HARVARD UNIVERSITY
Graduate School of Arts and Sciences

DISSERTATION ACCEPTANCE CERTIFICATE

The undersigned, appointed by the

Division of Medical Sciences

in the subject of Cell and Developmental Biology

have examined a dissertation entitled

Inhibition of Cdc42 during mitotic exit is required for cytokinesis in Saccharomyces cerevisiae

presented by Benjamin David Atkins

candidate for the degree of Doctor of Philosophy and hereby certify that it is worthy of acceptance.

Signature: _____

Typed Name: Dr. Samara Reck-Peterson

Signature: _____

Typed Name: Dr. Dannel McCollum

Signature: _____

Typed Name: Dr. David Burgess

_____

Dr. David Van Vactor, Program Head

Date: December 11, 2013

_____

Dr. David Lopes Cardozo, Director of Graduate Studies

Inhibition of Cdc42 during mitotic exit is required for cytokinesis in *Saccharomyces cerevisiae*

A dissertation presented

by

Benjamin David Atkins

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

In the subject of

Cell and Developmental Biology

Harvard University

Cambridge, Massachusetts

December 2013

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

Dissertation Advisor: Prof. David Pellman Benjamin David Atkins

Inhibition of Cdc42 during mitotic exit is required for cytokinesis in *Saccharomyces cerevisiae*

Abstract

Rho GTPases are highly conserved regulators of cell polarity and the actin cytoskeleton. The role of the Rho GTPase Cdc42 and its regulation during cell division is not well understood. Using biochemical and imaging approaches in budding yeast, I demonstrate that Cdc42 activation peaks during the G_1/S transition and during anaphase, but drops during mitotic exit and cytokinesis. Cdc5/Polo kinase is an important upstream cell cycle regulator that suppresses Cdc42 activity. Failure to downregulate Cdc42 during mitotic exit prevents the normal localization of key cytokinesis regulators – Iqg1 and Inn1– at the division site, and results in an abnormal septum. The effects of Cdc42 hyperactivation are largely mediated by the Cdc42 effector p21-activated kinase (PAK) kinase, Ste20. Inhibition of Cdc42 and related Rho GTPases may be a general feature of cytokinesis in eukaryotes.

Table of Contents

Title Page……..……i

Copyright page…….……ii

Abstract……...……iii

Table of contents…….iv

List of figures and tables.....v

Acknowledgments……..…...vi

Chapter 1: Overview of Rho GTPase control of cell polarity and cytokinesis……1

Chapter 2: Cell cycle regulation of Cdc42 activity in *S. cerevisiae*……..51

Chapter 3: Inhibition of Cdc42 during mitotic exit is required for cytokinesis…….79

Chapter 4: Conclusions and future directions…….....122

List of figures and tables

Figure 1-1: Key Cdc42 regulators, effectors, and functions in *S. cerevisiae* 5
Figure 1-2: Bud site selection in *S. cerevisiae* 8
Figure 1-3: Actin polarization changes across the *S. cerevisiae* cell cycle 17
Figure 1-4: Role of Cdc42 in mitotic exit signaling networks 22
Figure 2-1: GST-CRIB pulldown detects active GTP-Cdc42 from yeast lysates 56
Figure 2-2: GTP-Cdc42 levels peak at G_1/S and anaphase but fall during mitotic exit ... 58
Figure 2-3: Local Cdc42 activity at the bud neck during cytokinesis is low compared to the site of polarization during G_1/S 60
Figure 2-4: Cdc5/Polo kinase suppresses Cdc42 activity, likely through Cdc42-GAPs .. 63
Table 2-1: Yeast strains used in Chapter 2 70
Table 2-2 Plasmids used in Chapter 2 70
Figure 3-1: Genetic evidence that Cdc42 inhibits cytokinesis 85
Figure 3-2: *CDC42*^{Q61L} expression causes a cell separation defect 88
Figure 3-3: Inactivation of the Cdc42-GAP Bem2 causes a cell separation defect 90
Figure 3-4: *bem2-ts* inactivation in sensitized genetic backgrounds 92
Figure 3-5: *bem2-ts* cells have altered chitin deposition but not chitin synthase or Ace2 localization 94
Figure 3-6: Rho1 activation and Myo1 contraction appear normal in *bem2-ts* cells 97
Figure 3-7: *bem2-ts* cells have a defect in septum formation 100
Figure 3-8: Inefficient localization of Iqg1 and Inn1 to the bud neck in *bem2-ts* cells .. 102
Figure 3-9: Ste20 and Iqg1 mediate the inhibitory effect of active Cdc42 on cytokinesis and cell separation 105
Table 3-1: Yeast strains used in Chapter 3 111
Table 3-2: Plasmids used in Chapter 3 113

