δ*) and to SC-Leu plates containing the indicated sugars (to identify segregants carrying P_{GAL}-*clb2ΔDB*). No viable *myo1Δ* segregants were observed in 24 tetrads dissected on 0.5% galactose and 18 tetrads dissected on 2% galactose. (E) Strain KO360 was streaked onto YP plates containing the indicated sugars. The absence of white sectors (none were observed in >300 colonies observed) indicates an inability to form viable subclones that have lost the *MYO1 ADE3* plasmid even when Clb2pΔDB is expressed.

Figure 2.5. Suppression of *myo1Δ* phenotypes by overexpression of *IQG1* or *CYK3*. The genes were carried on *LEU2*-marked low-copy (pRS315-based) or high-copy (pRS425- or YEplac181-based) plasmids. (A and B) Suppression of *myo1Δ* lethality in the W303 background. (A) Suppression of spore inviability. Tetrads were dissected from strain RNY509 (*myo1Δ::kanMX6/+*) carrying either an empty vector (top two rows) or an *IQG1* or *CYK3* plasmid (bottom four rows). With the empty vectors, no viable *myo1Δ* segregants were observed in 43 tetrads dissected (or in 67 tetrads of strain RNY509 dissected in the absence of a plasmid). With the *IQG1* and *CYK3* plasmids, 34 viable *myo1Δ* segregants (scored by growth on YP+G418) were observed in 168 tetrads dissected; all such segregants also carried an *IQG1* or *CYK3* plasmid (scored by growth on SC-Leu). (B) Suppression demonstrated by a colony-sectoring assay. Strain RNY798 (*myo1Δ ade2 ade3*; *MYO1 ADE3* plasmid) was transformed with the indicated plasmids and streaked onto SC-Leu plates. Only transformants carrying an *IQG1* or *CYK3* plasmid could form viable subclones (sectors) that had lost the *MYO1 ADE3* plasmid and thus were white due to the loss of Ade3p activity. (C) Suppression of clustering phenotype in the S288C background. Strains RNY1006, RNY1007, RNY1001, RNY1002, RNY1003, and RNY1004 were grown overnight on SC-Leu plates, and clustering was scored as described in Materials and Methods. Values shown are the means of three counts; standard deviations are also indicated.

Figure 2.6. Increased Iqg1p levels in M-phase cells and in APC/C mutants. All strains expressed either Iqg1p-TAP or Iqg1p-3HA from the chromosomal *IQG1* promoter. (A) Domain structure of Iqg1p. CH, calponin-homology domain; DB1 (191RFELQDLYN199) and DB2 (276RSGLIKDFN284) (black lines), putative destruction-box sequences; KEN (600-602) (black line), putative KEN-box; GKEN (627-630) (black line), putative KEN-box or GxEN motif; IQ, IQ repeats; GRD, GAP-related domain. (B) Cell-cycle dependence of Iqg1p levels. Strains BY4741 (lane 1; untagged control) and IQG1-TAP (lanes 2 and 3) were grown to exponential phase in YP medium at 30˚C, and strain IQG1-TAP was then treated for 2 h either with 100 ng/ml α-factor or with 50 μg/ml benomyl to arrest cells in G1 or M phase. Proteins were then extracted, precipitated, and analyzed by SDS-PAGE and Western blotting as described in Materials and Methods. (C) Regulation of Iqg1p degradation by APC/C^{Cdh1}. Strains DOY138, DOY139, DOY140, and DOY141 were grown to exponential phase in YP medium. Strains DOY138 (top panel) and DOY139 (third panel) were then

treated for 2 h with 100 ng/ml α-factor to arrest cells in G1 phase; the last 30 min of this incubation was at 37˚C in order to inactivate the temperature-sensitive APC/C subunit Cdc23-1p in DOY139 while providing an appropriate control. Strain DOY140 (second panel) was arrested in M phase by shifting to YP medium containing 2% galactose + 2% raffinose for 2 h at 30˚C to induce expression of the nondegradable $Pds1p^{mdb}$. Strain DOY141 (bottom panel) was arrested in G1 phase by shifting to 37˚C for 2 h to inactivate Cdc28-13p. After these incubations, translation in all four strains was blocked by addition of 50 μg/ml cycloheximide. Samples were taken at the time of cycloheximide addition and at 10-min intervals thereafter, and proteins were extracted and analyzed as described in panel B. The blot in the top panel was exposed ~10 times longer than the others in order to allow visualization of the much lower amount (*cf.* panel B) of Iqg1p-TAP in the wild-type G1 cells at time 0. (D) Increased Iqg1p levels in APC/C-mutant strains. Strains KO474, KO470, KO460, and KO465 (left four lanes) are in the S288C background; strains KO490, KO486, KO477, and KO482 (right four lanes) are in the W303 background. Each strain was grown to exponential phase in SC-His medium, and proteins were extracted and analyzed by SDS-PAGE, Western blotting, and quantitation as described in Materials and Methods. Iqg1p-3HA levels are expressed relative to those in the appropriate wild type, using the actin band for normalization.

Figure 2.7. Cell-cycle regulation of Iqg1p levels and the effect of APC/C mutations on this regulation. All strains expressed Iqg1p-3HA from the chromosomal *IQG1* promoter. Strains were grown, synchronized in G1 by treating with α-factor, and sampled as described in Materials and Methods. Iqg1p-3HA levels were assessed as described in Figure 2.6D (but without quantitation in this case), and the percentages of anaphase cells were assessed by DAPI staining as described in Materials and Methods. (A) S288C-background strains KO672 and KO671. (B) W303-background strains KO589 and KO588.

Figure 2.8. Stronger (A) and more persistent (B) Iqg1p-GFP signal in APC/C mutants. Strains KO566 (wild type), KO563 (*doc1Δ*), and KO625 (*swm1Δ*) are in the S288C background and express Iqg1p-GFP from the chromosomal *IQG1* promoter. Cells were grown and observed as described in Materials and Methods. (A) For each strain, all medium- and large-budded cells with detectable Iqg1p-GFP signal at the mother-bud neck were imaged using identical exposure times (4 sec) and scaling factors. Representative images are shown together with a histogram of average signal intensities (determined as described in Materials and Methods) for 80-100 of the imaged cells of each strain; standard errors of the mean are also indicated. (B) Unbudded cells of strains KO566 and KO563 were imaged using identical exposure times (4 sec) and scaling factors. Representative fluorescence images are shown.

Figure 2.9. Ubiquitination of Iqg1p by APC/C^{Cdh1} and identification of a novel APC/C^{Cdh1}-recognition motif. (A and B) Ubiquitination of Iqg1p *in vitro* and its dependence on Iqg1p residues 33-42. Full-length Pds1p and various Iqg1p fragments (as indicated) were synthesized *in vitro* and incubated with E2-methyl-ubiquitin and purified APC/C^{Cdh1} as described in Materials and Methods. Proteins were then separated by SDS-PAGE and visualized using a PhosphorImager. The slower-migrating proteins seen in the presence of APC/C^{Cdh1} (lanes A2, A4, B2, B4, B6, B8, and B10) are Pds1p or Iqg1p ubiquitination

products. In B, the point mutations introduced into Iqg1p(33-250) are indicated (see Materials and Methods); 3A denotes the triple mutation R34A,S37A,N42A, and 4A denotes the quadruple mutation R34A,S37A,K40A,N42A. (C and D) Dependence of Iqg1p turnover *in vivo* on the N-terminal APC/C^{Cdh1}-recognition sequence. (C) Strains GT24 (pGAL-IQG1-TAP) and GT70 (pGAL-iqg1Δ42-TAP) were grown to exponential phase in YP medium containing 2% raffinose as sole carbon source and arrested in G1 phase by treatment with 1 μg/ml α-factor for 4 h. Galactose (2%) was then added to induce Iqg1p-TAP or Iqg1Δ42p-TAP for 1 h, after which glucose (2%) and cycloheximide (100 μg/ml) were added to block both transcription and translation of the *IQG1* constructs. Samples were taken at the time of glucose and cycloheximide addition and at intervals thereafter, and Iqg1p-TAP was extracted and analyzed by Western blotting as described in Materials and Methods. (D) Strains IQG1-TAP (*IQG1-TAP*) and GT124 (*iqg1Δ42-TAP*) were grown to exponential phase in YP medium at 30°C, arrested in G1 phase by treatment with 10 μg/ml α-factor for 4 h, and released from the arrest by washing once and resuspending in fresh YP medium. When 90% of the cells had budded, 10 μg/ml α-factor was added again to prevent the initiation of new cell cycles. Samples were taken at the time of release from α-factor arrest and at intervals thereafter, and proteins (including Clb2p as a control) were analyzed by Western blotting as described in Materials and Methods.

Figure 2.10. Suppression of *myo1Δ* phenotypes by expression of a nondegradable Iqg1p at endogenous levels. (A) Suppression of *myo1Δ* lethality in the W303 background. Tetrads were dissected from two strains (KO1228 and KO1229) that are heterozygous for both *myo1Δ::kanMX6* and *iqg1Δ42:LEU2*. From 44 tetrads dissected, 34 viable *myo1Δ* segregants were recovered, as shown by growth on YP+G418; each of these also carried *iqg1Δ42*, as shown by growth on SC-Leu. (B) Suppression of clustering phenotype in the S288C background. Strains YEF473A, KO1248, and KO1249 were grown overnight on YP plates and clustering was scored as described in Materials and Methods.

Table 2.1. Strain List of Chapter 2

Strain [a,b]	Genotype	Source
YEF473 [a]	***a/α*** *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/ lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52*	Bi and Pringle, 1996
YEF473A [a]	***a*** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Segregant from YEF473
YEF473B [a]	*α his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Segregant from YEF473
BY4741 [a,c]	***a*** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Brachmann *et al*., 1998
IQG1-TAP [a]	as BY4741 except *IQG1-TAP:His3MX6*	Ghaemmaghami *et al*., 2003
W1588-4C [b]	***a*** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	Zhao *et al*., 1998
JMY314.1 -4b [b]	*α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	T. Petes
AFS92 [b]	as W1588-4C except *bar1Δ*	Jaspersen *et al*., 1998
DOY138 [b]	as W1588-4C except *bar1Δ IQG1-TAP:His3MX6*	This study [d]
DOY139 [b]	as W1588-4C except *bar1Δ cdc23-1:URA3 IQG1-TAP:His3MX6*	This study [d]
DOY140 [b]	as W1588-4C except *bar1Δ IQG1-TAP:His3MX6 leu2-3,112:P_{GAL}-pds1-mdb:LEU2*	This study [d]
DOY141 [b]	as W1588-4C except *cdc28-13:TRP1 cdh1Δ::LEU2 IQG1-TAP:His3MX6*	This study [d]
GT24 [b]	as W1588-4C except *bar1Δ* [pGAL-IQG1-TAP]	This study [e]
GT70 [b]	as W1588-4C except *bar1Δ* [pGAL-iqg1Δ42-TAP]	This study [e]
GT112 [b]	as W1588-4C except *bar1Δ MYO1-GFP:His3MX6 iqg1Δ42:LEU2*	This study [f]
GT124 [a]	as BY4741 except *iqg1Δ42-TAP:His3MX6:LEU2*	This study [g]
RNY112 [a]	as YEF473 except *myo1Δ::kanMX6/+*	Nishihama *et al*., 2007
RNY501 [b]	***a/α*** *ade2-1/ade2-1 can1-100/can1-100 his3-11,15/ his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1*	W1588-4C X JMY314.1-4b
RNY509 [b]	as RNY501 except *myo1Δ::kanMX6/+*	Nishihama *et al*., 2007
RNY798 [b]	as W1588-4C except *myo1Δ::kanMX6 ade3Δ::His3MX6* [pTSV31A-MYO1]	This study [h]
RNY1001 [a]	as YEF473B except *myo1Δ::kanMX6* [pRS315-IQG1]	This study [i]

RNY1002 [a]	as YEF473B except *myo1Δ::kanMX6* [YEp181-IQG1]	This study [i]
RNY1003 [a]	as YEF473B except *myo1Δ::kanMX6* [pRS315-CYK3]	This study [i]
RNY1004 [a]	as YEF473B except *myo1Δ::kanMX6* [pRS425-CYK3]	This study [i]
RNY1006 [a]	as YEF473B except *myo1Δ::kanMX6* [pRS315]	This study [i]
RNY1007 [a]	as YEF473B except *myo1Δ::kanMX6* [pRS425]	This study [i]
KO139 [a]	as YEF473A except *myo1Δ::kanMX6 pds1Δ::TRP1*	This study [j]
KO199 [a]	as YEF473 except *myo1Δ::kanMX6/+ net1Δ::TRP1/+*	This study [j]
KO213 [b]	as RNY501 except *myo1Δ::kanMX6/+ cdc26Δ::TRP1/+*	This study [j]
KO216 [b]	as RNY501 except *myo1Δ::kanMX6/+ apc9Δ::TRP1/+*	This study [j]
KO246 [b]	as RNY501 except *myo1Δ::kanMX6/+ pds1Δ::TRP1/+*	This study [j]
KO257 [a]	as YEF473A except *myo1Δ::kanMX6 cdh1Δ::TRP1*	This study [j]
KO275 [a]	as YEF473 except *myo1Δ::kanMX6/+ net1Δ::TRP1/+ apc9Δ::His3MX6/+*	This study [k]
KO296 [b]	as W1588-4C	Segregant from RNY509
KO313 [b]	as W1588-4C except *myo1Δ::kanMX6 ade3Δ::His3MX6 apc9Δ::TRP1* [pTSV31A-MYO1]	This study [l]
KO336 [b]	as RNY501 except *myo1Δ::kanMX6/+ P_{GAL}-clb2ΔDB:LEU2*	This study [m]
KO342 [b]	as RNY501 except *P_{GAL}-clb2ΔDB:LEU2*	This study [m]
KO346 [b]	as RNY501 except *myo1Δ::kanMX6/+ swm1Δ::TRP1/+*	This study [j]
KO351 [b]	as RNY501 except *myo1Δ::kanMX6/+ mnd2Δ::TRP1/+*	This study [j]
KO354 [b]	as RNY501 except *myo1Δ::kanMX6/+ cdh1Δ::TRP1/+*	This study [j]
KO360 [b]	as W1588-4C except *myo1Δ::kanMX6 ade3Δ::His3MX6 P_{GAL}-clb2ΔDB:LEU2* [pTSV31A-MYO1]	This study [m]
KO369 [b]	as W1588-4C except *P_{GAL}-clb2ΔDB:LEU2*	Segregant from KO342
KO372 [b]	as W1588-4C	Segregant from KO342
KO388 [a]	as YEF473 except *P_{GAL}-clb2ΔDB:LEU2*	This study [m]
KO397 [a]	as YEF473A except *myo1Δ::kanMX6 cdc26Δ::TRP1*	This study [j]
KO399 [a]	as YEF473A except *doc1Δ::TRP1*	This study [j]
KO401 [a]	as YEF473A except *myo1Δ::kanMX6 doc1Δ::TRP1*	This study [j]
KO405 [a]	as YEF473A except *myo1Δ::kanMX6 swm1Δ::TRP1*	This study [j]
KO415 [a]	as YEF473A except *myo1Δ::kanMX6 mnd2Δ::TRP1*	This study [j]
KO421 [a]	as YEF473A except *myo1Δ::kanMX6 apc9Δ::TRP1*	This study [j]
KO460 [a]	as YEF473B except *IQG1-3HA:His3MX6 doc1Δ::TRP1*	This study [n]
KO463 [a]	as YEF473A except *IQG1-3HA:His3MX6 swm1Δ::TRP1*	This study [n]
KO465 [a]	as YEF473B except *IQG1-3HA:His3MX6 swm1Δ::TRP1*	This study [n]
KO470 [a]	as YEF473B except *IQG1-3HA:His3MX6 cdc26Δ::TRP1*	This study [n]
KO471 [a]	as YEF473A except *IQG1-3HA:His3MX6*	This study [n]
KO474 [a]	as YEF473B except *IQG1-3HA:His3MX6*	This study [n]
KO477 [b]	as JMY314.1-4b except *IQG1-3HA:His3MX6 apc9Δ::TRP1*	This study [n]

KO481 [b]	as W1588-4C except *IQG1-3HA:His3MX6 swm1Δ::TRP1*	This study [n]
KO482 [b]	as JMY314.1-4b except *IQG1-3HA:His3MX6 swm1Δ::TRP1*	This study [n]
KO486 [b]	as JMY314.1-4b except *IQG1-3HA:His3MX6 cdc26Δ::TRP1*	This study [n]
KO487 [b]	as W1588-4C except *IQG1-3HA:His3MX6*	This study [n]
KO490 [b]	as JMY314.1-4b except *IQG1-3HA:His3MX6*	This study [n]
KO492 [a]	as YEF473A	Segregant from KO388
KO494 [a]	as YEF473A except P_{GAL}*-clb2ΔDB:LEU2*	Segregrant from KO388
KO529 [b]	as W1588-4C except *cdh1Δ::TRP1*	Segregant from KO354
KO561 [a]	as YEF473 except *myo1Δ::kanMX6/+* P_{GAL}*-clb2ΔDB:LEU2*	This study [m]
KO563 [a]	as YEF473A except *IQG1-GFP:His3MX6 doc1Δ::TRP1*	This study [o]
KO566 [a]	as YEF473A except *IQG1-GFP:His3MX6*	This study [o]
KO570 [a]	as YEF473A except *myo1Δ::kanMX6* P_{GAL}*-clb2ΔDB:LEU2*	Segregant from KO561
KO588 [b]	as W1588-4C except *IQG1-3HA:His3MX6 swm1Δ::TRP1 bar1Δ::kanMX6*	This study [p]
KO589 [b]	as W1588-4C except *IQG1-3HA:His3MX6 bar1Δ::kanMX6*	This study [p]
KO608 [a]	as YEF473A except *myo1Δ::kanMX6*	Segregant from RNY112
KO625 [a]	as YEF473A except *IQG1-GFP:His3MX6 swm1Δ::TRP1*	This study [o]
KO671 [a]	as YEF473A except *IQG1-3HA:His3MX6 swm1Δ::TRP1 bar1Δ::kanMX6*	This study [p]
KO672 [a]	as YEF473A except *IQG1-3HA:His3MX6 bar1Δ::kanMX6*	This study [p]
KO1226 [a]	***a***/α *his3-Δ200/his3Δ1 leu2-Δ1/leu2Δ0 lys2-801/+ met15Δ0/+ trp1-Δ63/+ ura3-52/ura3Δ0 myo1Δ::kanMX6/+ iqg1Δ42-TAP:His3MX6:LEU2/+*	This study [q]
KO1228 [b]	as RNY501 except *bar1Δ/+ iqg1Δ42:LEU2/+ myo1Δ::kanMX6/MYO1-GFP:His3MX6*	This study [q]
KO1229 [b]	as RNY501 except *bar1Δ/+ iqg1Δ42:LEU2/+ myo1Δ::kanMX6/MYO1-GFP:His3MX6*	This study [q]
KO1248 [a]	as YEF473A except *myo1Δ::kanMX6*	Segregant from KO1226
KO1249 [a]	as YEF473A except *myo1Δ::kanMX6 iqg1Δ42-TAP:His3MX6:LEU2*	Segregant from KO1226

[a] Denotes strains of the S288C genetic background (Mortimer and Johnston, 1986).

[b] Denotes strains of the W303 genetic background (Thomas and Rothstein, 1989). Note that although the original W303 contained a *rad5* mutation, all of the W303-family strains used here are *RAD5*$^+$ like W1588-4C (Zhao *et al.*, 1998).

[c] American Type Culture Collection (Manassas, VA) strain ATCC201388 or ATCC4040002.

[d] Strains YJB14 and YJB115 (Burton and Solomon, 2000), OCF1517.2 (Cohen-Fix *et al.*, 1996), and YJB368 (Burton and Solomon, 2001) were transformed with an *IQG1-TAP:His3MX6* C-terminal-tagging cassette (see Materials and Methods).

[e] Strain AFS92 was transformed with the indicated plasmids (Table 2.2).

[f] Strain AFS92 was transformed with a *MYO1-GFP:His3MX6* C-terminal-tagging cassette and then with pGT04 (Table 2.2) after cutting with *Nhe*I to target integration to the *IQG1* coding sequence. The site of integration was confirmed by PCR.

[g] Strain IQG1-TAP was transformed with pGT04 after cutting with *Nhe*I, and the site of integration was confirmed by PCR.

[h] Strain RNY509 was transformed with pTSV31A-MYO1 (Table 2.2). A *myo1Δ::kanMX6* segregant carrying the plasmid was isolated by tetrad dissection and transformed with an *ade3Δ::His3MX6* deletion cassette.

[i] Strain RNY112 was transformed with pRS316-MYO1. A *myo1Δ::kanMX6* segregant carrying the plasmid was then isolated by tetrad dissection and transformed with the indicated plasmids (Table 2.2); pRS316-MYO1 was then eliminated by growth on 5-FOA.