Acknowledgments

I am extremely grateful to my graduate advisor, Dr. David Pellman, for offering me the opportunity to study with him. David has been a very generous scientific mentor. He always had time to discuss experiments and help prioritize which directions were most exciting and would contribute to a fuller understanding of my scientific questions. His approach to performing the most convincing experiments possible to test hypotheses sets a high bar that draws out the best from his trainees. At the same time, while having an excellent nose for when an exciting result was in the making, he gave me tremendous freedom to learn how to do experiments, as well as patience during my learning process. David has also been unfailingly generous in his financial support for experiments and for giving me the opportunity to present my work at conferences. Getting to observe his process of doing science first-hand, along with being exposed to his innovative and broad scientific thinking has been both stimulating and rewarding. David's ability to guide so many diverse projects in the lab is truly impressive. I am thankful and proud to be a student of David's.

David's passion for science and for training the next generation of scientists is obvious, as demonstrated by his ability to consistently fill the lab with an outstanding group of students, post-docs, technicians, and others, as well as in his support for his trainees. Throughout my years in the Pellman laboratory I've been very fortunate to learn from many people. First among these I thank Dr. Satoshi Yoshida for his mentorship, excitement, for showing me the awesome power of yeast genetics and cell biology, as well as for collaborating with me on this work. I also thank Dr. Keiko Kono for being my

first introduction both to the lab and to budding yeast during my rotation, and for her advice and insight after I joined.

Many other labmates over the years gave me friendship, enjoyable discussions, and collegial advice, both on scientific matters and on life in general. In particular, I'm grateful to my lunch-buddy Dr. Susana Godinho. Her excitement for science, friendship, and support were invaluable and greatly contributed to the pleasant atmosphere of the Pellman lab. Her recent lunch-successor Dr. Yosef Kaplan has also performed admirably in this position. I thank my fellow PhD student Hugo Arellano-Santoyo and Dr. Alexander Spektor for many adventures in and out of the lab. I am also grateful for having had the chance to work with Drs. Xiaolei Su, Shawnna Buttery, Karen Crasta, Neil Ganem, and many others.

I am also thankful to the leadership and administration of the Biological and Biomedical Sciences program at Harvard Medical School for giving me the chance to study in such a stimulating environment. Further, I'm grateful to my dissertation advisory committee, including Drs Fred Winston, Ulrike Eggert, and David Levin for their advice and guidance over the course of this work.

Of course, this work would also not have been possible without the constant love and support of my family, starting with my parents David and Ann Atkins. Their hard work provided me with the opportunities necessary to pursue my goals in life, and along with my sister Kaitlin they have provided me the true ideal of a loving family. My gratitude and love extend to my grandparents, Frank and Mary Jane Velesz and Dewey and Ellen Atkins, and our family on both sides. Finally, I'd like to thank my friends, notably Sam Nigh, Adam Couture, and Basia Niebudek, for their bonhomie and support.