[j] Strains RNY112 and RNY509 were transformed with various *TRP1*-marked deletion cassettes (see Materials and Methods). Single-mutant and double-mutant segregants from the RNY112 derivatives were isolated by tetrad dissection.

[k] Strain KO199 was transformed with an *apc9Δ::His3MX6* deletion cassette.

[l] Strain RNY798 was transformed with an *apc9Δ::TRP1* deletion cassette

[m] Strains YEF473, RNY112, RNY501, RNY509, and RNY798 were transformed with plasmid YIp-*GAL-clb2ΔDB* (Table 2.2) after cutting with *Kpn*I to target integration to the *LEU2* locus. Although the actual sites of integration were not checked, each diploid

transformant was heterozygous for a single copy of the P_{GAL}-*clb2ΔDB:LEU2* allele, as shown by subsequent tetrad analysis.

[n] Strains YEF473A and KO296 were transformed with an *IQG1-3HA:His3MX6* C-terminal-tagging cassette (see Materials and Methods). The resulting strains were crossed to various APC/C mutant strains, and segregants were isolated by tetrad dissection.

[o] Strain YEF473A was transformed with an *IQG1-GFP:His3MX6* C-terminal-tagging cassette (see Materials and Methods). The resulting strain was crossed to *doc1Δ* and *swm1Δ* strains (from the same tetrads that yielded KO401 and KO405), and single-mutant and double-mutant segregants were isolated by tetrad dissection.

[p] Strains KO463, KO471, KO481, and KO487 were transformed with a *bar1Δ::kanMX6* deletion cassette (see Materials and Methods).

[q] Strains RNY112 and RNY509 were transformed with plasmids pRS316-MYO1 and pTSV31A-MYO1, respectively. A *myo1Δ::kanMX6* segregant carrying the plasmid was then isolated from each strain by tetrad dissection and mated to strain GT124 or GT112, as appropriate to maintain the S288C or W303 genetic background. pRS316-MYO1 or pTSV31A-MYO1 was then eliminated by growth on 5-FOA.

Table 2.2. Plasmid List of Chapter 2

Plasmid	Description	Source
pRS315	*CEN6 ARS4 LEU2* (low copy)	Sikorski and Hieter, 1989
pRS425	2μ, *LEU2* (high copy)	Christianson *et al*., 1992
YEp181-IQG1	2μ, *LEU2 IQG1* (high copy)	Nishihama *et al*., 2007
pRS315-IQG1	*IQG1* in pRS315	Nishihama *et al*., 2007
pRS315-CYK3	*CYK3* in pRS315	Nishihama *et al*., 2007
pRS425-CYK3	*CYK3* in pRS425	Nishihama *et al*., 2007
pRS316-MYO1	*CEN6 ARS4 URA3 MYO1*	Nishihama *et al*., 2007
pTSV31A-MYO1	2μ, *URA3 ADE3 MYO1*	See text
YIp-*GAL-clb2ΔDB*	*LEU2* P_{GAL}*-clb2ΔDB* (integrating)	S. Reed
pGT04	*LEU2 iqg1Δ42* (integrating)	See text
pGAL-IQG1-TAP	2μ, *URA3* P_{GAL}*-IQG1-TAP*	See text
pGAL-iqg1Δ42-TAP	2μ, *URA3* P_{GAL}*-iqg1Δ42-TAP*	See text

Table 2.3. Primer List of Chapter 2

Primer	Purpose	Primer sequence
5'TTEF	Internal check primer for the *His3MX6* or *kanMX6* marker	5'-TATTTTTTTTCGCCTCGACATCATC TGCCC-3'
3'PTEF	Internal check primer for the *His3MX6* or *kanMX6* marker	5'-GTATGGGCTAAATGTACGGGCGAC AGTCAC-3'
5'TRP1	Internal check primer for the *TRP1* marker	5'-TAAAAGACTCTAACAAAATAGCAA ATTTCG-3'
3'TRP1	Internal check primer for the *TRP1* marker	5'-GTGCTTAATCACGTATACTCACGTG CTCAA-3'
APC9dF	5' primer to construct *apc9Δ*	5'-CAAATTCAGGAAGGAAAGAAATA GCTGTGACAAAGTAAGCGGATCCCCG GGTTAATTA-3'
APC9dR	3' primer to construct *apc9Δ*	5'-GATATATATACACGTGAAGCAAAA AAGGCATTGCCTCTGGAATTCGAGCT CGTTTAAAC-3'
APC9dK	Upstream check primer for *apc9Δ*	5'-TAAACCGTGGCATTCAACTAGCAC AAGACT-3'
APC9dC	Downstream check primer for *apc9Δ*	5'-GATTTCGAGTATTATGTCCGATCC TAAGTA-3'
CDC26dF	5' primer to construct *cdc26Δ*	5'-GAAGAGAAAAAAAAAACAGCAAC AATAACATCGCACTTGCGGATCCCCG GGTTAATTA-3'
CDC26dR	3' primer to construct *cdc26Δ*	5'-TGCGCGTGTGCGTGTGCGTGTGCG TATGCGGATATGCTAGAATTCGAGCT CGTTTAAAC-3'
CDC26dK	Upstream check primer for *cdc26Δ*	5'-CCAACAAACATTTGAACATACCTG ATTTGAGG-3'
CDC26dC	Downstream check primer for *cdc26Δ*	5'-GCGTGAATGATGCTGTCCAGACGC TACTGCAA-3'
CDH1dF	5' primer to construct *cdh1Δ*	5'-GTCACCCTTCCTTCTAGTCTTCATC CTAAATTTAGTTGCCGGATCCCCGGG TTAATTA-3'
CDH1dR	3' primer to construct	5'-GAATTTTTGAGATGATATTACTAC

	cdh1Δ	TATGAAAACCCTTTAGAATTCGAGCT CGTTTAAAC-3'
CDH1dK	Upstream check primer for *cdh1Δ*	5'-TTTTGGTTTTCCCTACCCAGTTATT TACCC-3'
CDH1dC	Downstream check primer for *cdh1Δ*	5'-TCATTTAGGTTTTTGTCAACATTGA TGTAA-3'
DOC1dF	5' primer to construct *doc1Δ*	5'-CGTGGAGATATCAGATGTAAAGTA GTTAGAGTAGAAAAACGGATCCCCG GGTTAATTA-3'
DOC1dR	3' primer to construct *doc1Δ*	5'-GGTAATAGCAAGGAAGAAAATAT CATAAACGGCATATTAGAATTCGAG CTCGTTTAAAC-3'
DOC1dK	Upstream check primer for *doc1Δ*	5'-AACGGTGGATTTTTTGTCTAGTTTC CAAAT-3'
DOC1dC	Downstream check primer for *doc1Δ*	5'-ATGTGCCACTGTAAGGACACCTTT ATTCGT-3'
MND2dF	5' primer to construct *mnd2Δ*	5'-ACCGACAACCATAAACAGCGGCG AAGAGAAGACAAAGCACGGATCCCC GGGTTAATTA-3'
MND2dR	3' primer to construct *mnd2Δ*	5'-AATAGATAGATGAATTTTGGTGCT GAGGAGAGCATTTTTGAATTCGAGCT CGTTTAAAC-3'
MND2dK	Upstream check primer for *mnd2Δ*	5'-TTTTCTAAATCTTTTAGAACATTCA AATCA-3'
MND2dC	Downstream check primer for *mnd2Δ*	5'-CAAGTTAGATCCATCGGGCAGCGA ATTGGT-3'
NET1dF	5' primer to construct *net1Δ*	5'-ACAGAGAGAAGTCGATTACTGTAC ACCGTGCCTCCTTCTCGGATCCCCGG GTTAATTA-3'
NET1dR	3' primer to construct *net1Δ*	5'-AGCTTTCTGTGACGTGTATTCTACT GAGACTTTCTGGTAGAATTCGAGCTC GTTTAAAC-3'
NETdK	Upstream check primer for *net1Δ*	5'-CCTCTCTTCAAGTTTCCTATACCCG AGCTT-3'
NETdC	Downstream check primer for *net1Δ*	5'-ATTGCTTTGATTCTGGCAATCCAA ATGGTT-3'
PDS1dF	5' primer to construct *pds1Δ*	5'-CGGTACCAAGCTAGATTAAGTGCT AGATAATAAACCTTTCGGATCCCCGG GTTAATTA-3'

PDS1dR	3' primer to construct *pds1Δ*	5'-CGTGTATATATGTTGTGTGTATGT GAATGAGCAGTGGATGAATTCGAGCT CGTTTAAAC-3'
PDS1dK	Upstream check primer for *pds1Δ*	5'-GGTGTGAAGGACGGAGTCTTCAAT GGTGAA-3'
PDS1dC	Downstream check primer for *pds1Δ*	5'-TATTGGGGTATTTACTGAATGAGA AAAGGA-3'
SWM1dF	5' primer to construct sw*m1Δ*	5'-TCAGAGAAGTGGGGTGAGCAAAG TATAACAACCACGATTCGGATCCCCG GGTTAATTA-3'
SWM1dR	3' primer to construct sw*m1Δ*	5'-AATTTCTGACTAATGATCAGCATA TACGTCACGTTCTGCGAATTCGAGCT CGTTTAAAC-3'
SWM1dK	Upstream check primer for *swm1Δ*	5'-TTACCGTGACCGTGTGAGACAATT CATTGA-3'
SWM1dC	Downstream check primer for sw*m1Δ*	5'-CAAGAAATACTTAATTAGCGTTTC AAATGC-3'
IQG1tF	5' primer to construct *IQG1-3HA* or *IQG1-GFP*	5'-CATTTGATTGTCAGTTTTTTCTATA AAAGGAACGCTTTGCGGATCCCCGG GTTAATTA-3'
IQG1-R1	3' primer to construct *IQG1-3HA* or *IQG1-GFP*	5'-TAACAGCTTTTGCCCAATATGCTC AAAACCGAGTTATCTAGAATTCGAG CTCGTTTAAAC-3'
IQG1tK	Upstream check primer for C-terminal tags of *IQG1*	5'-AGAGGAGTGCTTAAAACCATCAG GGGTGAA-3'
IQG1-6000R	Downstream check primer for C-terminal tags of *IQG1*	5'-CCAAAAATGCTGCCCATTCCTAGT-3'
MSO1855	5' primer to amplify *IQG1-TAP:His3MX6*	5'-GTTGAACCAAAAATCGAGACGTG-3'
MSO1856	3' primer to amplify *IQG1-TAP:His3MX6*	5'-TATCGCCAAATTACAGCACATGC-3'
pGT04-BamHI	5' primer for fragment (-262 to +3) of pGT04	5'-GAGACGGATCCCGCTTTATATTGA GCTACGCATCGC-3'
pGT04-linker3	3' primer for fragment (-262 to +3) of pGT04	5'-TATGTTTAACGAAGACATTGTTTTT GCACCACCTTGTTACATATAATTG-3'
pGT04-linker5	5' primer for fragment (+127 to +277) of pGT04	5'-GGTGGTGCAAAAACAATGTCTTCG TTAAACATAGCGTCGCC-3'

pGT04-XbaI	3' primer for fragment (+127 to +277) of pGT04	5'-GTCTCTCTAGACTACGGTATATTTTCCTACGAGTTTGTTAG-3'
Gal-5'-IQG1	5' primer to construct pGAL-IQG1-TAP	5'-GGAATTCCAGCTGACCACCATGACAGCATATTCAGGCTCTCCTTCGAAACCAGGCAATAA-3'
Gal-5'-iqg142	5' primer to construct pGAL-iqg1Δ42-TAP	5'-GGAATTCCAGCTGACCACCATGTCTTCGTTAAACATAGCGTCGCCAAGTCATTTAAAGAC-3'
Gal-3'-IQG1	3' primer to construct pGAL-IQG1-TAP or pGAL-iqg1Δ42-TAP	5'-CGATCCCCGGGAATTGCCATGCAAAGCGTTCCTTTTATAGAAAAAACTGACAATCAAATG-3'
IVT5'-T7-Kozak	5' primer to add T7 promoter sequences and a Kozak site to each fragment for ubiquitination assay	5'-GGGGGCCCCCGGGGGCCCCCTAATACGACTCACTATAGGGAATTGTGAGCGGATAACAATTCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATATCGCCGCCGCCATGGGG-3'
IVT5'-1aa	5' primer to make fragment that starts at amino acid 1 for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGATGACAGCATATTCAGGCTCTCCTTCGAAACCAGGC-3'
IVT3'-750aa	3' primer to make fragments that end at amino acid 750 for ubiquitination assay	5'-TTAAGCCCAAAGACTTGGATTTGGACAGTC-3'
IVT5'-43aa	5' primer to make fragment that starts at amino acid 43 for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGATGTCTTCGTTAAACATAGCGTCGCCAAGTC-3'
IVT5'-400aa	5' primer to make fragment that starts at amino acid 400 for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGATGTCTCATTATTCTCCCATGAGAAGAGAAAGGATGA-3'
IVT3'-1100aa	3' primer to make fragment that ends at amino acid 1100 for ubiquitination assay	5'-TTACACATTATAAAGGACATCTTTGATATGAGGC-3'
IVT5'-741aa	5' primer to make fragment that starts at amino acid 741 for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGATGCTAGACTGTCCAAATCCAAGTCTTTGGGCTGTCA-3'
IVT3'-1495aa	3' primer to make fragment that ends at amino acid 1495 for ubiquitination assay [a]	5'-GCAAGAGCCCTCAGCTTCTC-3'
IVT3'-250aa	3' primer to make fragments that end at amino acid 250 for ubiquitination assay	5'-TTAAGGCCATGCTTTTTTACAAGCTGCG-3'
IVT5'-33aa	5' primer to make fragment	5'-GATATATCGCCGCCGCCATGGGGA

	that starts at amino acid 33 (no mutation) for ubiquitination assay	TGCTGAGACCGCAGTCAAGTTCCAAA ATC-3'
IVT5'-33aa -R34A	5' primer to make fragment that starts at amino acid 33 (mutation R34A) for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGA TGCTGGCACCGCAGTCAAGTTCCAAA ATC-3'
IVT5'-33aa -S37A	5' primer to make fragment that starts at amino acid 33 (mutation S37A) for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGA TGCTGAGACCGCAGGCAAGTTCCAAA ATC-3'
IVT5'-33aa -S37L	5' primer to make fragment that starts at amino acid 33 (mutation S37L) for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGA TGCTGAGACCGCAGCTAAGTTCCAAA ATC-3'
IVT5'-33aa -K40A	5' primer to make fragment that starts at amino acid 33 (mutation K40A) for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGA TGCTGAGACCGCAGTCAAGTTCCGCA ATC-3'
IVT5'-33aa -3A	5' primer to make fragment that starts at amino acid 33 (triple mutation) for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGA TGCTGGCACCGCAGGCAAGTTCCAAA ATCGCCTCTT-3'
IVT5'-33aa -4A	5' primer to make fragment that starts at amino acid 33 (quadruple mutation) for ubiquitination assay	5'-GATATATCGCCGCCGCCATGGGGA TGCTGGCACCGCAGGCAAGTTCCGCA ATCGCCTCTT -3'

[a] No stop codon is needed in the primer because the natural *IQG1* stop codon is included in the amplified fragment.

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CHAPTER 3

IDENTIFICATION OF ADDITIONAL PROTEINS INVOLVED IN SEPTIN-DEPENDENT, ACTOMYOSIN-RING-INDEPENDENT CYTOKINESIS IN *SACCHAROMYCES CEREVISIAE*

ABSTRACT

In the budding yeast *Saccharomyces cerevisiae*, a ring of myosin II (Myo1p) forms in a septin-dependent manner at the presumptive budding site in late G1. At the onset of cytokinesis, actin is recruited to the Myo1p ring to form an actomyosin ring, which soon contracts, and a septum of cell wall (including a chitinous primary septum synthesized by Chs2p) forms concurrently to complete cytokinesis. Three other proteins involved in cytokinesis are Iqg1p, Cyk3p, and Hof1p. Iqg1p is the only *S. cerevisiae* IQGAP and is required for actomyosin-ring formation, primary-septum formation, and hence normal cytokinesis. Because the actomyosin ring itself is not essential for primary-septum formation, Iqg1p must have at least one other cytokinetic function(s), which may involve stimulating Cyk3p and Hof1p to function in a septin-dependent and actomyosin-ring-independent pathway. To identify additional proteins involved in this pathway, we have conducted screens for dosage suppressors of the *myo1Δ cyk3Δ* and *myo1Δ hof1Δ* synthetic growth defects. We have identified several genes including *EGT2* (which encodes a cell-wall endoglucanase that may be involved in cell separation), *ECM33* (which encodes a GPI-anchored protein that may regulate cell-wall organization), and a truncated *CDC24* (which encodes the GEF for the Rho-type GTPase Cdc42p) that lacks the C-terminal PB1 domain.

Overexpression of Ecm33p or the truncated Cdc24p can also suppress the *iqg1Δ* and *chs2Δ* growth defects and restore the formation of primary septum in *iqg1Δ* cells. Further studies showed that deletion of *ECM33* causes minor cytokinetic defects at 37˚C, and that the suppression by the truncated Cdc42p is likely to be Cdc42p-independent and may involve targeting Rho1p, Rho2p, Rho5p, and/or an unknown factor(s).

INTRODUCTION

Cytokinesis is a late stage of cell division whereby the cell surface and cytoplasm of one cell is divided into two cells. Although the molecular understanding of cytokinesis has improved recently, only some of the participating proteins have been identified so far. Among them are the septins, which are GTP-binding proteins that have been found at the division site in all fungal and animal cells examined (Longtine *et al*., 1996; Hall and Russell, 2004; Gladfelter, 2006). Surprisingly, although the septins are required for cytokinesis in some cell types, they are not in others (Longtine *et al*., 1996; Adam *et al*., 2000; Nguyen *et al*., 2000; Kinoshita and Noda, 2001; An *et al*., 2004). Functionally, the septins have been proposed as both a diffusion barrier and a scaffold for the organization and localization of other components (Gladfelter *et al*., 2001; Longtine and Bi, 2003; Dobbelaere and Barral, 2004; Versele and Thorner, 2005; Spiliotis and Nelson, 2006). One such septin-dependent component in the budding yeast *Saccharomyces cerevisiae* is the contractile actomyosin ring. Whereas the septins are essential for cytokinesis in this organism, the actomyosin ring is not in most strain backgrounds (Bi *et al*., 1998; Schmidt *et al*., 2002; Nishihama *et al*., 2007). Thus, the septins must have other roles in cytokinesis that are independent of the actomyosin ring. One current objective is to assess these roles and identify the involved proteins in yeast and perhaps in other cell types where the actomyosin ring may be dispensable (Uyeda *et al*., 2000; Nagasaki *et al*., 2002; Kanada *et al*., 2005).

In *S. cerevisiae*, the initial recruitment of the septins to the presumptive budding site has been indicated to depend on the activation of Rho-type GTPase Cdc42p by its guanine-nucleotide-exchange factor (GEF) Cdc24p (Gladfelter *et al*., 2001; Iwase *et al*., 2006), which appears to require its Dbl-homology (DH) domain for the GEF activity (Shimada *et al*.,

2004) and an interaction with Bem1p (Chenevert *et al*., 1992) through its Phox-Bem1-homology (PB1) domain (Butty *et al*., 2002; Shimada *et al*., 2004). Cdc42p and other Rho-type GTPases (Rho1p-Rho5p) have been shown to be involved in pathways regulating cell polarization and integrity (Pringle *et al*., 1995; Drgonova *et al*., 1996; Qadota *et al*., 1996; Madden and Snyder, 1998; Pruyne and Bretscher, 2000; Guo *et al*., 2001; Schmitz *et al.*, 2002; Fernandes *et al*., 2006). After the initial recruitment, the septins appear to assemble into a ring structure (Iwase *et al*., 2006), and Myo1p (the only type II myosin in *S. cerevisiae*) then forms a ring within the septin ring in late G1 (Bi *et al*., 1998; Lippincott and Li, 1998). This ring remains at the mother-bud neck until the onset of cytokinesis, when actin and other proteins are recruited to it to form the mature actomyosin ring, which soon contracts. Around the same time, the plasma membrane invaginates and the primary septum (composed of chitin) is synthesized, principally by the chitin synthase Chs2p (Cabib *et al*., 2001; Schmidt *et al*., 2002). Secondary septa (composed mainly of other cell-wall materials, such glucans and mannoproteins) are then deposited on both sides of the primary septum to form the mature trilaminar septum. Thus, for a normal septum to be formed, mechanisms that are required for normal cell-wall synthesis, such as the glycosylation and anchoring of mannoproteins, may need to be fully functional (Schmidt *et al*., 2005). A GPI-anchored protein Ecm33p and its homolog Pst1p have recently been shown to participate in such mechanisms (Pardo *et al*., 2004). Finally, to complete cell separation, chitin in the primary septum is hydrolyzed by a chitinase (Kuranda and Robbins, 1991; Colman-Lerner *et al*., 2001) and some glucans are removed by glucanases, such as Egt2p (Kovacech *et al*., 1996).

In addition to the actomyosin ring, some of the other septin-dependent components of cytokinesis in *S. cerevisiae* include Iqg1p, Cyk3p, and Hof1p. Iqg1p is the only known

IQGAP (Brown and Sacks, 2006) in *S. cerevisiae* and has been shown to be indispensable for actomyosin-ring formation, primary-septum formation, and normal cytokinesis (Epp and Chant, 1997; Shannon and Li, 1999; Luo *et al*., 2004; Nishihama *et al*., 2007; see Chapter 2). Because the actomyosin ring itself is not essential for cytokinesis, Iqg1p must have at least one other cytokinetic function(s) that is independent of the actomyosin ring. To date, the exact function of Cyk3p is unclear, but its overexpression suppresses the *iqg1Δ* growth defect without restoring the actomyosin ring, and *myo1Δ cyk3Δ* double mutant is synthetically sick, suggesting that Cyk3p also promotes cytokinesis through an actomyosin-ring-independent pathway (Korinek *et al*., 2000; Nishihama *et al*., 2007). Although the exact cellular function of Hof1p remains as a mystery, this protein has been shown to be a member of the Pombe-Cdc15-Homology (PCH) family, which includes *S. pombe* Cdc15p and mammalian proteins PSTPIP1 and PSTPIP2 (Chitu and Stanley, 2007). PCH proteins have been suggested to function at the membrane-cytoskeleton interface by coordinating changes in actin cytoskeleton and membrane morphology. Thus, Hof1p may function in cytokinesis as an adapter linking the primary-septum-synthesis machinery to the actomyosin ring (Vallen *et al*., 2000). Because the *myo1Δ hof1Δ* double mutant is synthetically inviable, Hof1p may also participate in an actomyosin-ring-independent pathway of cytokinesis.

To investigate the mechanisms and to identify additional components of septin-dependent, actomyosin-ring-independent cytokinesis in *S. cerevisiae*, we have been conducting dosage-suppressor screens starting with *myo1Δ cyk3Δ* and *myo1Δ hof1Δ* double mutants. From these studies, we have identified several known and novel proteins, including Ecm33p and a truncated Cdc24p without its PB1 domain. Our analysis suggests that both Ecm33p and the truncated Cdc24p can promote the formation of primary-septum-like

structures in the absence of the actomyosin ring. We also present evidence that this novel function of the truncated Cdc24p may be GEF-dependent but Cdc42p-independent and involve targeting Rho1p, Rho2p, Rho5p, and/or an unknown factor(s).

MATERIALS AND METHODS

Strains, Plasmids, Growth Conditions, and Genetic Methods

The strains and plasmids used in this study are listed in Tables 3.1 and 3.2; their construction is described below or in the tables. Except as noted, yeast were grown at 23˚C on liquid or solid synthetic complete (SC) medium lacking specific nutrients as needed to select transformants or to maintain plasmids, YP rich solid medium, or YM-P rich, buffered liquid medium (Lillie and Pringle, 1980; Guthrie and Fink, 1991). 2% glucose was used as carbon source except for experiments involving induction of gene expression under *GAL* promoter control (for which 1% raffinose plus 2% galactose was used) and recovery of viable *iqg1Δ* or *chs2Δ* spore colonies on rich medium without dosage-suppressor plasmids (for which 2% glycerol was used). The antibiotic geneticin (G418; Cambrex, Walkersville, MD) was used to select for cells containing the kan^R marker, and 5-fluoroorotic acid (5-FOA; Research Products International, Mt. Prospect, IL) was used to select for *ura3*-mutant cells. Standard procedures were used for growth of *Escherichia coli*, genetic manipulations, polymerase chain reaction (PCR), and other molecular biological procedures (Sambrook *et al*., 1989; Guthrie and Fink, 1991; Ausubel *et al*., 1995).

Strain and Plasmid Constructions

Genes were deleted using the PCR method (Baudin *et al*., 1993; Longtine *et al*., 1998) and the primers indicated in Table 3.4; in each case, the entire coding region was deleted. The success of each deletion was confirmed by two PCR tests that used check primers that were upstream and downstream of the deleted region together with primers internal to the selectable markers (Longtine *et al*., 1998; Table 3.4).

Plasmids YEp13-ECM33, YEp13-EGT2, YEp13-cdc24-PB1Δ, YEp13-RHO1,

YEp13-RGD2, YEp13-RPL30, YEp13-NAB6, YEp13-MNN9, and YEp13-YJL055W were isolated in two dosage-suppressor screens using a *myo1Δ cyk3Δ* or *myo1Δ hof1Δ* double-mutant strain and a genomic-DNA library (DeMarini *et al*., 1997) constructed by cloning *Sau*3A fragments into the *Bam*HI site of plasmid YEp13 (high-copy, *LEU2*; Broach *et al*., 1979). Plasmid pRS315-CDC24 (kindly provided by E. Bi, University of Pennsylvania, Philadelphia, PA) was constructed in two steps. First, an *Xba*I-*Hpa*I-cut fragment, containing *CDC24*, from plasmid YEp352-CDC24 (Bi *et al*., 2000) was cloned into *Xba*I-*Sma*I-cut plasmid YIplac211 (Gietz and Sugino, 1988). In the second step, an *Xba*I-*Sac*I-cut fragment, containing nucleotides from -1961 relative to the start site of *CDC24* to +313 relative to its stop codon, from the resulting plasmid of the first step, was cloned into *Xba*I-*Sma*I-cut plasmid pRS315 (Sikorski and Hieter, 1989). Plasmid YEp13-CDC24 was constructed in two steps. First, a fragment containing nucleotides from +2296 to +2859 relative to the start site of *CDC24* plus +1 to +40 relative to the *Sph*I of YEp13 was PCR-amplified using pRS315-CDC24 as template and primers CDC24P3 and CDC24P4 (Table 3.4). In the second step, the PCR product from the first step was purified and transformed together with *Sph*I-cut YEp13-cdc24-PB1Δ into wild-type cells, and transformants containing plasmids resulting from homologous recombination were selected on SC-Leu plates. Plasmid YEp13-cdc24-DH8 was constructed in two steps. First, a fragment containing nucleotides from +764 to +1659 relative to the start site of *CDC24* and two mutations (N452G and E453G) within the DH domain was PCR-amplified using plasmid pRS414-3xmycCdc24-DH8 (kindly provided by R. Arkowitz, Universite De Nice, France) as template and primers CDC24P7 and CDC24P8 (Table 3.4). In the second step, the PCR product from the first step was purified and transformed together with *Bbv*CI-cut YEp13-

cdc24-PB1Δ into wild-type cells, and transformants containing plasmids resulting from homologous recombination were selected on SC-Leu plates. Plasmid pGAL-6xHIS-CDC42 was constructed in two steps. First, a fragment containing *CDC42* was PCR-amplified using plasmid pQE-6xHIS-CDC42 (see below) as template and primers pQErev and SalI-HIS (Table 3.4). In the second step, the PCR product from the first step was purified, cut with *Sal*I and *Xba*I, and cloned into the corresponding sites of plasmid YCpIF2 (Foreman and Davis, 1994). pQE-6xHIS-CDC42 was constructed in three steps. First, a fragment containing *CDC42* was PCR-amplified using plasmid pRS314-CDC42 (Zhang *et al*., 2001) as template and primers T3 and BamHI-CDC42 (Table 3.4). Second, the PCR product from the first step was purified, cut with *Bam*HI and *Sal*I, and cloned into the corresponding sites of plasmid YCplac33 (Gietz and Sugino, 1988). Third, a *Bam*HI-*Sal*I-cut fragment from the resulting plasmid of the second step was then cloned into the corresponding sites of plasmid pQE-30 (QIAGEN Sciences, Valencia, CA). Plasmids pGAL-RHO1, pGAL-RHO2, pGAL-RHO3, pGAL-RHO4, and pGAL-RHO5 were all constructed by similar methods. First, a fragment containing the *RHO1*, *RHO2*, *RHO3*, *RHO4*, or *RHO5* gene was PCR-amplified using either a high-copy plasmid containing the gene (kindly provided by D. Lew, Duke University, Durham, NC) or genomic DNA from a *His3MX6:*P_{GAL}*-RHO5* strain as template and primers set GAL-RHO1 plus 3'IF-RHO1, GAL-RHO2 plus 3'IF-RHO2, GAL-RHO3 plus 3'IF-RHO3, GAL-RHO4 plus 3'IF-RHO4, or GAL1-seq plus 3'IF-RHO5 (Table 3.4). Second, the PCR product from the first step was purified and transformed together with *Sal*I-cut YCpIF2 into wild-type cells, and transformants containing plasmids resulting from homologous recombination were selected on SC-Leu plates.

Growth Rates, Microscopy, and WGA Labeling

To determine growth rates, exponential-phase cultures ($OD_{600} \approx 0.4$) in the indicated medium were diluted two-fold with fresh medium, and incubation was continued at 23˚C or 37˚C, as indicated. The times needed to return to the original OD_{600} were recorded as the doubling times.

DIC microscopy was performed using a Nikon (Tokyo, Japan) Eclipse E600-FN microscope and an ORCA-2 cooled CCD camera (Hamamatsu Photonic Systems, Bridgewater, NJ). Electron microscopy (EM) was performed using a JEOL (Tokyo, Japan) JEM-1230 microscope and a Gatan Peltier-cooled Bioscan (Gatan Inc., Pleasanton, CA). Cells were grown to exponential phase in either YM-P (for wild-type), SC-His (for *iqg1Δ* and *chs2Δ* mutants), or SC-Leu (to select for plasmids) medium (50 ml). Cells were collected on a 0.2 μm filter, resuspended in 10 ml of primary fixative buffer [3% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in 0.1 M sodium cacodylate, pH6.8 (Electron Microscopy Sciences, Hatfield, PA)], and incubated at 23˚C for 15 min. Cells were recollected by centrifugation, resuspended again in 10 ml of primary fixative buffer, incubated at 23˚C for 45 min, and then at 4˚C overnight. After washing three times with water by centrifugation, cells were further fixed with 1 ml of 4% potassium permanganate (Electron Microscopy Sciences) at 4˚C for 2 h with shaking, followed by five washes with water. Fixed cells were stained with 1 ml of 2% uranyl acetate solution (Electron Microscopy Sciences) at 23˚C for 1 hr, followed by two washes with water. At 23˚C, cells were then dehydrated through a graded series (50%, 70%, 85%, 90%, 95%, and 100%) of ethanol for 5 min each and infiltrated with increasing concentrations (1:1 dilution of LR White:100% ethanol, overnight; 3:1 dilution, 2 h) of LR White (Sigma-Aldrich), followed by

two suspensions in pure LR White (3 h for each). Cell suspension was transferred to gelatin capsules, which were then cured in incubator at 55˚C for 3 days to polymerize the resin. Thin sections for EM were cut with a Leica Ultracut S (Leica Microsystems, Bannockburn, IL). Sections were placed on EM grids and stained with aqueous uranyl acetate and lead citrate (Sigma-Aldrich) for 5 min each before observation.

For WGA labeling, sections on grids were pre-incubated in PBS for 5 min, incubated in 1:50 to 1:5 dilutions of WGA-gold (15 nm; EY Laboratories, Inc., San Mateo, CA) in PBS for 15 min, jet-rinsed with water, and stained with aqueous uranyl acetate and lead citrate for 5 min each before observation.

RESULTS

Dosage Suppressors of the myo1Δ cyk3Δ *and* myo1Δ hof1Δ *Synthetic Growth Defects*

To identify additional proteins involved in cytokinesis in *S. cerevisiae*, particularly those that may participate in a septin-dependent and actomyosin-ring-independent pathway, we have used a high-copy, *LEU2*-marked genomic library to screen for dosage suppressors of the 5-FOA-induced synthetic growth defect of a *myo1Δ cyk3Δ* [*URA3 MYO1* plasmid] or *myo1Δ hof1Δ* [*URA3 MYO1* plasmid] strain (Vallen *et al*., 2000; Nishihama *et al*., 2007; see Materials and Methods). To date, 25,000 colonies have been screened in each of the two screens. 17 (*myo1Δ cyk3Δ*) and 11 (*myo1Δ hof1Δ*) plasmids that could suppress the growth defects have been isolated, five and two of the 17 plasmids contained *CYK3* and *MYO1*, and four and one of the 11 contained *HOF1* and *MYO1*, indicating that the screens could successfully identify bona fide suppressors. The remaining plasmids were sequenced and subcloned (data not shown) to determine the responsible suppressor genes. The identified dosage suppressors from each screen are shown in Figure 3.1. A plasmid containing *ECM33* was pulled out twice and four times in the *myo1Δ cyk3Δ* and *myo1Δ hof1Δ* screens, a plasmid containing *RPL30* was pulled out twice, and all other plasmids were pulled out once each. Besides Ecm33p or Egt2p, overexpressing Nab6p or Yjl055Wp could also suppress the 5-FOA-induced growth defects of both strains (data not shown). The strong suppression by Yjl055Wp was shown to be independent of cytokinesis and has been indicated to directly suppress the inviability of *URA3*$^{+}$ cells in the presence of 5-FOA (see Chapter 4).

Suppression of the iqg1Δ *and* chs2Δ *Growth Defects*

Because Myo1p, Cyk3p, and Hof1p might all function downstream of Iqg1p, and Chs2p is required for the synthesis of a normal septum, it seemed possible that the isolated dosage

suppressors could also suppress the *iqg1Δ* and/or *chs2Δ* growth defects. Indeed, overexpression of Cdc24-PB1Δp (without the C-terminal PB1 domain) or Ecm33p could suppress the *iqg1Δ* and *chs2Δ* spore inviability on YPD (Figure 3.2A; Table 3.3) and the inability of vegetative *iqg1Δ* and *chs2Δ* cells to lose an *IQG1* or *CHS2* plasmid (Figure 3.2B). Overexpressing Egt2p could do the same, except it could not suppress the *chs2Δ* spore inviability (Figure 3.2, A and B; Table 3.3). Finally, all the other isolated suppressors could not suppress the *iqg1Δ* spore inviability (Table 3.3), but were not tested for the *chs2Δ* growth defects.

In addition to the suppressions described above, we wanted to test if overexpression of Cdc24-PB1Δp, Ecm33p, or Egt2p could also suppress the slow-growth phenotype (Epp and Chant, 1997; Schmidt *et al*., 2002; Nishihama *et al*., 2007) of *iqg1Δ* and *chs2Δ* cells. To explore this possibility, we first needed to isolate viable *iqg1Δ* and *chs2Δ* strains without using any dosage suppressors. Previous studies (Bulawa and Osmond, 1990) have indicated that *chs2Δ* spores are sometimes viable, but grow very poorly, on YP+glycerol plate. We wanted to confirm this result and test whether the same would be true for *iqg1Δ* spores. Indeed, viable but very slow-growing *iqg1Δ* and *chs2Δ* spore colonies could be recovered on YP+glycerol (Figure 3.3A). Similar spore colonies carrying empty vector YEp13 (Table 3.2) were also isolated using this method (data not shown). Finally, compared to these very slow-growing mutant cells, *iqg1Δ* and *chs2Δ* cells overexpressing Cdc24-PB1Δp, Ecm33p, or Egt2p grew significantly faster (Figure 3.3B).

Primary-Septum-Like Structures Induced by Dosage Suppressors

To understand how exactly could the overexpression of Cdc24-PB1Δp, Ecm33p, or Egt2p suppress the *iqg1Δ* and *chs2Δ* growth defects, we examined the septum structures of the

suppressed cells with electron microscopy (EM). As reported previously (Schmidt *et al.*, 2002), wild-type cells formed trilaminar septa (Figure 3.4, panels A1-A4). Using the strains recovered from YP+glycerol, we found that both *iqg1Δ* (Figure 3.4, panels B1-B4) and *chs2Δ* cells (Figure 3.5, panels A1-A4) contained no primary septa, and instead of a trilaminar-septum structure, mutant cells could only synthesize thick secondary septa. Similarly, *chs2Δ* cells overexpressing Cdc24-PB1Δp (Figure 3.5, panels B1-B4) and *iqg1Δ* and *chs2Δ* cells overexpressing Egt2p (Figure 3.4, panels E1-E4; Figure 3.5, panels C1-C4) could only form thick septa. In contrast, we found that in addition to forming thick septa (Figure 3.4, panels C1 and D1; Figure 3.5, panel D1), *iqg1Δ* cells overexpressing Cdc24-PB1Δp (Figure 3.4, panels C2-C4) or Ecm33p (Figure 3.4, panels D2-D4) and *chs2Δ* cells overexpressing Ecm33p (Figure 3.5, panels D2-E3) could occasionally form primary-septum-like structures (represented by a sharp-white stripe(s) within a thick septum). In the case of *chs2Δ* cells overexpressing Ecm33p, the sharp-white stripes appeared to be lesser sharp and sometimes revolved around a block of darker stained materials (probably made of cell wall) within the thick septum (Figure 3.5, panel E3) and seemed to be directly involved in cell separation (Figure 3.5, panel E4).

The apparent existence of primary-septum-like structures in some *chs2Δ* cells overexpressing Ecm33p was especially surprising because *chs2Δ* cells should lack the chitin to build the primary septum. To test whether the observed primary-septum-like structures were made of chitin, we labeled them with Wheat-Germ-Agglutinin (WGA). Indeed, the sharp-white stripes of *iqg1Δ* cells overexpressing Cdc24-PB1Δp or Ecm33p were composed at least partially of chitin (Figure 3.6, panels A1-B2), but those (including the lesser-sharp ones that revolved around a block of darker stained materials) of *chs2Δ* cells overexpressing

Ecm33p seemed not to be composed of chitin (Figure 3.6, panels C1 and C2). This means that cells overexpressing Ecm33p can form primary-septum-like structures, even in the absence of the actomyosin ring or chitin synthase II, suggesting that Ecm33p may have a role in the construction of primary septum during cytokinesis.

Involvement of Ecm33p and Pst1p in Cytokinesis

To address whether Ecm33p and its only known homolog Pst1p may be involved in cytokinesis, we constructed *ecm33Δ, pst1Δ, and ecm33Δ pst1Δ* strains. At 23˚C, cells of all three mutants seemed to grow as well as wild-type cells (Figure 3.7A, four left lanes); and at 37˚C, *ecm33Δ* and *ecm33Δ pst1Δ*, but not *pst1Δ*, cells seemed to grow slightly slower than wild-type cells (Figure 3.7A, four right lanes) and form multi-cell clusters (Figure 3.7B) at a very low frequency (data not shown), suggesting that Ecm33p may have a minor role in cytokinesis at 37˚C. Consistent with this result, at 37˚C, *ecm33Δ hof1Δ*, *ecm33Δ pst1Δ hof1Δ*, and *ecm33Δ pst1Δ myo1Δ* cells grew more poorly than *hof1Δ* and *myo1Δ* cells (Figure 3.7C, right panel). Interestingly, even at 23˚C, *ecm33Δ pst1Δ hof1Δ* cells grew more poorly than *hof1Δ* and *ecm33Δ hof1Δ* cells (Figure 3.7C, left panel), suggesting perhaps Pst1p can sometimes substitute for Ecm33p in cytokinesis.

The Cdc42p-Independent, GEF-Dependent Suppression by Cdc24-PB1Δp May Involve Other Rho-GTPase(s) and/or Unknown Factor(s)

Based on sequencing, Cdc24-PB1Δp should contain amino acids 1-780 of Cdc24p plus 299 amino acids of YEp13-vector sequence (Figure 3.8A). Without a PB1 domain to interact with Bem1p, Cdc24-PB1Δp might not be a fully functional GEF for the Cdc42p-GTPase. Indeed, unlike wild-type Cdc24p, expressing Cdc24-PB1Δp could not rescue the temperature-sensitive lethality of *cdc24-4* (Toenjes *et al*., 1999) (Figure 3.8B). Consistent with this result, overexpression of Cdc42p could not suppress the *iqg1Δ* spore inviability on

YPD (Table 3.3). Thus, the suppression of cytokinetic growth defects by overexpressing Cdc24-PB1Δp is probably independent of Cdc42p and may involve other factor(s). We also constructed a plasmid overexpressing Cdc24-DH8p, which is Cdc24-PB1Δp plus two mutations within the DH domain that should deactivate the GEF activity. Overexpressing Cdc24-DH8p could not suppress the *iqg1Δ* spore inviability (Table 3.3), indicating that the suppression by Cdc24-PB1Δp still requires its GEF activity.

Because the suppression by Cdc24-PB1Δp is GEF-dependent, we wanted to see if other known Rho-type GTPases could be involved. Indeed, overexpression of Rho1p, Rho2p, or Rho5p, but not Rho3p nor Rho4p, could suppress the *iqg1Δ* spore inviability (Table 3.3), suggesting that Cdc24-PB1Δp, at least when overexpressed, may function as a GEF for Rho1p, Rho2p, and/or Rho5p. However, overexpression of Cdc24-PB1Δp could still suppress the *iqg1Δ* spore inviability even in the absence of Rho2p and/or Rho5p (Figure 3.8C), indicating that although Rho2p and Rho5p may be targets of Cdc24-PB1Δp, they are not required for the described suppression. Furthermore, we found that overexpressing Cdc24-PB1Δp could not suppress the synthetic lethality of deletions of *TUS1* (Schmelzle *et al*., 2002) and *ROM2* (Ozaki *et al*., 1996), which encode two known GEFs of Rho1p (Figure 3.8D), indicating that although Cdc24-PB1Δp may function as a GEF for Rho1p, it cannot adequately function as the main one.