Chapter 1

Overview of Rho GTPase control of cell polarity and cytokinesis

1.1 Overview 2

1.2 Rho GTPases are master regulators of the actin cytoskeleton and cell polarity 2

1.3 Control of cell polarization by Cdc42 and other Rho GTPases in budding yeast 4

1.3.1 Cdc42 is essential for actin polarization and bud emergence 4

1.3.2 Mechanisms to determine the site of Cdc42-dependent budding 7

1.3.3 Cdc42-dependent bud growth and septin assembly 11

1.3.4 Role of Rho1 and other Rho GTPases besides Cdc42 in cell polarization 13

1.3.5 Coordination of Rho GTPase activity in S. cerevisiae 15

1.4 Cell cycle regulation of cell polarization in budding yeast 18

1.5 Function of Cdc42 during mitosis 20

1.6 Control of cytokinesis by Rho GTPases 24

1.6.1 Rho1/RhoA activation at the division site is required for cytokinesis 24

1.6.2 Rho-dependent contractile actin ring assembly 26

1.6.3 CAR contraction and furrow ingression 27

1.6.4 Does Rho function in abscission? 28

1.6.5 Role of other Rho family GTPases in cytokinesis 30

1.7 Goals of this dissertation 32

1.8 References 32

1.1 Overview

Nearly all cells are polarized – that is, non-symmetric along at least one axis. This fundamental property is critical for many cellular processes, including motility, membrane trafficking, developmental differentiation, and cell division. Many cells undergo changes in polarity during their cell division cycle, but the underlying molecular mechanisms remain poorly understood. The overall goal of this dissertation is to explore the connection between the cell cycle and cell polarization at a molecular level, and to understand how these connections are important for cell division. Specifically, I will address the cell cycle regulation of Cdc42, a conserved master regulator of cell polarization, as well as the effects of Cdc42 activation on cytokinesis, the final step of cell division.

1.2 Rho GTPases are master regulators of the actin cytoskeleton and cell polarity

One approach to polarize harnessed by most cells is to create organized arrays of cytoskeletal polymers such as actin or microtubules. These arrays can serve as tracks for vesicular trafficking, confer shape and rigidity to cells, and exert force to move or deform cellular structures. Two classic examples of cytoskeletal arrays are the microtubule-based mitotic spindle, which separates the duplicated genetic material into two distinct masses, and the contractile actin ring, which facilitates plasma membrane ingression during cytokinesis to physically divide the cell into two daughter cells (Dumont and Mitchison, 2009; Fededa and Gerlich, 2012).

A family of proteins called Rho guanosine triphosphatases (GTPases) control actin assembly and cell polarity across the eukaryotic lineage. Rho GTPases use the

energy from GTP hydrolysis to cycle between molecular conformations. When bound to GTP, Rho GTPases can interact with and signal to their effector proteins, which typically contain domains such as the Cdc42/Rac Interactive Binding (CRIB) domain that preferentially recognize the GTP-bound conformation (Burbelo et al., 1995). In the canonical 'switch' model, the signal is terminated by GTP hydrolysis (Cherfils and Zeghouf, 2013). Most Rho GTPases have slow intrinsic GTPase activity, which must be promoted by GTPase activating proteins (GAPs) to occur on physiologically relevant time scales (Moon and Zheng, 2003). Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by catalyzing the rate-limiting step of release of GDP + inorganic phosphate, which in turn allows GTP to bind due to the higher intracellular concentration of GTP compared to GDP (Rossman et al., 2005).

Most Rho GTPases are peripheral membrane proteins with a post-translationally added hydrophobic lipid tail (Adamson et al., 1992a; Adamson et al., 1992b; Katayama et al., 1991). Rho GTPases can be extracted from the plasma membrane by binding to Rho guanine nucleotide dissociation inhibitor (Rho-GDI) proteins. Rho-GDI binding masks the hydrophobic tail of Rho GTPases and sequesters them in the cytoplasm (Cherfils and Zeghouf, 2013; Garcia-Mata et al., 2011). While Rho GTPases can associate with a number of intracellular membranes, their activity at the plasma membrane is considered to be most important for their function in actin polarization, cytokinesis, and motility. Accordingly, many GEFs, GAPs, and effectors contain phosphoinositide-binding domains or motifs that can promote their localization to the plasma membrane; this localization is often modulated in order to control these Rho regulators in space and time

(Gulli et al., 2000; Matthews et al., 2012; Shimada et al., 2000; Strochlic et al., 2010; Takahashi and Pryciak, 2007).