DISCUSSION

To identify additional proteins involved in septin-dependent, actomyosin-ring-independent cytokinesis, we have been conducting dosage-suppressor screens starting with *myo1Δ cyk3Δ* and *myo1Δ hof1Δ* double mutants. We have isolated *ECM33*, *EGT2*, *cdc24-PB1Δ*, *RHO1*, *RGD2*, *RPL30*, *NAB6*, and *MNN9*. Subsequent analyses indicated that overexpression of Ecm33p, Egt2p, or Cdc24-PB1Δp could also suppress the *iqg1Δ* and *chs2Δ* growth defects. Furthermore, overexpressing Ecm33p in *iqg1Δ* and *chs2Δ* cells or overexpressing Cdc24-PB1Δp in *iqg1Δ* cells could induce the formation of primary-septum-like structures. Deletions of *ECM33* and its homolog *PST1* seemed to cause minor defects in cytokinesis, particularly at 37˚C. Finally, the suppression by Cdc24-PB1Δp was found to be Cdc42p-independent and GEF-dependent and might involve Rho1p, Rho2p, Rho5p, and/or an unknown factor(s).

Suppressions by Overexpressing Ecm33p

The primary-septum-like structures in *iqg1Δ* and *chs2Δ* cells overexpressing Ecm33p probably help mutant cells to grow better by providing a more efficient method of cell separation. However, the formation of these structures in *chs2Δ* cells was rather surprising because Chs2p synthesizes the chitin for primary septum, but the lack of proper WGA labeling on these structures suggests that they are composed mainly of cell-wall materials other than chitin, perhaps of glucans and mannoproteins. This is consistent with a previous report (Pardo *et al*., 2004) that Ecm33p is involved in the N-linked glycosylation and cell-wall anchoring of mannoproteins. Perhaps Ecm33p participates in an actomyosin-ring-independent and Iqg1-Cyk3-involved pathway of primary-septum construction by regulating mannoproteins and other cell-wall materials. Among the remaining mysteries is that the

observed primary-septum-like structures often revolve around a block of darker stained cell-wall material, and that cell separation seems to occur via the hydrolysis of these structures. To date, this phenotype has not been observed in other mutant cells and seems to be unique to the overexpression of Ecm33p. Understanding the molecular basis of these structures may help us to understand the role of Ecm33p in primary-septum formation.

Because the observed primary-septum-like structures did not appear very frequently (approximately one in every 20 cytokinetic cells observed; data not shown), the overexpression of Ecm33p probably can also suppress the *iqg1Δ* and *chs2Δ* growth defects via another mechanism(s). One possibility is that overexpressing Ecm33p may help mutant cells with cytokinetic defects to construct better thick secondary septa. After germinating on YPD, *iqg1Δ* and *chs2Δ* spores often go through several rounds of cell division before a whole colony is lysed (data not shown). Consistent with this observation, *iqg1Δ* and *chs2Δ* cells sometimes appear to be lysed (EM analysis; data not shown), and the source of the lysis often appears to be near the thick septa. Thus, the thick septa may often be poorly constructed and can easily cause cell lysis, especially during cell separation when parts of the thick septa are dissolved. Overexpressing Ecm33p can anchor more mannoproteins and other cell-wall materials to construct a more stable and stronger thick septum.

Regardless of the exact mechanism of suppression by Ecm33p, it is likely that Ecm33p only plays a minor role in normal cytokinesis, because the deletion of *ECM33* did not cause any detectable cytokinetic defect at 23˚C and only caused minimal multi-cell clustering at 37˚C, which probably led to the slightly slower growth rate. In addition, all observed *ecm33Δ* cells, including those in clusters at 37˚C, could synthesize the trilaminar septum with a normal primary septum (data not shown). One interesting observation of

ecm33Δ cells was the existence of small sharp-white stripes in the secondary septa, running perpendicularly and connected to the primary septum (data not shown) at both 23˚C and 37˚C. Furthermore, these small sharp-white stripes were sometimes present throughout the entire cell wall of *ecm33Δ* cells (data not shown). It is possible that these structures could have interfered with proper cell separation, thus at 37˚C, where cell cycle would progress faster, *ecm33Δ* cells could occasionally form clusters. It will be interesting to test if these small sharp-white stripes are made of chitin and/or other cell-wall materials. Understanding the molecular basis of these structures may help us to understand the role of Ecm33p in primary-septum formation and cell-wall organization.

Initial analysis of Pst1p, a homolog of Ecm33p, has shown that it appears to play no direct role in cytokinesis, because *pst1Δ* cells did not form clusters and had normal looking septum at both 23˚C or 37˚C (data not shown). However, although *ecm33Δ* single and *ecm33Δ pst1Δ* double mutant cells looked similar under EM, the small sharp-white stripes in the double mutant seemed to be more abundant (data not shown). Furthermore, *ecm33Δ pst1Δ* double mutations, but not *ecm33Δ* or *pst1Δ* single mutation, are synthetic lethal in combination with *myo1Δ* at 37˚C. Taken together, these results suggest that in the absence of Ecm33p, Pst1p may partially substitute for its function, which is consistent with a previous report (Pardo *et al*., 2004).

In summary, Ecm33p appears to play a role in cytokinesis, especially at 37˚C or when normal cytokinesis is compromised. It will be interesting to test if Cyk3p promotes primary-septum synthesis partly through Ecm33p. One experiment is to test if overexpressing Cyk3p can restore primary-septum formation in *iqg1Δ ecm33Δ* cells. Also, it will be interesting to determine if Ecm33p localizes more intensely to the bud necks in *iqg1Δ* and *chs2Δ* cells.

Suppressions by Overexpressing Cdc24-PB1Δp

The formation of primary-septum-like structures in *iqg1Δ* cells, but not in *chs2Δ* cells, overexpressing Cdc24-PB1Δp suggests that the suppressions by Cdc24-PB1Δp may involve more than one mechanism. Perhaps the overexpression of Cdc24-PB1Δp can help mutant cells to produce primary-septum-like structures (only in the presence of chitin made by Chs2p), build better thick septa (by over-activating the cell-integrity pathways), and/or conduct more efficient hydrolysis of cell-wall materials during cell separation. Regardless of the exact mechanism, the suppressions are likely to be independent of Cdc42p but dependent of the GEF activity and functioning through Rho1p, Rho2p, Rho5p, and/or an unknown factor(s).

Rho1p is the most probable target because it has been shown to participate in multiple pathways of cell polarization and integrity (see Introduction). It is possible that Cdc24-PB1Δp, without its PB1 domain to interact with Bem1p and Cdc42p, is free to promote Rho1p-GTP, which then hyper-activates its many downstream targets. The non-suppression of *tus1Δ rom2Δ* synthetic lethality by the overexpression of Cdc24-PB1Δp does not necessarily reject this model, because it is possible that Cdc24-PB1Δp simply cannot act as the main or sole GEF to promote the normal essential functions of Rho1p. To verify this model, one test is to see if Cdc24-PB1Δp has GEF activity for Rho1p via *in vitro* assay. It is also possible to test if overexpressing Cdc24-PB1Δp can suppress the *iqg1Δ* growth defect when Rho1p is partially compromised (using a hypomorphic *rho1* allele). Rho2p is also a possible target, but because overexpressing Cdc24-PB1Δp could still suppress the *iqg1Δ* defect even without Rho2p, it cannot be the only target. One possibility is that Cdc24-PB1Δp can activate both Rho1p and Rho2p, which then both activate their downstream

targets. One test is to see if Cdc24-PB1Δp has GEF activity for Rho2p via *in vitro* assay. Rho5p is another possible candidate, but its reported role as a negative regulator (Schmitz *et al*., 2002) of the Rho1p-cell-integrity pathway is discordant with the model that Cdc24-PB1Δp may activate downstream cell-integrity pathways to construct a better septum. As Rho2p, Rho5p was not required for the suppression of the *iqg1Δ* defect by overexpressing Cdc24-PB1Δp. Furthermore, the suppression of the *iqg1Δ* defect by overexpressing Rho5p appeared to be milder than the suppression by Rho1p, Rho2p, or Cdc24-PB1Δp, and is also discordant with the isolation of Rgd2p (GAP for Rho5p; Roumanie *et al*., 2001) as a dosage suppressor of the *myo1Δ cyk3Δ* growth defect. Again, one test is to see if Cdc24-PB1Δp has GEF activity for Rho5p via *in vitro* assay. The possible unknown factor(s) may either be an unidentified Rho-type GTPase in *S. cerevisiae* or a protein that is activated by Cdc24-PB1Δp independently of the GEF activity. If the latter case is true, then the two mutations in the DH domain of Cdc24-DH8p probably cause defects in addition to GEF inactivation. In that case, wild-type Cdc24p may have a minor GEF-independent role, which becomes dominant in the absence of the PB1 domain.

Finally, the suppression of the *iqg1Δ* growth defect by overexpressing Cdc24-PB1Δp requires the PB1 domain to be absent, because overexpressing wild-type Cdc24p could not suppress the same defect. However, Cdc24p without its PB1 domain has been reported previously (Shimada *et al*., 2004) to be nonfunctional and likely degraded. Consistent with this result, when the 299 amino acids (from the YEp13 vector) of Cdc24-PB1Δp were replaced with a stop codon, the resulting construct could no longer suppress the *iqg1Δ* spore inviability on YPD (data not shown), suggesting that the 299 amino acids might act a stabilizer that protects Cdc24-PB1Δp from degradation. One experiment is to test if Cdc24-

PB1Δp, with the 299 amino acids replaced by a GFP or 3HA epitope, can still suppress the *iqg1Δ* spore inviability.

Suppressions by Overexpressing Egt2p or Other Dosage Suppressors

The suppression of the *iqg1Δ* and *chs2Δ* growth defects by overexpressing Egt2p appears to be independent of primary-septum formation. One possibility is that overexpressing an endoglucanase like Egt2p can help mutant cells with thick septa to conduct a more efficient hydrolysis of cell-wall materials during cell separation. More efficient cell separations lead to fewer multi-cell clusters, and cells not in clusters can probably progress into the next cell cycle faster, because there is less limitation on its cell surface area. An alternative explanation is that Egt2p may have a glucanase-independent function(s) that promotes the construction of a better thick septum. Finally, the investigation of other isolated dosage suppressors, especially *NAB6* and *RPL30*, are in progress.

FIGURES (1 to 8)