Although the human genome encodes approximately twenty Rho GTPases, three in particular – RhoA, Rac1, and Cdc42 -- have been intensively studied since their discoveries more than twenty years ago due their high level of conservation and significant effects on cell function (Didsbury et al., 1989; Johnson et al., 1987; Madaule and Axel, 1985; Polakis et al., 1989a; Polakis et al., 1989b). These proteins often play conserved roles in actin regulation in different systems, as demonstrated by the fact that expression of human Cdc42 or RhoA can complement budding yeast mutants lacking the function of the homologous gene (Munemitsu et al., 1990; Qadota et al., 1994). Because Rho GTPases have both dedicated and shared effectors, proper coordination of their local and temporal activation is required for normal cell polarization, motility, and cytokinesis (Boulter et al., 2012; Etienne-Manneville and Hall, 2002; Ridley and Hall, 1992; Ridley et al., 1992).

1.3 Control of cell polarization by Cdc42 and other Rho GTPases in *S. cerevisiae*

1.3.1 Cdc42 is essential for actin polarization and bud emergence

The budding yeast *Saccharomyces cerevisiae* is the system where we have perhaps the most detailed understanding of how Cdc42 and other Rho GTPases control cell polarization at a mechanistic level. Pringle and colleagues discovered the *CDC42* gene as a multi-copy suppressor of a temperature-sensitive (ts) *cdc24* mutant amidst a flurry of work showing that *CDC42* was essential for bud formation and actin polarization (Adams et al., 1990; Bender and Pringle, 1989; Johnson and Pringle, 1990).