Figure 3.1. Figure 1 of Chapter 3

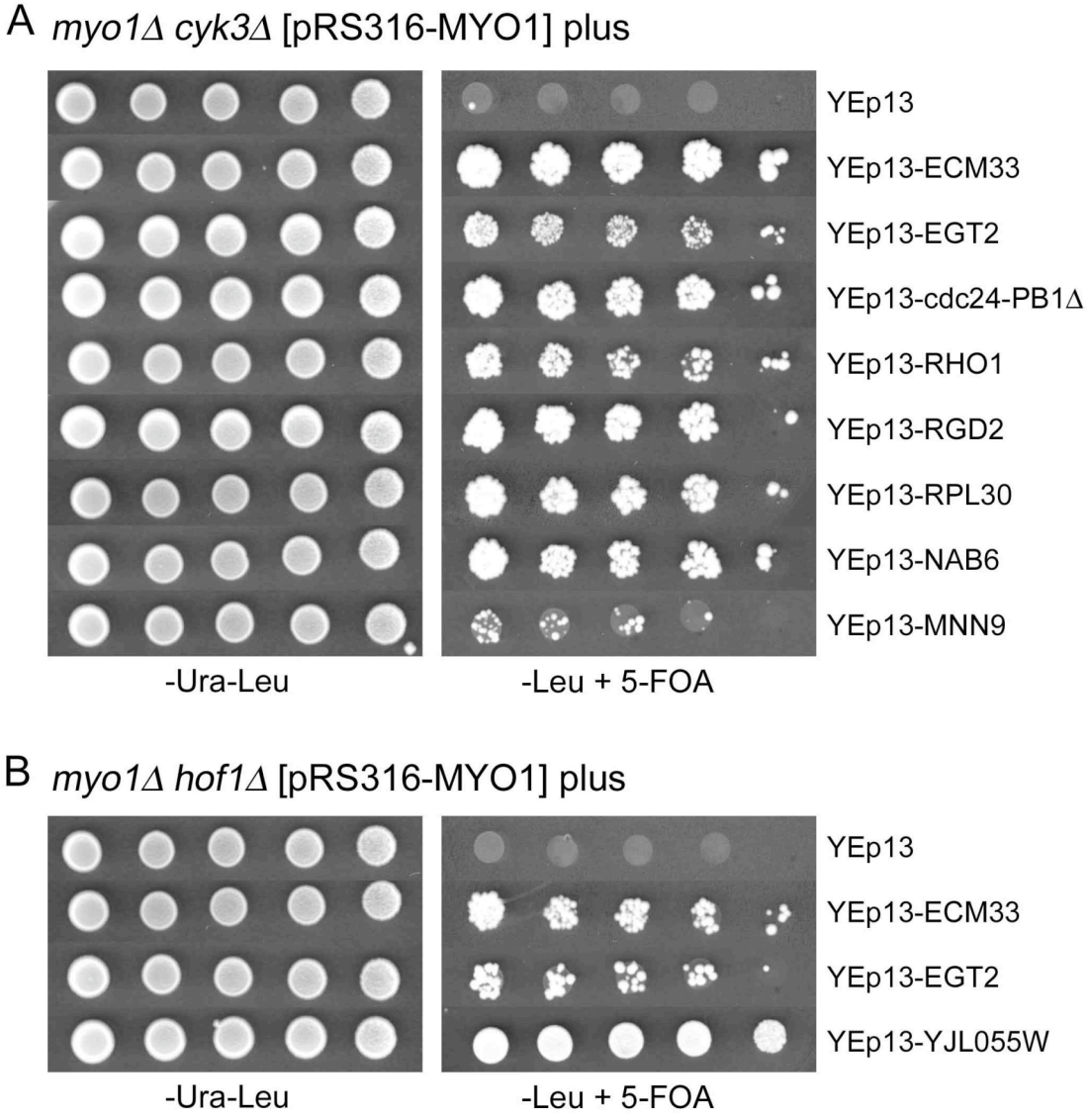

Figure 3.2. Figure 2 of Chapter 3

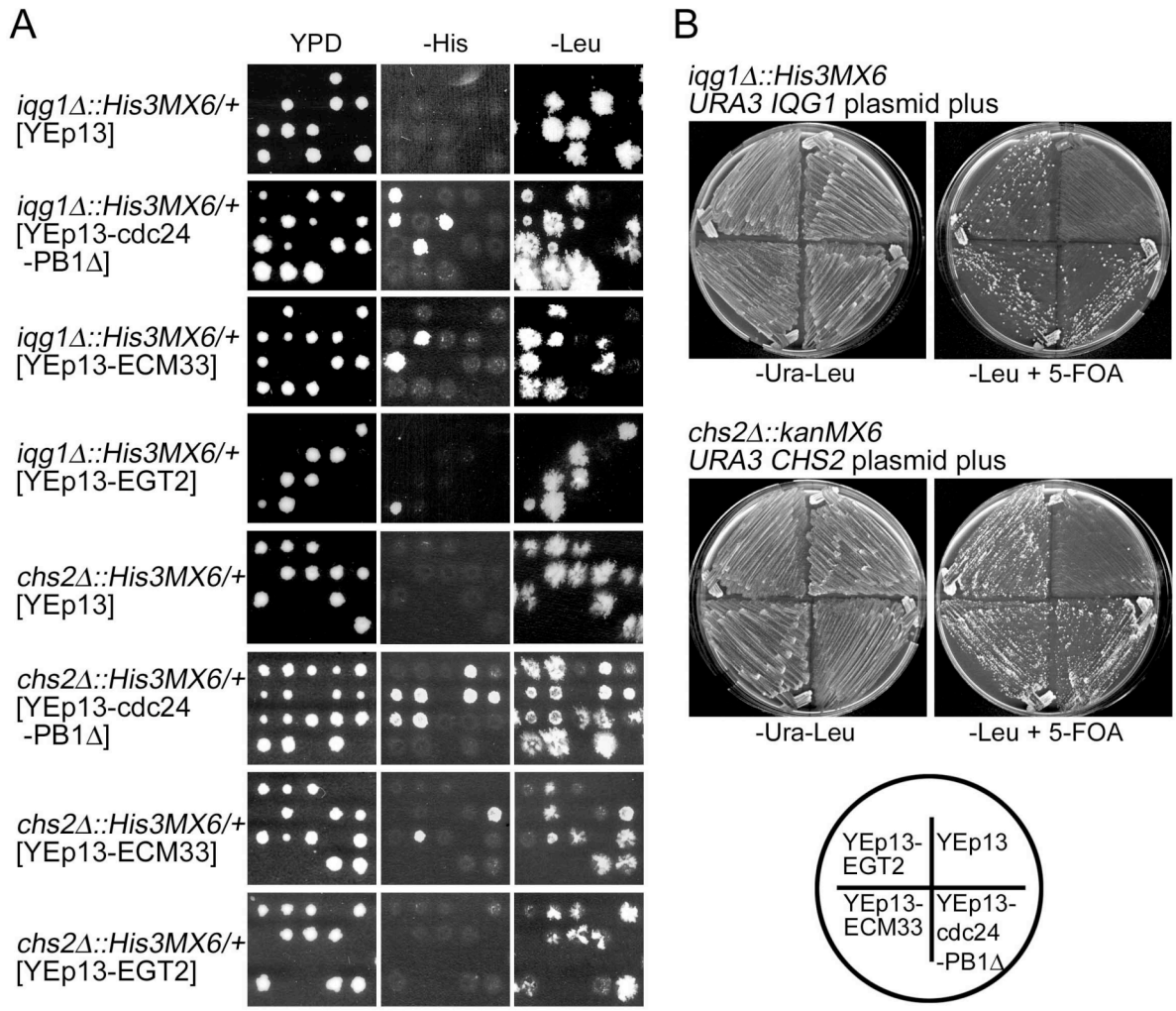

Figure 3.3. Figure 3 of Chapter 3

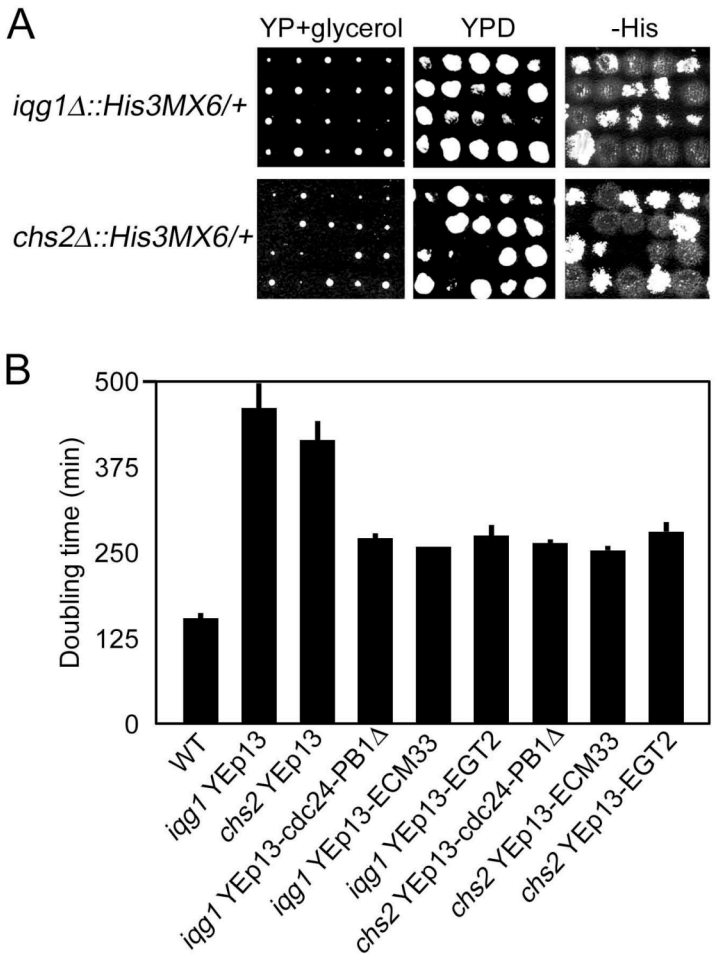

Figure 3.4. Figure 4 of Chapter 3

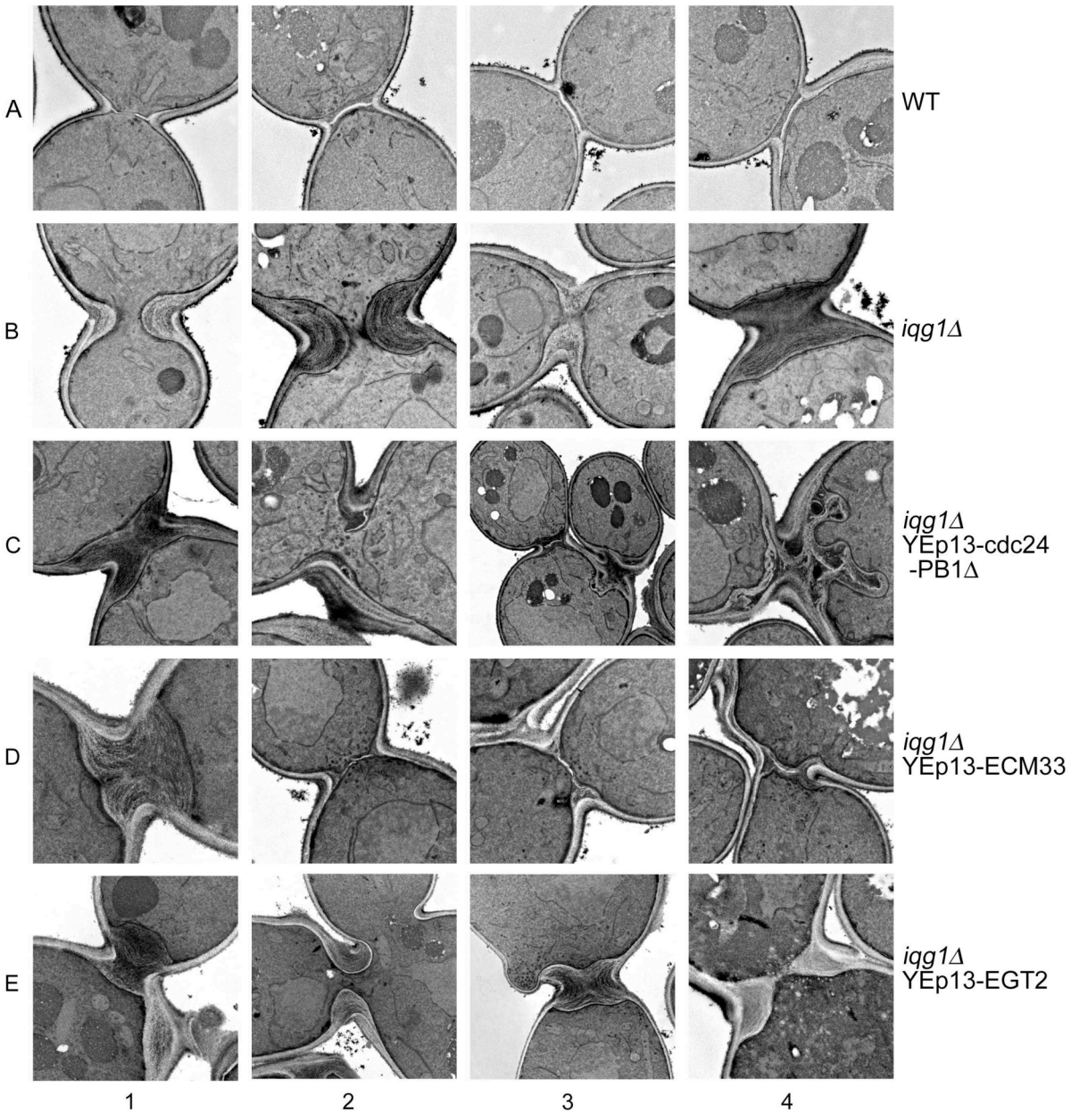

Figure 3.5. Figure 5 of Chapter 3

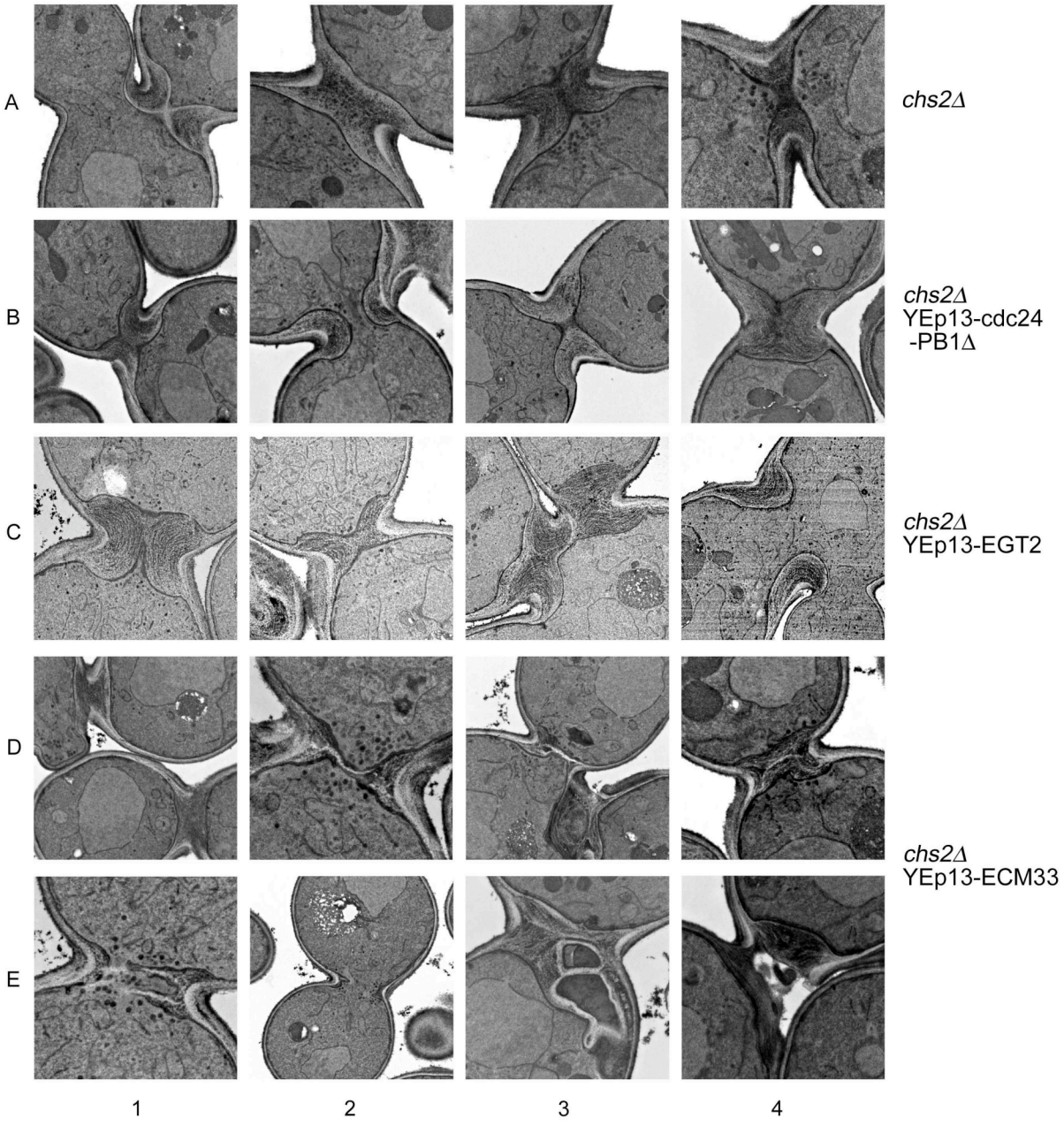

Figure 3.6. Figure 6 of Chapter 3

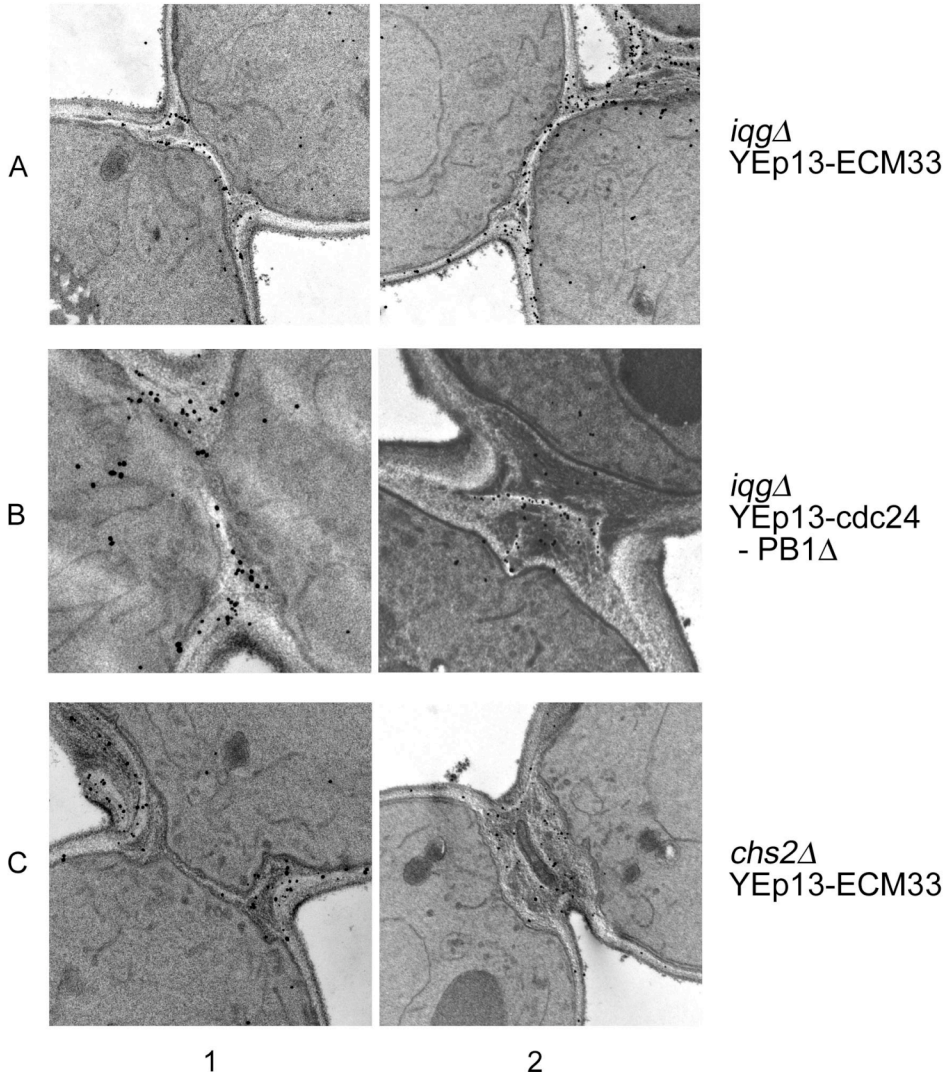

Figure 3.7. Figure 7 of Chapter 3

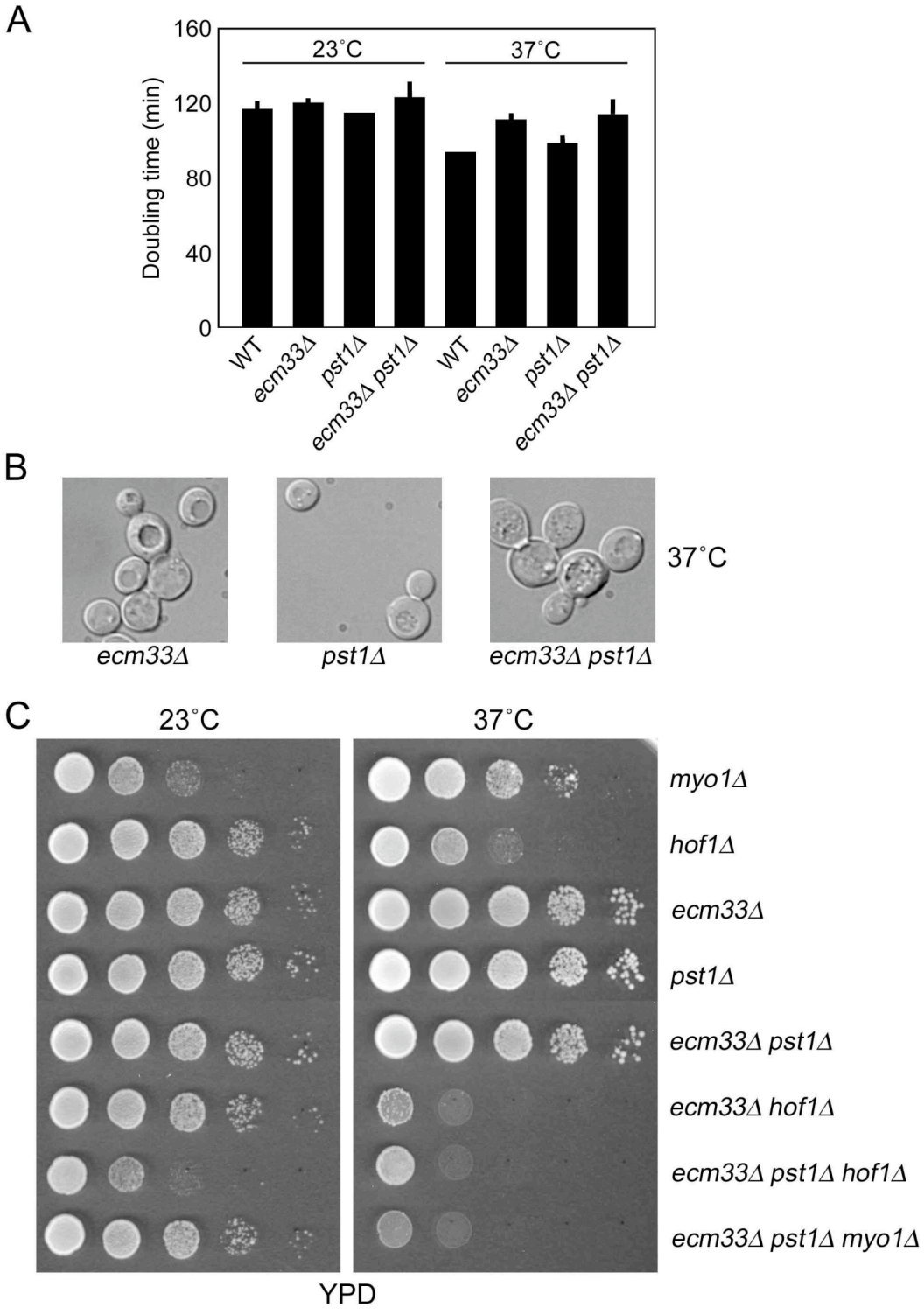

Figure 3.8. Figure 8 of Chapter 3

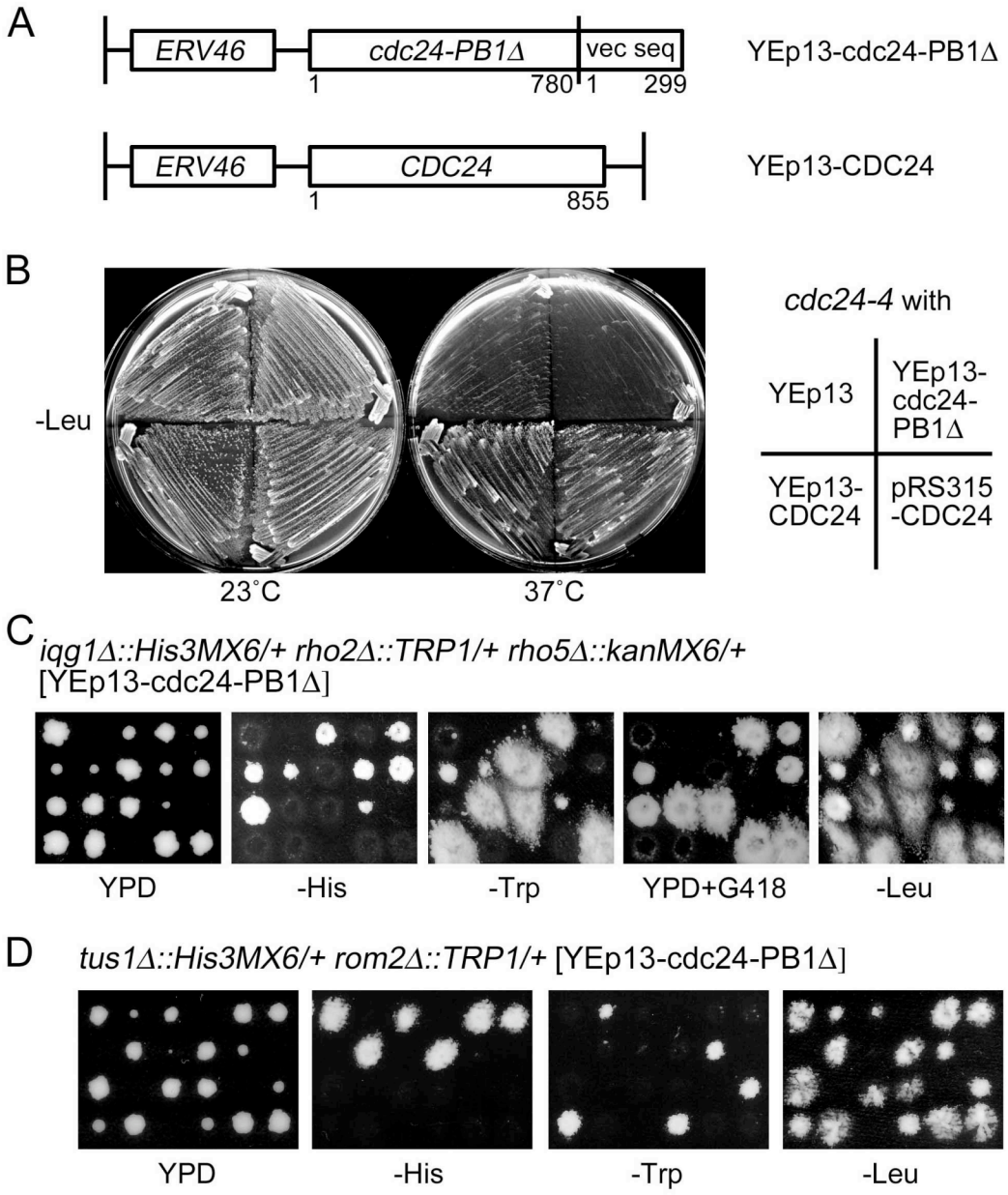

FIGURE LEGENDS (1 to 8)

Figure 3.1. Dosage suppression of the *myo1Δ cyk3Δ* or *myo1Δ hof1Δ* synthetic growth defect. (A) Strain RNY598 (*myo1Δ::kanMX6 cyk3Δ::TRP1* [*URA3 MYO1* plasmid]) was transformed with plasmid YEp13 or suppressor plasmids. Strains KO725, KO1206, KO1207, KO1208, KO1209, KO1210, KO1211, KO1212, and KO1213 were grown to exponential phase ($OD_{600} \approx 0.4$) in SC-Ura-Leu medium, and 5-μl aliquots of a dilution series (undiluted, 2x, 4x, 8x, and 80x diluted) were spotted onto SC-Ura-Leu and SC-Leu+5-FOA plates and grown at 30˚C for 4 days. (B) Strain RNY757 (*myo1Δ::kanMX6 hof1Δ::TRP1* [*URA3 MYO1* plasmid]) was transformed with plasmid YEp13 or suppressor plasmids. Strains KO723, KO1214, KO1215, and KO1216 were grown to exponential phase and dilution series were performed as described in A.

Figure 3.2. Suppression of the *iqg1Δ* or *chs2Δ* spore inviability and vegetative growth defect. (A) Suppression of the spore inviability on YPD. Tetrads were dissected on YPD from strains heterozygous for *iqg1Δ::His3MX6* (top four rows) or *chs2Δ::His3MX6* (bottom four rows) and carrying either plasmid YEp13 (KO836 or KO1190) or *cdc24-PB1Δ* (KO939 or KO997), *ECM33* (KO840 or KO995), or *EGT2* (KO839 or KO993) plasmid. With YEp13, no viable *iqg1Δ* or *chs2Δ* segregants were observed in more than 24 tetrads dissected for each strain. With the *cdc24-PB1Δ* or *ECM33* plasmid, all viable *iqg1Δ* and *chs2Δ* segregants (scored by growth on SC-His) also carried the corresponding plasmids (scored by growth on SC-Leu). With the *EGT2* plasmid, only viable *iqg1Δ* (carrying the plasmid) and no *chs2Δ* segregrants were isolated. The numbers of viable segregants and tetrads dissected are described in Table 3.3. (B) Suppression of the inability of vegetative *iqg1Δ* or *chs2Δ* cells to lose an *IQG1* or *CHS2* plasmid. Strains RNY970 (top row) and RNY1419 (bottom row) were transformed with either plasmid YEp13 or *cdc24-PB1Δ*, *ECM33*, or *EGT2* plasmid. Strains KO1130, KO1134, KO1132, KO1138, KO1095, KO1142, KO1140, and KO1097 were streaked onto SC-Ura-Leu and SC-Leu+5-FOA plates and grown for 5 days.