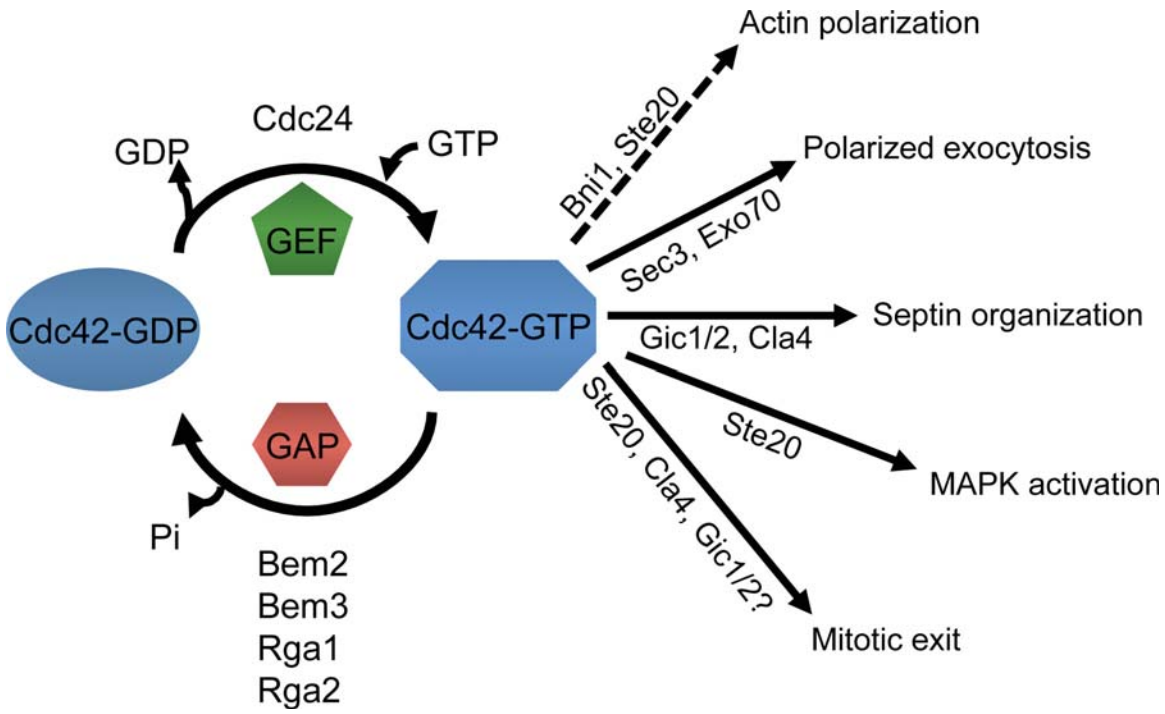


Figure 1-1: Key Cdc42 regulators, effectors, and functions in *S. cerevisiae*

Subsequent genetic screens and biochemistry identified most of the other players involved in cell polarization and Cdc42 function (including the sole GEF Cdc24 and several GAPs) as well as downstream effectors (Bi and Park, 2012; Cvrckova et al., 1995; Kim et al., 1994; Peterson et al., 1994; Wang and Bretscher, 1995; Zheng et al., 1994; Zheng et al., 1993), Fig1-1.

This section will focus on the mechanism of cell polarization during the mitotic cell division cycle by Cdc42 because Cdc42 is considered the most upstream Rho GTPase for cell polarization in yeast, but the function of the other Rho family members and connections with Cdc42 will be briefly described as well. Cdc42 is also essential for polarization in other contexts, most notably mating and pseudohyphal growth, which are beyond the scope of this chapter. Several recent reviews have explored these topics in detail (Arkowitz and Bassilana, 2011; Bi and Park, 2012; Cullen and Sprague, 2012).

Growth of the daughter cell, or bud, begins at the G_1/S transition after cells commit to a new cell cycle and requires polarization of the actin cytoskeleton, which is comprised of actin cables and actin patches (Adams and Pringle, 1984; Bi and Park, 2012; Kilmartin and Adams, 1984). Actin cables are formin-nucleated linear tracks for myosin V (Myo2)-dependent directed trafficking of post-Golgi vesicles, which are also required for bud growth (Evangelista et al., 2002; Pruyne et al., 1998; Sagot et al., 2002). Actin patches are sites of Arp2/3-dependent actin polymerization that are required for endocytosis (Kaksonen et al., 2003; Kubler and Riezman, 1993; Mulholland et al., 1994). Polarized organization of actin cables, actin patches, and exocytosis all depend on Cdc42, suggesting that Cdc42 controls polarization through a multi-pronged approach (Adamo et al., 2001; Adams et al., 1990; Dong et al., 2003), Fig 1-1.

1.3.2 Mechanisms to determine the site of Cdc42-dependent budding

A universal assumption of all models for cell polarization in budding yeast is that activation of Cdc42 at a single site determines the presumptive bud site (Bi and Park, 2012). Cdc42 itself clusters in a polarized fashion at the presumptive bud site prior to bud emergence (Richman et al., 2002; Ziman et al., 1993). Interestingly, Cdc42 localization to the cluster is very dynamic, with a half-life of around 5 seconds (Slaughter et al., 2009; Wedlich-Soldner et al., 2004). How Cdc42 is clustered and activated at bud emergence without being lost by diffusion has therefore been the driving question in the field. The proposed mechanisms discussed below involve localization of the Cdc42-GEF Cdc24 by the bud site selection machinery, extraction of Cdc42 by the Rho-GDI Rdi1, and positive feedback involving an autocatalytic mechanism or polarized trafficking of Cdc42.

The mating type of the cell dictates the site of budding (Freifelder, 1960; Hicks et al., 1977). Haploid cells bud in an axial fashion adjacent to the previous division site, while diploid cells bud in a bipolar fashion, where the mother buds either adjacent to the previous division site or at the opposite pole, and the daughter almost always buds opposite the previous division site (Fig 1-2A, Bi and Park 2012). A set of proteins called the bud site selection machinery is required for this pattern (Bender and Pringle, 1989; Chant and Herskowitz, 1991). These proteins include mating-type specific integral membrane proteins that function as landmarks for the Ras-related GTPase Rsr1/Bud1 and its GEF and GAP (Fig 1-2B). The bud site selection machinery is thought to guide the site of Cdc42 activation through an interaction between GTP-bound Rsr1 and the Cdc42-GEF Cdc24 that promotes localization and perhaps activation of Cdc24 to the presumptive bud site (Fig 1-2B); interactions

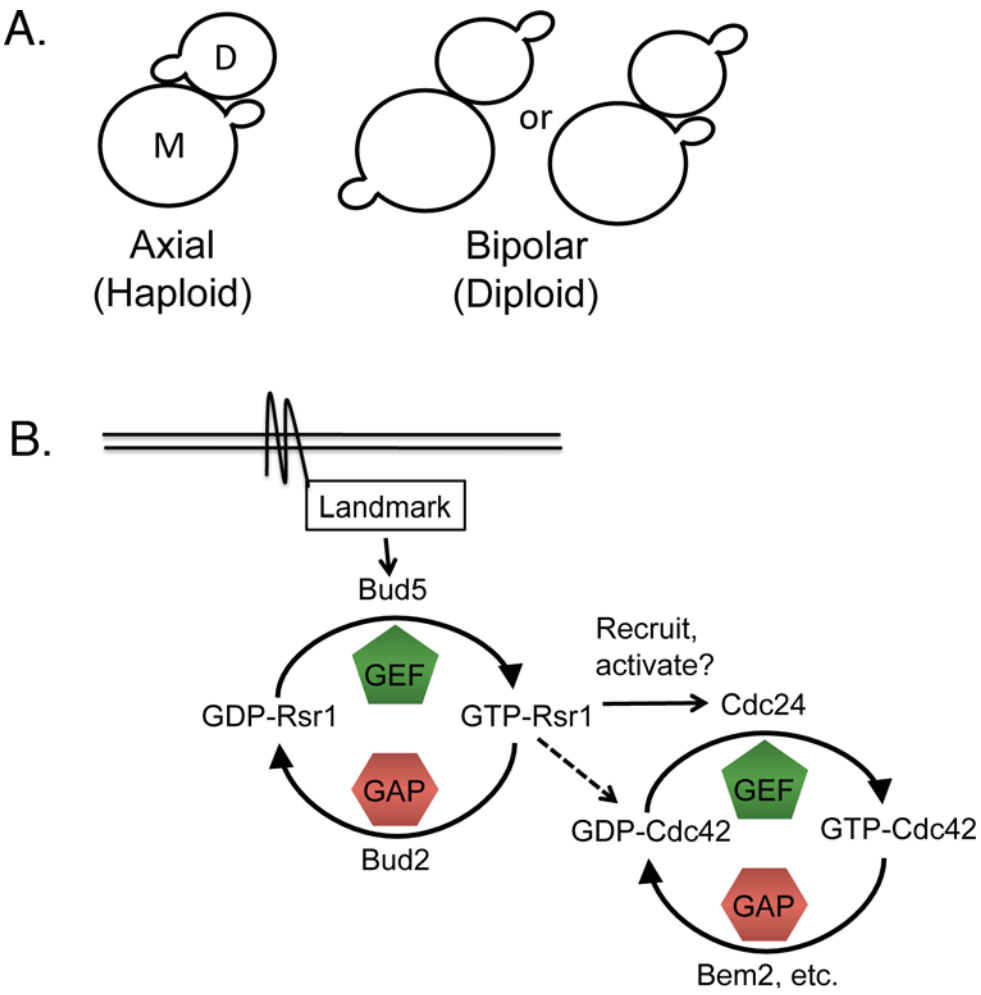


Figure 1-2: Bud site selection in *S. cerevisiae*

(A) Haploid (MATa or MATα) cells bud in an axial fashion, with both mother (M) and daughter (D) budding directly adjacent to but not within the previous division site. Diploid (MATa/α) cells bud in a bipolar fashion, where the mother buds either opposite or adjacent to the previous division site, while daughters almost exclusively bud opposite the previous division site (Bi and Park, 2012).

(B) Landmark proteins (the landmark used depends on mating type) are thought to recruit the GEF for the small GTPase Rsr1 to promote local GTP-loading, which interacts with and may activate Cdc24 to promote local Cdc42 GTP-loading at the correct budding site.

between Rsr1 and Cdc42 itself may also contribute (Kozminski et al., 2003; Park et al., 1997; Park et al., 2002; Shimada et al., 2004; Zheng et al., 1995).

Localized GEF activity could explain local Cdc42 GTP-loading but is unlikely to be sufficient for polarity cluster formation due to rapid diffusion of Cdc42 (for a discussion see Howell et al., 2012). Another mechanism proposed to concentrate Cdc42 is through extraction and recycling of Cdc42 from membranes by the Rho-GDI Rdi1. Human Rho-GDI promotes selective extraction of GDP-bound Cdc42 from membranes compared to GTP-Cdc42 (Freisinger et al., 2013; Johnson