Figure 3.3. Suppression of the *iqg1Δ* or *chs2Δ* spore inviability by glycerol and slow-growth phenotype. (A) *iqg1Δ* and *chs2Δ* spores are viable on YP+glycerol plates. Tetrads were dissected on YP+glycerol from strains heterozygous for *iqg1Δ::His3MX6* (RNY704) or *chs2Δ::His3MX6* (ICY29). After 14 days, all viable spore colonies were relocated onto YPD, grown for three more days, and then replicated onto SC-His to identify the *iqg1Δ* or *chs2Δ* segregants. (B) Suppression of the slow-growth phenotype. Strains scored were KO4, KO1243, KO1271, KO967, KO883, KO887, KO1016, KO1014, and KO1274. Strains were grown in SC or SC-Leu (to select for plasmid) medium, and the doubling times shown are the averages of two measurements made as described in Materials and Methods.

Figure 3.4. Formation of primary-septum-like structures in *iqg1Δ* cells overexpressing Cdc24-PB1Δp or Ecm33p. Strains KO4, KO969, KO967, KO883, and KO887 were grown to exponential phase in YM-P, SC-His, or SC-Leu medium and then collected for EM analysis as described in Materials and Methods. At least 500 cytokinetic cells were observed for each strain.

Figure 3.5. Formation of primary-septum-like structures in *chs2Δ* cells overexpressing Ecm33p. Strains KO1255, KO1016, KO1014, and KO1105 were grown to exponential phase in SC-His or SC-Leu medium and then collected for EM analysis as described in Materials and Methods. At least 500 cytokinetic cells were observed for each strain.

Figure 3.6. WGA labeling of *iqg1Δ* cells overexpressing Cdc24-PB1Δp or Ecm33p and *chs2Δ* cells overexpressing Ecm33p. Strains KO883, KO967, and KO1014 were grown to exponential phase in SC-Leu medium and then collected for WGA labeling and EM analysis as described in Materials and Methods. WGA (black dots; labels chitin) stained the primary-septum-like structures (sharp-white stripes) of *iqg1Δ* cells overexpressing Cdc24-PB1Δp or Ecm33p, but seemed not to stain those of *chs2Δ* cells overexpressing Ecm33p. At least 300 *chs2Δ* cytokinetic cells with WGA-labeling were examined.

Figure 3.7. Single deletion of *ECM33* and double deletions of *ECM33* and *PST1* may cause cytokinetic defects at 37˚C. (A) *ecm33Δ* and *ecm33Δ pst1Δ* cells grow slightly slower than wild-type cells at 37˚C. Strains scored were KO4, KO819, KO975, and KO981. Strains were grown in YM-P medium, and the doubling times shown are the averages of two measurements made as described in Materials and Methods. (B) Presence of multi-cell clusters in an *ecm33Δ* or *ecm33Δ pst1Δ* strain at 37˚C. Strains KO819, KO975, and KO981 were grown overnight in YM-P medium at 37˚C, collected by centrifugation, resuspended in water, and observed by DIC microscopy. (C) Strains KO608, KO61, KO819, KO975, KO981, KO875, KO1056, and KO982 were grown to exponential phase ($OD_{600} \approx 0.4$) in YM-P medium, and 5-μl aliquots of a dilution series (undiluted, 10x, 100x, 1000x, and 10000x diluted) were spotted onto YPD plates and grown at the indicated temperatures for 4 days.

Figure 3.8. The suppression by Cdc24-PB1Δp may involve Rho1p, Rho2p, Rho5p, and/or an unknown factor(s), but not Cdc42p. (A) The inserts of the relevant plasmids. The PB1 domain (which is absent in the *cdc24-PB1Δ* plasmid) of wild-type Cdc24p is from amino acids 781-855. (B) Cdc24-PB1Δp cannot function as wild-type Cdc24p. Strain YEF313 (*cdc24-4*) was transformed with the indicated plasmids, streaked onto SC-Leu plates, and grown for 3 days at the indicated temperatures. (C) The suppression of the *iqg1Δ* spore inviability in the absence of Rho2p and/or Rho5p. Tetrads were dissected on YPD from a strain (KO1445) carrying the *cdc24-PB1Δ* plasmid and heterozygous for *iqg1Δ::His3MX6*, *rho2Δ::TRP1*, and *rho5Δ::kanMX6*. In 32 tetrads dissected, five *iqg1Δ*, eight *iqg1Δ rho2Δ*, 12 *iqg1Δ rho5Δ*, and four *iqg1Δ rho2Δ rho5Δ* viable segregants carrying the plasmid were recovered. (D) The overexpression of Cdc24-PB1Δp does not suppress the synthetic lethality of *tus1Δ rom2Δ*. Tetrads were dissected on YPD from strains (KO1391 and KO1392) heterozygous for *tus1Δ::His3MX6* and *rom2Δ::TRP1*. In 44 tetrads dissected, no viable *tus1Δ rom2Δ* segregants were recovered.

Table 3.1. Strain List of Chapter 3

Strain [a]	Genotype	Source
YEF473	***a/α*** *his3-Δ200/his3-Δ200 leu2-Δ1/leu2-Δ1 lys2-801/ lys2-801 trp1-Δ63/trp1-Δ63 ura3-52/ura3-52*	Bi and Pringle, 1996
YEF473A	***a*** *his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Segregant from YEF473
YEF473B	*α his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	Segregant from YEF473
YEF313	as YEF473A except *cdc24-4*	Shimada *et al.* 2004
ICY29	as YEF473 except *chs2Δ::His3MX6/+*	This study [b]
RNY598	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1]	Nishihama *et al*., 2007
RNY704	as YEF473 except *iqg1Δ::His3MX6/+*	Nishihama *et al*., 2007
RNY757	as YEF473A except *myo1Δ::kanMX6 hof1Δ::TRP1* [pRS316-MYO1]	This study [c]
RNY970	as YEF473A except *iqg1Δ::His3MX6* [p1868]	Nishihama *et al*., 2007
RNY1232	as YEF473 except *iqg1Δ::kanMX6/+*	This study [b]
RNY1233	as YEF473 except *iqg1Δ::kanMX6/+*	This study [b]
RNY1419	as YEF473A except *chs2Δ::kanMX6* [pJC328]	Nishihama *et al*., 20007
KO4	as YEF473A	This study
KO61	as YEF473A except *hof1Δ::TRP1*	This study [d]
KO608	as YEF473A except *myo1Δ::kanMX6*	see Chapter 2
KO723	as YEF473A except *myo1Δ::kanMX6 hof1Δ::TRP1* [pRS316-MYO1] [YEp13]	This study [c]
KO725	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13]	This study [c]
KO819	as YEF473A except *ecm33Δ::His3MX6*	This study [d]
KO836	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13]	This study [e]
KO838	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-YJL055W]	This study [e]
KO839	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-EGT2]	This study [e]
KO840	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-ECM33]	This study [e]
KO853	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-YJL055W]	This study [e]
KO854	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-ECM33]	This study [e]
KO855	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-EGT2]	This study [e]
KO875	as YEF473B except *hof1Δ::TRP1 ecm33Δ::His3MX6*	This study [d]
KO883	as YEF473A except *iqg1Δ::His3MX6* [YEp13-ECM33]	Segregant from KO840

KO887	as YEF473B except *iqg1Δ::His3MX6* [YEp13-EGT2]	Segregant from KO839
KO933	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-RGD2]	This study [e]
KO934	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-RGD2]	This study [e]
KO935	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-RHO1]	This study [e]
KO936	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-RHO1]	This study [e]
KO937	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-RPL30]	This study [e]
KO938	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-RPL30]	This study [e]
KO939	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-cdc24-PB1Δ]	This study [e]
KO940	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-cdc24-PB1Δ]	This study [e]
KO941	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-MNN9]	This study [e]
KO942	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-MNN9]	This study [e]
KO964	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-NAB6]	This study [e]
KO965	as YEF473 except *iqg1Δ::His3MX6/+* [YEp13-NAB6]	This study [e]
KO967	as YEF473A except *iqg1Δ::His3MX6* [YEp13-cdc24-PB1Δ]	Segregant from KO939
KO969	as YEF473B except *iqg1Δ::His3MX6*	This study [f]
KO975	as YEF473B except *pst1Δ::TRP1*	This study [d]
KO981	as YEF473A except *ecm33Δ::His3MX6 pst1Δ::TRP1*	This study [d]
KO982	as YEF473A except *myo1Δ::kanMX6 ecm33Δ::His3MX6 pst1Δ::TRP1*	This study [d]
KO993	as YEF473 except *chs2Δ::His3MX6/+* [YEp13-EGT2]	This study [e]
KO994	as YEF473 except *chs2Δ::His3MX6/+* [YEp13-EGT2]	This study [e]
KO995	as YEF473 except *chs2Δ::His3MX6/+* [YEp13-ECM33]	This study [e]
KO996	as YEF473 except *chs2Δ::His3MX6/+* [YEp13-ECM33]	This study [e]
KO997	as YEF473 except *chs2Δ::His3MX6/+* [YEp13-cdc24-PB1Δ]	This study [e]
KO998	as YEF473 except *chs2Δ::His3MX6/+* [YEp13-cdc24-PB1Δ]	This study [e]
KO1014	as YEF473B except *chs2Δ::His3MX6* [YEp13-ECM33]	Segregant from KO995
KO1016	as YEF473A except *chs2Δ::His3MX6* [YEp13-cdc24-PB1Δ]	Segregant from KO997
KO1040	as YEF473 except *iqg1Δ::His3MX6/+* [pGAL-6xHIS-CDC42]	This study [e]
KO1041	as YEF473 except *iqg1Δ::His3MX6/+* [pGAL-6xHIS-CDC42]	This study [e]
KO1042	as YEF473 except *iqg1Δ::His3MX6/+* [YEp51(42)]	This study [e]
KO1043	as YEF473 except *iqg1Δ::His3MX6/+* [YEp51(42)]	This study [e]
KO1056	as YEF473A except *hof1Δ::TRP1 ecm33Δ::His3MX6 pst1Δ::kanMX6*	This study [d]
KO1095	as YEF473A except *chs2Δ::kanMX6* [pJC328] [YEp13]	This study [e]

KO1097	as YEF473A except *chs2Δ::kanMX6* [pJC328] [YEp13-EGT2]	This study [e]
KO1105	as YEF473A except *chs2Δ::kanMX6* [YEp13-EGT2]	This study [g]
KO1130	as YEF473A except *iqg1Δ::His3MX6* [p1868] [YEp13]	This study [e]
KO1132	as YEF473A except *iqg1Δ::His3MX6* [p1868] [YEp13-ECM33]	This study [e]
KO1134	as YEF473A except *iqg1Δ::His3MX6* [p1868] [YEp13-cdc24-PB1Δ]	This study [e]
KO1138	as YEF473A except *iqg1Δ::His3MX6* [p1868] [YEp13-EGT2]	This study [e]
KO1140	as YEF473A except *chs2Δ::kanMX6* [pJC328] [YEp13-ECM33]	This study [e]
KO1142	as YEF473A except *chs2Δ::kanMX6* [pJC328] [YEp13-cdc24-PB1Δ]	This study [e]
KO1190	as YEF473 except *chs2Δ::His3MX6/+* [YEp13]	This study [e]
KO1206	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-ECM33]	This study [c]
KO1207	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-EGT2]	This study [c]
KO1208	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-cdc24-PB1Δ]	This study [c]
KO1209	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-RHO1]	This study [c]
KO1210	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-RGD2]	This study [c]
KO1211	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-RPL30]	This study [c]
KO1212	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-NAB6]	This study [c]
KO1213	as YEF473A except *myo1Δ::kanMX6 cyk3Δ::TRP1* [pRS316-MYO1] [YEp13-MNN9]	This study [c]
KO1214	as YEF473A except *myo1Δ::kanMX6 hof1Δ::TRP1* [pRS316-MYO1] [YEp13-ECM33]	This study [c]
KO1215	as YEF473A except *myo1Δ::kanMX6 hof1Δ::TRP1* [pRS316-MYO1] [YEp13-EGT2]	This study [c]
KO1216	as YEF473A except *myo1Δ::kanMX6 hof1Δ::TRP1* [pRS316-MYO1] [YEp13-YJL055W]	This study [c]
KO1243	as YEF473A except *iqg1Δ::His3MX6* [YEp13]	This study [f]
KO1255	as YEF473A except *chs2Δ::His3MX6*	This study [f]
KO1271	as YEF473A except *chs2Δ::His3MX6* [YEp13]	This study [f]
KO1274	as YEF473A except *chs2Δ::kanMX6* [YEp13-EGT2]	This study [g]
KO1324	as YEF473A except *cdc24-4* [YEp13-CDC24]	This study [h]
KO1332	as YEF473A except *cdc24-4* [YEp13]	This study [h]
KO1340	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-CDC24]	This study [e]

KO1341	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-CDC24]	This study [e]
KO1342	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-CDC24]	This study [e]
KO1343	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-CDC24]	This study [e]
KO1344	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-CDC24]	This study [e]
KO1345	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-CDC24]	This study [e]
KO1346	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-cdc24-DH8]	This study [e]
KO1348	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-cdc24-DH8]	This study [e]
KO1350	as YEF473 except *iqg1Δ::kanMX6/+* [YEp13-cdc24-DH8]	This study [e]
KO1368	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO5]	This study [e]
KO1369	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO5]	This study [e]
KO1381	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO1]	This study [e]
KO1382	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO1]	This study [e]
KO1383	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO2]	This study [e]
KO1384	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO2]	This study [e]
KO1385	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO3]	This study [e]
KO1386	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO3]	This study [e]
KO1387	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO4]	This study [e]
KO1388	as YEF473 except *iqg1Δ::kanMX6/+* [pGAL-RHO4]	This study [e]
KO1391	as YEF473 except *tus1Δ::His3MX6/+ rom2Δ::TRP1/+* [YEp13-cdc24-PB1Δ]]	This study [i]
KO1392	as YEF473 except *tus1Δ::His3MX6/+ rom2Δ::TRP1/+* [YEp13-cdc24-PB1Δ]]	This study [i]
KO1445	as YEF473 except *iqg1Δ::His3MX6/+ rho2Δ::TRP1/+ rho5Δ::kanMX6/+* [YEp13-cdc24-PB1Δ]	This study [j]
KO1462	as YEF473A except *cdc24-4* [YEp13-cdc24-PB1Δ]	This study [h]
KO1466	as YEF473A except *cdc24-4* [pRS315-CDC24]	This study [h]

[a] Denotes strains of the S288C genetic background (Mortimer and Johnson, 1986)

[b] Strain YEF473 was transformed with an *iqg1Δ::kanMX6* or *chs2Δ::His3MX6* deletion cassette.

[c] Tetrads were dissected on YPD from two strains (carrying pRS316-MYO1) doubly heterozygous either for *myo1Δ::kanMX6* and *cyk3Δ::TRP1* or *myo1Δ::kanMX6* and *hof1Δ::TRP1*. Viable double mutant segregants carrying the plasmid were isolated and transformed with either plasmid YEp13 or a high-copy YEp13-based genomic library.

[d] Tetrads were dissected on YPD from a strain either heterozygous for *hof1Δ::TRP1*, doubly heterozygous for *ecm33Δ::His3MX6* and *hof1Δ::TRP1*, triply heterozygous for *ecm33Δ::His3MX6*, *hof1Δ::TRP1*, and *pst1Δ::kanMX6*, or triply heterozygous for

ecm33Δ::His3MX6, *myo1Δ::kanMX6*, and *pst1Δ::TRP1*. Viable single, double, and triple mutant segregants were isolated.

[e] RNY704, RNY1232, RNY1233, ICY29, RNY970, and RNY1419 were transformed with the indicated plasmids.

[f] Tetrads were dissected on YP+glycerol plates from four strains carrying either no plasmid or plasmid YEp13 and heterozygous either for *iqg1Δ::His3MX6* or *chs2Δ::His3MX6*. After 2 weeks at 23˚C, viable single mutant segregants carrying either no plasmid or YEp13 were isolated.

[g] Strain KO1097 was streaked onto SC-Leu+5-FOA for single colonies. Viable *chs2Δ* colonies carrying only YEp13-EGT2 were isolated.

[h] Strain YEF313 was transformed with the indicated plasmids.

[i] A strain heterozygous for *tus1Δ::His3MX6* and *rom2Δ::TRP1* was transformed with YEp13-cdc24-PB1Δ.

[j] Strain KO939 was transformed with a *rho2Δ::TRP1* and *rho5Δ::kanMX6* deletion cassettes.

Table 3.2. Plasmid List of Chapter 3

Plasmid	Description	Source
YEp13	2μ, *LEU2* (high copy)	Broach *et al*., 1979
pRS316-MYO1	*CEN6 URA3 MYO1* (low copy)	Nishihama *et al*., 2007
pJC328	*CEN6 URA3 CHS2myc* (low copy)	Chuang and Schekman, 1996
p1868	*CEN6 URA3 IQG1* (low copy)	Korinek *et al*., 2000
YEp13-ECM33	*ECM33* in YEp13	See text
YEp13-EGT2	*EGT2* in YEp13	See text
YEp13-cdc24-PB1Δ	*cdc24-PB1Δ* in YEp13	See text
YEp13-CDC24	*CDC24* in YEp13	See text
pRS315-CDC24	*CEN6 LEU2 CDC24* (low copy)	See text
YEp13-cdc24-DH8	*cdc24-DH8* in YEp13	See text
pGAL-6xHIS-CDC42	*CEN4 LEU2* P_{GAL}*-CDC42* (low copy)	See text
YEp51(42)	2μ, *LEU2* P_{GAL}*-CDC42* (high copy)	Johnson and Pringle, 1990
pGAL-RHO1	*CEN4 LEU2* P_{GAL}*-RHO1* (low copy)	See text
pGAL-RHO2	*CEN4 LEU2* P_{GAL}*-RHO2* (low copy)	See text
pGAL-RHO3	*CEN4 LEU2* P_{GAL}*-RHO3* (low copy)	See text
pGAL-RHO4	*CEN4 LEU2* P_{GAL}*-RHO4* (low copy)	See text
pGAL-RHO5	*CEN4 LEU2* P_{GAL}*-RHO5* (low copy)	See text
YEp13-RHO1	*RHO1* in YEp13	See text
YEp13-RGD2	*RGD2* in YEp13	See text
YEp13-RPL30	*RPL30* in YEp13	See text
YEp13-NAB6	*NAB6* in YEp13	See text
YEp13-MNN9	*MNN9* in YEp13	See text
YEp13-YJL055W	*YJL055W* in YEp13	See text

Table 3.3. Suppression of the *iqg1Δ* or *chs2Δ* spore inviability

Dosage Suppressor	Tetrads dissected	Total viable spores	*iqg1Δ* or *chs2Δ* spores	Carrying plasmid
[A] *ECM33*	33	75	12	52
[C] *ECM33*	22	49	5	35
[A] *EGT2*	33	39	2	20
[C] *EGT2*	22	40	0	22
[A] *cdc24-PB1Δ*	22	65	20	62
[C] *cdc24-PB1Δ*	21	62	18	57
[A] *CDC24*	66	131	0	96
[A] *cdc24-DH8*	31	58	0	45
[B] *CDC42*	43	85	0	59
[A] *RHO1*	22	42	0	36
[B] *RHO1*	22	50	12	30
[B] *RHO2*	22	61	17	41
[B] *RHO3*	22	42	0	27
[B] *RHO4*	22	38	0	28
[B] *RHO5*	22	51	6	37
[A] *RGD2*	20	40	0	34
[A] *RPL30*	22	42	0	11
[A] *NAB6*	43	85	0	30
[A] *MNN9*	21	34	0	18
[A] *YJL055W*	11	21	0	16

[A] A strain heterozygous for *iqg1Δ* was transformed with a high-copy plasmid overexpressing an indicated dosage-suppressor gene, tetrads were then dissected on YPD from two genetically identical diploid transformants [except for *CDC24* (six identical) and *cdc24-DH8* (three identical)], and spore colonies were allowed to grow for at least 7 days before replicating to selective plates.

[B] A strain heterozygous for *iqg1Δ* was transformed with a low-copy plasmid overexpressing an indicated dosage-suppressor gene from a *GAL* promoter, tetrads were then dissected on YPGal/Raff from two genetically identical diploid transformants, and spore colonies were allowed to grow for at least 14 days before replicating to selective plates. In the case for *CDC42*, a low-copy and a high-copy plasmid (both overexpressing from a *GAL* promoter)

were used, and tetrads were dissected from four transformants.

[C] A strain heterozygous for *chs2Δ* was transformed with a high-copy plasmid overexpressing an indicated dosage-suppressor gene, tetrads were dissected on YPD from two genetically identical diploid transformants, and spore colonies were allowed to grow for at least 7 days before replicating to selective plates.

Table 3.4. Primer List of Chapter 3 [a]

Primer [a]	Purpose	Sequence
5'TTEF	Internal check primer of the *His3MX6* or *kanMX6* marker	5'-TATTTTTTTTCGCCTCGACATCATC TGCCC-3'
3'PTEF	Internal check primer of the *His3MX6* or *kanMX6* marker	5'-GTATGGGCTAAATGTACGGGCGAC AGTCAC-3'
5'TRP1	Internal check primer of the *TRP1* marker	5'-TAAAAGACTCTAACAAAATAGCAA ATTTCG-3'
3'TRP1	Internal check primer of the *TRP1* marker	5'-GTGCTTAATCACGTATACTCACGTG CTCAA-3'
ML176	5' primer to construct *chs2Δ::His3MX6*	5'-CATATAGACCCAAATAAAAACCAA AGAACCACATATAGAACGGATCCCC GGGTTAATTAA-3'
ML177	3' primer to construct *chs2Δ::His3MX6*	5'-AAAGAGGGAATGACGAGAAATTAG CTGAAAAATACTGGCAGAATTCGAGC TCGTTTAAAC-3'
CHS2-5'-656	Upstream check primer for *chs2Δ::His3MX6*	5'-ACTCGATCGAGCCTTCTCGCACT-3'
CHS2-3' -3370	Downstream check primer for *chs2Δ::His3MX6*	5'-ACTAGTTCTAGCGACACGTCCT-3'
ECM33dF	5' primer to construct *ecm33Δ*	5'-CTTTTAAAGATCTAGTTTTAATTTT ACTATTATTCCGCACGGATCCCCGGG TTAATTA-3'
ECM33dR	3' primer to construct *ecm33Δ*	5'-ACAATAAGAAATAAAAGAGATATT AACGACACTAAAATTGAATTCGAGCT CGTTTAAAC-3'
ECM33dK	Upstream check primer for *ecm33Δ*	5'-AGGTTGATTACTGCTTTATTTTCCT TTCTT-3'
ECM33dC	Downstream check primer for *ecm33Δ*	5'-CCAGCTACTCCAGCAACCCCAGCA ACCCCA-3
PST1dF	5' primer to construct *pst1Δ*	5'-AGAGAGAAGCAAAAAAAAAAAGC TCGCTATAAAAATATCCGGATCCCCG GGTTAATTA-3'
PST1dR	3' primer to construct	5'-GTACTAGTTATCAAAAATGAAGAA

	pst1Δ	TATTTCTTCATTCCAGAATTCGAGCTC GTTTAAAC-3'
PST1dK	Upstream check primer for *pst1Δ*	5'-CTCCTTTGGACTGAACCCGCATTA GTAATT-3'
PST1dC	Downstream check primer for *pst1Δ*	5'-AACAACCGTTAATATACAGGATGT TGAAGT-3
HOF1-F1	5' primer to construct *hof1Δ*	5'-TTGGAAAGTGTACTACTAATATTCA GAAAAAGGTGAAAGACGGATCCCCG GGTTAATTA-3'
HOF1-R1	3' primer to construct *hof1Δ*	5'-CTTTTATCAGAAAACTAGTAAAATT GATATACATCGAGAGAATTCGAGCTC GTTTAAAC-3'
HOF1/-500F	Upstream check primer for *hof1Δ*	5'-GTGTAATCGTCAGAGTGCACTATTT -3'
HOF1/3510R	Downstream check primer for *hof1Δ*	5'-TCCTGCCTAAATGGAGCCATATTCA -3'
RHO2dF	5' primer to construct *rho2Δ*	5'-TGAAACGTTCTGCTTTGGTTGTGCT TTTGATCCCGTACTCGGATCCCCGG GTTAATTA-3'
RHO2dR	3' primer to construct *rho2Δ*	5'-TGCTAAAAAGATAATGTATCATTT CAGTGTAAGTTTTTTGAATTCGAGCT CGTTTAAAC-3'
RHO2dK	Upstream check primer for *rho2Δ*	5'-TAGGACTTAACTTGGATTACTCGA CGTTTA-3'
RHO2dC	Downstream check primer for *rho2Δ*	5'-TTTTTAAGATATGTTCCAGTTGAGA AATTT-3
RHO5dF	5' primer to construct *rho5Δ*	5'-AATATTTATTAAATACAATATAGTA TACTAATAAGTCTGCGGATCCCCGGG TTAATTA-3'
RHO5dR	3' primer to construct *rho5Δ*	5'-ATATGTAATTATTTATTTACTTTTT CTGCCTCCTTGATAGAATTCGAGCTC GTTTAAAC-3'
RHO5dK	Upstream check primer for *rho5Δ*	5'-ATACTAAAGCTGCAGTGAGAGTAA ACCGGA-3'
RHO5dC	Downstream check primer for *rho5Δ*	5'-GTTTTTCGCAAGCAGCAAGCTACA ATTGGG-3
ROM2-F1	5' primer to construct *rom2Δ*	5'-TGCTGACTTAATTGGACAATTCATC TCTTTTCCTGCGGTTCGGATCCCCGG GTTAATTAA-3'

ROM2-R1	3' primer to construct *rom2Δ*	5'-ATTCTAAAGAAAATAAGGAAAGTC TATATACGTTGCTATCGAATTCGAGC TCGTTTAAAC-3'
ROM2/-501F	Upstream check primer for *rom2Δ*	5'-AGTGTTAGGATCTACAGGGAAAGT -C3'
ROM2/4590R	Downstream check primer for *rom2Δ*	5'-GAAGTAAGCTTGAAGCGAGTGAAG -A3'
TUS1-F1	5' primer to construct *tus1Δ*	5'-AACATTAAAACAAAAAACTTATTG AGTGCAGCAAGTTAACCGGATCCCCG GGTTAATTAA-3'
TUS1-R1	3' primer to construct *tus1Δ*	5'-TTACAACGATATTTACCATTAAAAG TGTCTATATCTTATAGAATTCGAGCT CGTTTAAAC-3'
TUS1/-481F	Upstream check primer for *tus1Δ*	5'-AACCAGTCATGCAAAGGAAATGCG -T3'
TUS1/4430R	Downstream check primer for *tus1Δ*	5'-GGTCGGGACAGGTCGCCTGGAAGA -G3'
CDC24P3	5' primer to construct YEp13-CDC24	5'-ATATCATATAATAACAACTCTAATA ATACCTCTAGTAGCG-3'
CDC24P4	3' primer to construct YEp13-CDC24	5'-TAGGTTGAGGCCGTTGAGCACCGC CGCCGCAAGGAATGGTTTTTTTCTTTT TGTTTTCACAGGACTCATT-3'
CDC24P7	5' primer to construct YEp13-cdc24-DH8	5'-ACAGCAAATCATGAACGCAGAAAA CCAACACCGACATCAG-3'
CDC24P8	3' primer to construct YEp13-cdc24-DH8	5'-GCTGCCATTGTTGTCCGTTATGTTC -3'
pQErev	5' primer to construct pGAL-6xHIS-CDC42	5'-AAGCTCAGCTAATTAAGC-3'
SalI-HIS	3' primer to construct pGAL-6xHIS-CDC42	5'-ACGCGTCGACATGAGAGGATCGCA TCAC-3'
T3	5' primer to construct pQE-6xHIS-CDC42	5'-ATTAACCTCACTAAAGGGA-3'
BamHI -CDC42	3' primer to construct pQE-6xHIS-CDC42	5'-CGGGATCCATGCAAACGCTAAAGT GTGT-3'
GAL-RHO1	5' primer to construct pGAL-RHO1	5'-TACCTCTATACTTTAACGTCAAGGA GAAAAAACTATAATGTCACAACAAG TTGGTAA-3'

3'IF-RHO1	3' primer to construct pGAL-RHO1	5'-CCCCGGGCTGCAGGAATTCGATAT CAAGCTTATCGATACCAATAGATGAT AATACTTACT-3'
GAL-RHO2	5' primer to construct pGAL-RHO2	5'-TACCTCTATACTTTAACGTCAAGGA GAAAAAACTATAATGTCTGAAAAGG CCGTTAG-3'
3'IF-RHO2	3' primer to construct pGAL-RHO2	5'-CCCCGGGCTGCAGGAATTCGATAT CAAGCTTATCGATACCTCCGTCGGAA ATTTTGCTTC-3'
GAL-RHO3	5' primer to construct pGAL-RHO3	5'-TACCTCTATACTTTAACGTCAAGGA GAAAAAACTATAATGTCATTTCTATG TGGGTC-3'
3'IF-RHO3	3' primer to construct pGAL-RHO3	5'-CCCCGGGCTGCAGGAATTCGATAT CAAGCTTATCGATACCTCGAGCACAT GCTGGAGGGG-3'
GAL-RHO4	5' primer to construct pGAL-RHO4	5'-TACCTCTATACTTTAACGTCAAGGA GAAAAAACTATAATGAATACACTATT ATTTAA-3'
3'IF-RHO4	3' primer to construct pGAL-RHO4	5'-CCCCGGGCTGCAGGAATTCGATAT CAAGCTTATCGATACCTGAATTATTA TGATAAAATT-3'
GAL1-seq	5' primer to construct pGAL-RHO5	5'-AACAAAAAATTGTTAATATACC-3'
3'IF-RHO5	3' primer to construct pGAL-RHO5	5'-GGCTGCAGGAATTCGATATCAAGC TTATCGATACCACTATTTGATAATAC GGAAAGAGAT-3'

[a] Primers that were used to construct *iqg1Δ*, *chs2Δ::kanMX6*, and *cyk3Δ* strains are listed in Nishihama *et al*., 2007 (Table 3.4).

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CHAPTER 4

CONTROL OF 5-FOA RESISTANCE BY *SACCHAROMYCES CEREVISIAE YJL055W*

ABSTRACT

In a *URA3*/5-FOA-based dosage-suppressor screen, we isolated a plasmid containing the little-characterized ORF *YJL055W*. Further analysis showed that this gene did not suppress the mutation of interest. Instead, overexpression of Yjl055Wp directly suppresses the inviability of $URA3^{+}$ cells in the presence of 5-FOA. However, deletion of *YJL055W* has no detectable effect on 5-FOA resistance. Based on these observations and a previous report that a *yjl055wΔ* mutant has increased sensitivity to purine-analog mutagens, we suggest that Yjl055Wp may function in one of several pathways for the detoxification of base analogs. However, its precise mechanism of action remains unknown

INTRODUCTION

In *Saccharomyces cerevisiae*, 5-fluoroorotic acid (5-FOA) has long been used in various genetic screens to select against *URA3*$^{+}$ cells (Boeke *et al*., 1984; Rothstein, 1991; Sikorski and Boeke, 1991). *URA3* encodes orotidine-5'-phosphate decarboxylase, an enzyme in the uracil biosynthetic pathway that can convert 5-FOA into 5-fluorouracil (5-FU). 5-FU is a pyrimidine analog that can be mis-incorporated into RNA and DNA in place of uracil or thymine, leading to interference with RNA and DNA processing and function and thus to cell death. *ura3* mutants require exogenous uracil for growth and are viable in medium containing 5-FOA. The presence of a *URA3*-marked plasmid makes *ura3* cells sensitive to 5-FOA, but they can survive on medium containing 5-FOA and uracil by spontaneously losing the plasmid. Thus, dosage-suppressor screens can be conducted by transforming a genomic or cDNA library in a vector with a non-*URA3* marker into a strain that harbors both a lethal mutation and a *URA3*-marked plasmid that contains a wild-type copy of the mutated gene. The original strain is inviable on 5-FOA medium, but the presence of a library plasmid that carries either a wild-type copy of the mutated gene or a dosage suppressor allows cells to lose the *URA3* plasmid and thus survive in the presence of 5-FOA. A potential problem with such a screen is that a gene might be isolated not because it suppresses the lethal mutation of interest but because it directly suppresses the lethality caused by 5-FOA in *URA3*$^{+}$ cells. We report here that the largely uncharacterized ORF *YJL055W* is such a gene.

MATERIALS AND METHODS

Strains, Growth Conditions, Growth-Rate Measurements, and Genetic Methods

The *S. cerevisiae* strains used in this study were RNY757 (*MAT**a** his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 myo1Δ::kanMX6 hof1Δ::TRP1* [pRS316-MYO1]) (see Chapter 3), DC-5 (*MAT**a** leu2-3,112 his3 can^R URA3$^+$*) (Broach *et al*., 1979), and KO1468 (as DC-5 except *yjl055wΔ::His3MX6*). To construct KO1468, the entire coding region of *YJL055W* in strain DC-5 was deleted using the PCR method (Baudin *et al*., 1993; Longtine *et al*., 1998) and the primers indicated in Table 4.1. The success of the deletion was confirmed by two PCR tests that used check primers that were upstream and downstream of the deleted region together with primers internal to the selectable marker (Longtine *et al*., 1998; Table 4.1). Yeast were grown on YP rich medium, synthetic complete (SC) medium, or SC medium lacking specific nutrients as needed to select plasmids or transformants (Guthrie and Fink, 1991). 2% glucose was used as carbon source. Liquid cultures were grown at 23˚C, and plates were incubated at 30˚C. 5-FOA (Research Products International, Mt. Prospect, IL) was used at 1 mg/ml (except as indicated) to select against *URA3*$^+$ cells. To determine growth rates, exponential-phase cultures ($OD_{600} \approx 0.4$) in SC-Ura medium were diluted two-fold with fresh SC-Ura, incubation was continued, and the times needed to return to the original OD_{600} were recorded as the doubling times. Standard procedures were used for growth of *Escherichia coli*, genetic manipulations, polymerase chain reaction (PCR), and other molecular biological procedures (Sambrook *et al*., 1989; Guthrie and Fink, 1991; Ausubel *et al*., 1995).

Plasmids

Plasmid pRS316-MYO1 contains the *MYO1* gene in the low-copy, *URA3*-marked vector pRS316 (Sikorski and Hieter, 1989; Nishihama *et al*., 2007). Plasmid p757-8-2 (see Figure 4.1A) was isolated in a dosage-suppressor screen using strain RNY757 and a genomic-DNA library (DeMarini *et al*., 1997) constructed by cloning *Sau*3A fragments into the *Bam*HI site of plasmid YEp13 (high-copy, *LEU2*; Broach *et al*., 1979). Plasmid p757-8-2-ΔYJL055W (see Figure 4.1A) was constructed using the PCR method (Baudin *et al*., 1993). Cells of a *leu2Δ his3Δ* strain containing p757-8-2 were transformed with a PCR fragment obtained using plasmid pFA6a-His3MX6 (Longtine *et al*., 1998) as template and primers YEp13sp168 and YJL55dsr2 (Table 4.1). This was expected to replace the region from nucleotide +258 relative to the start site of *YJL055W* to nucleotide +10 relative to the *Bam*HI cloning site of YEp13 with the *His3MX6* marker, and the success of the construction was confirmed by two PCR tests that used the isolated plasmid as template and check primers that were upstream and downstream of the deleted region together with primers internal to the selectable marker (Table 4.1). Plasmid YEp13-YJL055Wsub (see Figure 4.1A) was constructed in two steps. First, a fragment containing nucleotides from -621 relative to the start site of *YJL055W* to +521 relative to its stop codon was PCR-amplified using p757-8-2 as template and primers YJL055WF and YJL055WR (Table 4.1). YJL055WF contained nucleotides -49 to -11 relative to the *Bam*HI site of YEp13 plus nucleotides -621 to -601 relative to the start site of *YJL055W*, whereas YJL055WR contained nucleotides +49 to +11 relative to the *Bam*HI site of YEp13 plus nucleotides +521 to +501 relative to the stop codon of *YJL055W*. In the second step, the PCR product from the first step was purified and transformed together with *Bam*HI-cut YEp13 into *leu2Δ* yeast cells, and transformants (containing plasmids resulting

from homologous recombination) were selected on SC-Leu plates. Several independent plasmids were isolated and gave identical results in subsequent tests.

RESULTS AND DISCUSSION

To identify additional proteins involved in cytokinesis, we used a high-copy, *LEU2*-marked genomic library to screen for dosage suppressors of the 5-FOA-induced lethality of a *myo1Δ hof1Δ* [*URA3 MYO1* plasmid] strain (see Chapter 3, Materials and Methods). Among the several plasmids isolated, p757-8-2 displayed the strongest suppression (data not shown); it proved to contain the complete *YJL055W* ORF plus truncated copies of the neighboring genes (Figure 4.1A). We then asked if p757-8-2 could suppress a deletion of *IQG1* (whose product, like Myo1p and Hof1p, is involved in cytokinesis). p757-8-2 strongly suppressed the 5-FOA-induced lethality of an *iqg1Δ* [*URA3 IQG1* plasmid] strain, but it could not suppress the lethality of *iqg1Δ* spores on YP plates (data not shown). These discordant results suggested that p757-8-2 might not suppress the *myo1Δ hof1Δ* and *iqg1Δ* defects but instead interfere with the action of 5-FOA. Indeed, when we transformed plasmids p757-8-2 and YEp13-YJL055Wsub (which contains *YJL055W* plus small fragments of the neighboring genes; Figure 4.1A) into a strain that was wild type at the *URA3* chromosomal locus, the transformants were viable in the presence of 5-FOA (Figure 4.1B). In contrast, a plasmid in which most of *YJL055W* had been deleted could not rescue the same strain on a 5-FOA plate (Figure 4.1, A and B). We next tested if deletion of *YJL055W* would lead to an increased sensitivity to 5-FOA. *yjl055wΔ* cells appeared to grow slightly less well than wild-type *URA3*$^{+}$ cells in the presence of low concentrations of 5-FOA (Figure 4.1C, top four rows). However, this slight growth difference appeared to be independent of 5-FOA (Figure 4.1C, bottom row; also, in liquid SC-Ura medium, doubling times of 100 and 166 min were observed for wild-type and *yjl055wΔ* cells, respectively). Thus, although overexpression of

Yjl055Wp can suppress the inviability of *URA3*$^{+}$ cells in the presence of 5-FOA, deletion of *YJL055W* has little or no effect on 5-FOA resistance.

There has been little information available previously about the function of Yjl055Wp. BLASTP searches reveal homologs in Archaea, Eubacteria, fungi, and plants (but, interestingly, apparently not in animals), suggesting that at least some aspects of Yjl055Wp function are ancient in origin and have been conserved. Some of these homologs have been annotated as "possible lysine decarboxylases", based on a PGGxGTxxE motif that is shared with Yjl055Wp but whose association with lysine decarboxylase activity does not actually seem very clear, given that several bona fide enzymes of this type lack the motif (Kikuchi *et al*., 1997). Most of the Yjl055Wp homologs also share with Yjl055Wp a Rossmann-fold motif ($Gx_{1-2}GxxG$) that is indicative of possible nucleotide-binding activity (Kleiger and Eisenberg, 2002; Kukimoto-Niino *et al*., 2004). In the only previously published report on Yjl055Wp itself, Stepchenkova *et al*. (2005) showed that *yjl055w*Δ strains have modestly elevated sensitivities to the purine-analog mutagens 6-hydroxylaminopurine (HAP) and 2-amino-6-hydroxylaminopurine (AHA), which function by misincorporation into DNA (Pavlov *et al*., 1991; de Serres, 1991).

Because Yjl055Wp can protect cells from both pyrimidine and purine analogs and presumably from effects at the levels of both DNA and RNA, it seems unlikely that it functions by affecting the transport of analogs into cells, the levels of normal nucleotide pools, or DNA repair. Instead, it seems most likely to function through the metabolic detoxification of the base analogs. Because there are probably multiple pathways for such detoxification, it is not really surprising that the effects of deleting *YJL055W* are modest (HAP and AHA) or undetectable (5-FOA) despite the strong effect of *YJL055W*

overexpression on 5-FOA sensitivity. Because *yjl055w*Δ cells grow significantly more slowly than congenic wild-type cells even in the absence of added base analogs, we speculate that Yjl055Wp functions in the detoxification of base analogs that accumulate in the course of normal metabolism (e.g., see Simandan *et al*., 1998) and/or that its detoxification ability is a by-product of another metabolic activity. However, further studies will clearly be necessary to define the molecular details of Yjl055Wp function.

FIGURES (1)

Figure 4.1. Figure 1 of Chapter 4

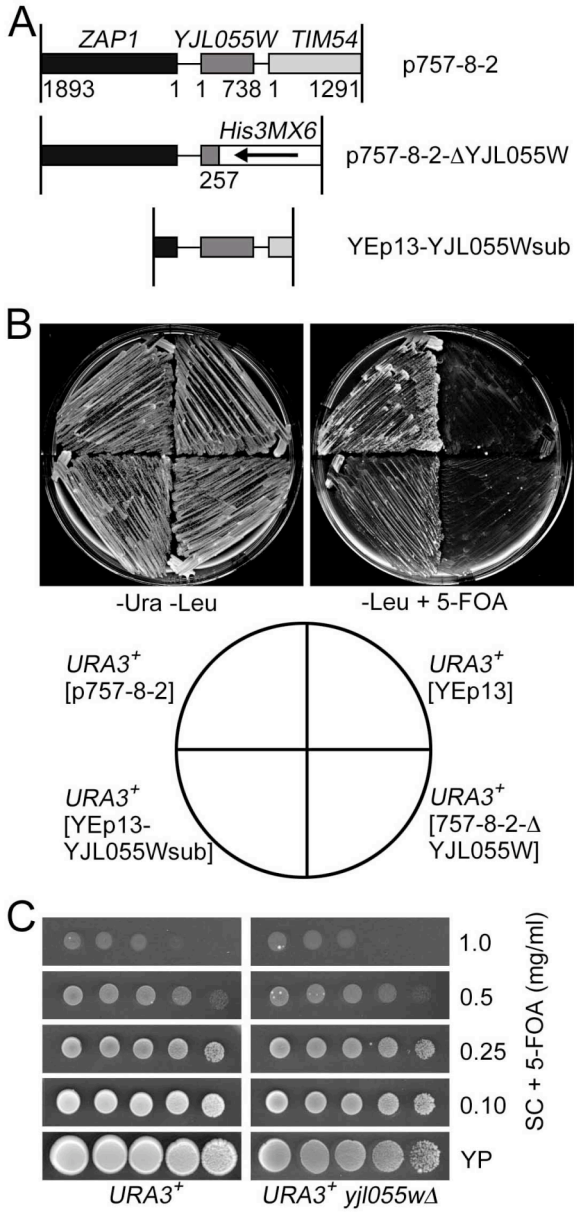

FIGURE LEGENDS (1)

Figure 4.1. Effects on 5-FOA-induced lethality of overexpression or deletion of *YJL055W*. (A) The inserts of the relevant plasmids. Plasmid p757-8-2 contains an N-terminal fragment of the 2643-nucleotide *ZAP1* ORF, the complete *YJL055W* ORF, and an N-terminal fragment of the 1437-nucleotide *TIM54* ORF. Plasmid p757-8-2-ΔYJL055W contains the same N-terminal *ZAP1* fragment, a 257-nucleotide N-terminal YJL055W fragment, and a *His3MX6* marker (the arrow indicates the direction of transcription). Plasmid YEp13-YJL055Wsub contains a 336-nucleotide N-terminal *ZAP1* fragment, the complete *YJL055W* ORF, and a 349-nucleotide N-terminal *TIM54* fragment. (B) Viability in the presence of 5-FOA of *URA3*$^+$ cells overexpressing Yjl055Wp. Strain DC-5 was transformed with the indicated plasmids and grown on SC-Ura-Leu (2 days) and SC-Leu+5-FOA (6 days) plates. Only the plasmids harboring the complete *YJL055W* ORF could confer 5-FOA resistance to the *URA3*$^+$ cells. (C) Similar sensitivities of wild-type and *yjl055wΔ* cells to 5-FOA. Strain DC-5 and its *yjl055wΔ* derivative KO1468 were grown to exponential phase ($OD_{600} \approx 0.4$), and 10-μl aliquots of a dilution series (undiluted and 4x, 10x, 100x, and 1000x diluted) were spotted onto YP plates (bottom row) or SC plates containing 5-FOA at the indicated concentrations (top four rows) and grown for 4 days.

Table 4.1. Primer List of Chapter 4

Primer	Purpose	Sequence
YJL55dF	5' primer to construct *yjl055wΔ*	5'-TAGAGTAAATCGCAGGCAATCCAG TACTAAGAAACGGCGCGGATCCCCG GGTTAATTA-3'
YJL55dR	3' primer to construct *yjl055wΔ*	5'-TACAGATCGTTTAAACTAGATACAT GACTTACGTTTCTAGAATTCGAGCTC GTTTAAAC-3'
YJL55dK	Upstream check primer for *yjl055wΔ*	5'-TGCAAAAGAATGATTATTAGAATT ATACGT-3'
YJL55dC	Downstream check primer for *yjl055wΔ*	5'-GCCCATCCGTCGATGCCAATATTTC CTTTC-3'
3'PTEF	Internal check primer of the *His3MX6* marker	5'-GTATGGGCTAAATGTACGGGCGAC AGTCAC-3'
5'TTEF	Internal check primer of the *His3MX6* marker	5'-TATTTTTTTTCGCCTCGACATCATC TGCCC-3'
YEp13sp168	5' primer to construct p757-8-2-ΔYJL055W	5'-CTTGGAGCCACTATCGACTACGCG ATCATGGCGACCACACGGATCCCCGG GTTAATTA-3'
YJL55dsr2	3' primer to construct p757-8-2-ΔYJL055W	5'-CTGATTTAAGCGGACAGGTTCACG GTATCATTCCAAATGGAATTCGAGCT CGTTTAAAC-3'
YEp13cp168	Upstream check primer for p757-8-2-ΔYJL055W	5'-GCATAGTACCGAGAAACTAGTGCG AAGTAG-3'
YEp13cp169	Downstream check primer for p757-8-2-ΔYJL055W	5'-CTGTCCCTGATGGTCGTCATCTACC TGCCT-3'
YJL055WF	5' primer to construct YEp13-YJL055Wsub	5'-CTTGGAGCCACTATCGACTACGCG ATCATGGCGACCACAACTCGAAATGT TGGCAGTCG-3'
YJL055WR	3' primer to construct YEp13-YJL055Wsub	5'-ACCGCACCTGTGGCGCCGGTGATG CCGGCCACGATGCGTGCATAATACAG TACAGGCTT-3'

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CHAPTER 5

DISCUSSION

Various fungi are excellent model systems for the study of cytokinesis, because fungal cytokinesis shares similarities with both animal and plant cytokinesis. Specifically, both animal and fungal cells divide by utilizing a contractile actomyosin ring, and both plant and fungal cells form a dividing structure made of cell-wall materials during cytokinesis. The central aim of this work is to investigate septin-dependent, actomyosin-ring-independent cytokinesis in the budding yeast *Saccharomyces cerevisiae*.

Synthetic-lethal and dosage-suppressor screens starting with septin and *myo1Δ* mutants have found that deletion of genes encoding nonessential subunits of the anaphase-promoting complex or cyclosome (APC/C) and overexpression of Iqg1p or Cyk3p can suppress the growth defects of *myo1Δ* cells. Furthermore, Iqg1p has been identified as a novel APC/C substrate, and the elevated levels of Iqg1p in APC/C mutants account for the suppression of the *myo1Δ* growth defects. In addition, dosage-suppressor screens starting with *myo1Δ cyk3Δ* and *myo1Δ hof1Δ* double mutants have found that overexpression of either Ecm33p or a truncated Cdc24p lacking its PB1 domain can promote the formation of primary-septum-like structures and possibly more organized thick septa in the absence of the actomyosin ring. Finally, unrelated to cytokinesis, dosage-suppressor screens have unexpectedly found that overexpression of Yjl055Wp can suppress the 5-FOA-induced lethality of $URA3^{+}$ cells. These results are described in details in Chapters 2-4.

As with any work of this magnitude, there are a number of unresolved issues that need to be addressed. The following sections represent some of these issues.

myo1Δ *Phenotypes in the S288C and W303 Strain Backgrounds*

One unresolved issue is the difference between the *myo1Δ* phenotypes in the S288C and W303 strain backgrounds (Bi *et al*., 1998; Tolliday *et al*., 2003; see Chapter 2). One possible explanation is that the actomyosin ring is essential in *S. cerevisiae*, and thus the viability observed in most genetic backgrounds, including S288C, is the result of some unidentified suppressor mutation(s). This explanation is not plausible because the viable *myo1Δ* spore colonies were isolated by tetrad dissections of several heterozygous *myo1Δ* diploid strains. Thus, it is doubtful that each of the heterozygous *myo1Δ* mutants or the *myo1Δ* spore colonies independently acquired a suppressor mutation. An alternative explanation is that the actomyosin ring is nonessential in *S. cerevisiae*, and thus the inviability observed in the W303 strain background is the result of some unidentified synthetic-lethal mutation(s), accumulated through polymorphisms. Consistent with this model, previous studies have shown that no actomyosin ring can be detected in viable *myo1Δ* cells, indicating that there must be an actomyosin-ring-independent mechanism to complete cytokinesis (Bi *et al*., 1998). Our recent studies have shown that Iqg1p and Cyk3p are involved in such mechanism, but sequence analysis of both genes in the W303 background has revealed no mutation (Nishihama *et al*., 2007). Thus, the unknown synthetic-lethal mutation(s) may be located within some other gene(s) involved in cytokinesis. Currently, a dosage-suppressor screen, using *myo1Δ* mutant in the W303 strain background, is being conducted to identify this gene(s).

Delay in Mitotic Exit and* myo1Δ *Phenotypes

This study has shown that *myo1Δ* and *net1Δ* are synthetic lethal, suggesting that the mechanism of suppression of the *myo1Δ* phenotypes by the APC/C mutations may involve a simple delay in mitotic exit, which gives mutant cells more time to build the partially functional septum. However, delaying mitotic exit by overexpressing a nondegradable mitotic cyclin does not suppress the same phenotypes. One explanation for this inconsistency is that the overexpression of a nondegradable mitotic cyclin causes a severe delay in mitotic exit, which may induce cytokinetic defects by itself. Conversely, mutation in a nonessential APC/C subunit causes only a slight delay, which gives *myo1Δ* cells enough extra time to carry out the inefficient cytokinesis without causing any additional cytokinetic defects. One problem with this explanation is that unlike a deletion of other nonessential APC/C subunits, *mnd2Δ* appears to have no detectable effect on cell-cycle progression in mitotic cells (Oelschlaegel *et al.,* 2005; Page *et al*., 2005), suggesting that the suppression of the *myo1Δ* phenotypes by *mnd2Δ* is likely to be independent of mitotic exit. An alternative explanation for the inconsistency is that *net1Δ* prematurely activates the APC/C-mediated degradation of Iqg1p. Premature degradation of Iqg1p compromises the actomyosin-ring-independent mechanism of cytokinesis, which is lethal in combination with the absence of the actomyosin ring. Consistent with this explanation, *net1Δ* single mutant cells appear have cytokinetic defects (data not shown). Finally, another explanation for the inconsistency is that *net1Δ* can cause Iqg1p-independent growth defects and that the synthetic lethality between *myo1Δ* and *net1Δ* is merely the result of two unrelated growth defects being too damaging for the mutant cells to overcome.

The Novel APC/C-Recognition Motif in Iqg1p

This study has demonstrated that the absence of a novel N-terminal recognition motif makes

Iqg1p nondegradable by the APC/C. Surprisingly, the two previously potential destruction-box (DB) sequences found in Iqg1p turned out to be unnecessary for the degradation of Iqg1p, demonstrating that the existence of DB sequence is often not a good indicator for identifying bona fide APC/C targets. As for the novel recognition motif, although it does not follow exactly the conserved sequence of a DB or KEN-box, it does contain similar conserved amino acids (arginine, lysine, and asparagine). Important tests are to see whether the other amino acids within this motif are required for the recognition and to determine the exact mechanism (as a DB, KEN-box, or something entirely different) by which this novel motif functions.

Significance of Timely APC/C-mediated Degradation of Iqg1p

Another issue that needs to be addressed is the significance of the APC/C-dependent proteolysis of Iqg1p. In other words, why does Iqg1p have to be degraded after cytokinesis? Recent studies have shown that *S. cerevisiae* cells are significantly sensitive to the cellular level of Mlc1p, which is the essential light chain for Iqg1p, Myo1p, and type V myosin Myo2p, and that the Mlc1p-Myo2p interaction is important for septum formation, cell separation, and new bud formation (Luo *et al*., 2004). Thus, heterozygous *mlc1Δ* diploid cells display haploinsufficiency that can be suppressed by reduced copy of *MYO2* (Stevens and Davis, 1998), and the overexpression of a Myo1p C-terminal tail (containing the Mlc1p-binding IQ motifs) leads to cytokinetic defects that can be alleviated by overexpression of Mlc1p (Tolliday *et al*., 2003). Furthermore, our recent studies have shown that overexpression of wild-type Iqg1p can cause minor cytokinetic defects (Nishihama *et al*., 2007) and that overexpression of the nondegradable Iqg1p from a *GAL* promoter causes severe cytokinetic defects. Taken together, these results suggest that the timely degradation

of Iqg1p may be important for providing Mlc1p to interact with Myo2p, that the association of Mlc1p with each binding partner may need to be fine-tuned temporally, and that the APC/C-dependent proteolysis of Iqg1p may contribute to this fine-tuning.

Possible Nuclear Localization of Iqg1p

Another unresolved issue is the exact location of the APC/C-Iqg1p interaction. In *S. cerevisiae*, the localization of the APC/C or the primary site of its activity is nuclear (Sikorski *et al*., 1993; Jaquenoud *et al*., 2002; Huh *et al*., 2003; Melloy and Holloway, 2004). Thus, in order to be properly ubiquitinated, many of the known APC/C substrates require transport into the nucleus. For examples, both Kip1p and Cin8p contain a nuclear localization sequence (NLS) (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001), and the proper degradation of Pds1p seems to require a certain nuclear transport protein (Bäumer *et al*., 2000). Another APC/C target, Hsl1p, has been shown to localize to the bud neck. Thus, Hsl1p is either transported into the nucleus or ubiquitinated as the nucleus passes through the bud neck during nuclear migration (Burton and Solomon, 2000; Burton and Solomon, 2001). Iqg1p has been shown to localize to the bud neck as a ring (Epp and Chant, 1997; Shannon and Li, 1999; Nishihama *et al*., 2007; see Chapter 2), and because Iqg1p appears to be fully functional until after cytokinesis, it is unlikely to be ubiquitinated as early as nuclear migration. Thus, Iqg1p is probably shuttled into the nucleus, either through its own possible NLS or by binding to another nuclear localizing protein, to be ubiquitinated after cytokinesis.

Possible Conserved Regulation of IQGAPs by the APC/C

Iqg1p is the only known member of the conserved IQGAP family in *S. cerevisiae* (Brown and Sacks, 2006), and because the APC/C is also a conserved complex, it is possible that IQGAPs in other organisms are also regulated by the APC/C, and these regulations are

evolutionarily conserved. This hypothesis is especially possible for Rng2p in *S. pombe* and IQGAP1 in mammalian cells, because they have also been shown to be involved in cytokinesis. Specifically, IQGAP1 was one of the proteins identified in midbodies isolated from Chinese Hamster Ovary cells, and it appears to be required for completion of meiotic and mitotic divisions as well as germline formation in *Caenorhabditis elegans*, based on RNAi studies (Skop *et al*., 2004). Furthermore, Rng2p has been indicated to be essential for actomyosin-ring formation and cytokinesis in *S. pombe* cells (Eng *et al*., 1998; Wu *et al*., 2003). Logical experiments are to determine if the overall protein level of Rng2p is cell-cycle-regulated and if this possible regulation is APC/C-dependent. Similar questions should be answered for IQGAP1 and any other IQGAPs that might play a role in cytokinesis.

Although it is possible that Rng2p and/or IQGAP1 are regulated by the APC/C and that each protein has a single DB consensus sequence, neither protein appears to contain any obvious sequence similar to the novel APC/C-recognition motif identified in Iqg1p. BLASTP searches with Iqg1p sequence has revealed that in two other yeast species, *Candida glabrata* and *Kluyveromyces lactis*, there are uncharacterized proteins (XP_446838.1 and XP_453638.1, NCBI protein database) that contain N-terminal sequences (a.a. 25-34 and a.a. 33-42, respectively) similar to the novel APC/C-recognition motif. Although these two uncharacterized proteins have yet to be verified functionally as IQGAPs, they appear to contain some of the other known domains of IQGAP. If they are indeed bona fide IQGAPs and their homologous N-terminal sequences are real APC/C-recognition motifs, then the regulation of IQGAPs by the APC/C appears to be conserved, at least among some yeast organisms. This potential finding would suggest that Rng2p and IQGAP1, without the novel recognition motif, might not be APC/C targets.

Suppressions of* iqg1Δ and chs2Δ *Defects by Overexpressing Ecm33p or Cdc24-PB1Δp

Another unresolved issue is the exact mechanism by which overexpression of Ecm33p or Cdc24-PB1Δp suppresses the *iqg1Δ* and *chs2Δ* growth defects. Studies with electron microscopy (EM) have shown that instead of a trilaminar-septum structure, *iqg1Δ* and *chs2Δ* single mutant cells can only form thick septa (Schmidt *et al*., 2002; Nishihama *et al*., 2007; see Chapter 3). Thus, the primary-septum-like structures in *iqg1Δ* and *chs2Δ* cells overexpressing Ecm33p probably help mutant cells to grow better by providing a more efficient method of cell separation. Ecm33p has been suggested to play a role in the N-linked glycosylation and cell-wall anchoring of mannoproteins (Pardo *et al*., 2004). Thus, it is possible that Ecm33p participates in an actomyosin-ring-independent and Iqg1p-Cyk3p-involved pathway of primary-septum construction by regulating mannoproteins and other cell-wall materials. Because the observed primary-septum-like structures do not appear very frequently, the overexpression of Ecm33p probably also suppresses the *iqg1Δ* and *chs2Δ* growth defects via another mechanism(s). One possibility is that overexpressing Ecm33p may help mutant cells defective in cytokinesis to construct more stable and stronger thick septa, which may prevent cell lysis, especially during cell separation. Because the deletion of *ECM33* does not cause any detectable cytokinetic defect at 23˚C and only causes minimal multi-cell clustering at 37˚C, it is likely that Ecm33p only plays a minor role in normal cytokinesis.

The formation of primary-septum-like structures in *iqg1Δ* cells, but not in *chs2Δ* cells, overexpressing Cdc24-PB1Δp suggests that the suppression may involve more than one mechanism. Perhaps the overexpression of Cdc24-PB1Δp can help mutant cells to produce primary-septum-like structures (only in the presence of chitin made by Chs2p), build more

stable thick septa (by over-activating the cell-integrity pathways), and/or conduct more efficient hydrolysis of cell-wall materials during cell separation. Furthermore, the suppression is likely to be independent of Cdc42p but dependent of the GEF activity of Cdc24-PB1Δp, which may function through another Rho-type GTPase(s) and/or an unknown factor(s). To verify this model, it is possible to test the GEF activity of Cdc24-PB1Δp for Rho1p, Rho2p, and/or Rho5p via *in vitro* assays.

Roles of Iqg1p and Cyk3p in Actomyosin-Ring-Independent Cytokinesis

A central goal of our investigation of actomyosin-ring-independent cytokinesis is to understand the cytokinetic roles of Iqg1p and its possible downstream targets, including Cyk3p. Based on EM analysis, *myo1Δ* cells can form both thick septa and disorganized primary-septa-like structures, which can be increased in number by the overexpression of Iqg1p or Cyk3p (Schmidt *et al*., 2002; Nishihama *et al*., 2007). The overexpression of Cyk3p can also induce the formation of primary-septa-like structures in *iqg1Δ* cells, and *cyk3Δ* cells appear to synthesize primary and secondary septa simultaneously, suggesting that there may be a delay in the construction of primary septum (Nishihama *et al*., 2007). Taken together, these results suggest that the formation of the actomyosin ring and the stimulation of the Cyk3p pathway are two functions of Iqg1p. Furthermore, Cyk3p may function in the construction of primary septum by controlling targeted vesicle secretion and membrane invagination at the bud neck and/or the synthesis of cell-wall chitin (Nishihama *et al*., 2007). Thus, even in the absence of the actomyosin ring, Cyk3p can complete primary-septum construction, albeit less efficiently. In other words, the actomyosin-ring contraction and the Cyk3p pathway are two mechanisms involved in the construction of primary septum. Perhaps because the mother-bud necks of *S. cerevisiae* cells are quite narrow, neither

mechanism by itself is essential for primary-septum formation, thus both *myo1Δ* and *cyk3Δ* single mutant cells can synthesize some primary septa or primary-septum-like structures. However, when both mechanisms are compromised, such as in *myo1Δ cyk3Δ* double and *iqg1Δ* single mutants, cells can only form thick septa, which often lead to severe growth defects.

Possible Detoxification of Nucleotide Analogs by Yjl055Wp

Yjl055Wp was identified as a dosage-suppressor of the inviability of $URA3^{+}$ cells in the presence of 5-FOA. Because this little-known protein can protect cells from both pyrimidine and purine analogs and presumably from effects at the levels of both DNA and RNA, it seems unlikely that it functions by affecting the transport of analogs into cells, the levels of normal nucleotide pools, or DNA repair. Instead, it seems most likely to function through the metabolic detoxification of the nucleotide analogs. However, further studies will clearly be necessary to define the molecular details of Yjl055Wp function.

Final Remarks

In conclusion, the results described in this work, suggest that Iqg1p, a novel APC/C target, may stimulate Cyk3p and other proteins (such as those involved in targeted membrane addition and/or cell-wall synthesis/integrity) in a cell-cycle-regulated manner to promote the construction of a more normal septum through the actomyosin-ring-independent mechanism of cytokinesis in *S. cerevisiae*. However, many of the pathways and components of actomyosin-ring-independent cytokinesis remain to be identified, and the precise molecular roles that Iqg1p and Cyk3p play in actomyosin-ring-independent cytokinesis remain to be elucidated.

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