et al., 2009). Assuming that budding yeast Rdi1 behaves similarly, mathematical modeling suggests that selective extraction of GDP-Cdc42 combined with localized GEF activity is sufficient for formation of a concentrated Cdc42 polarity cluster (Goryachev and Pokhilko, 2008). In this model, localized GEF activity will act as a 'trap' by converting the extractable GDP-Cdc42 into less-extractable GTP-Cdc42. Consistently, fluorescence recovery after photobleaching (FRAP) studies have shown that GFP-Cdc42 in the polarity cluster is 4-5 fold less mobile in *rdi1Δ* cells compared to wild-type (Slaughter et al., 2009). However, this mechanism cannot completely account for Cdc42 polarization, because *rdi1Δ* cells grow as well as wild-type and are still able to polarize and cluster Cdc42 (Boulter et al., 2010; Masuda et al., 1994; Slaughter et al., 2009; Tiedje et al., 2008).

In the absence of the bud site selection machinery, cells are still able to polarize Cdc42 and form a single bud at a random site (Bender and Pringle, 1989; Chant and Herskowitz, 1991). This process is termed 'symmetry breaking', and has become an important model for the mechanism of local Cdc42 activation (Johnson et al., 2011). In the *rsr1Δ* background, the scaffold protein Bem1 is required for viability and for Cdc42

polarization, which occurs in an actin-independent fashion (Ayscough et al., 1997; Irazoqui et al., 2003). A tri-partite complex between Bem1, the Cdc42-GEF Cdc24, and a PAK kinase (either Cla4 or Ste20) appears to be critical for symmetry breaking (Bose et al., 2001; Gulli et al., 2000; Kozubowski et al., 2008). This complex has been proposed to locally amplify Cdc42 GTP-loading through a positive-feedback mechanism, where a stochastically activated GTP-Cdc42 molecule recruits the complex by binding the CRIB-domain of the PAK kinase (Kozubowski et al., 2008). This binding will localize the complex, and the GEF Cdc24 will activate nearby GDP-Cdc42 molecules, which will in turn recruit more of the Bem1-Cdc24-PAK complex. This model predicts competition for a limiting amount of the complex by nascent polarity foci (Goryachev and Pokhilko, 2008; Howell et al., 2009). This competition has been observed by rapid time lapse microscopy; interestingly, the process appears oscillatory, suggesting the involvement of negative feedback (Howell et al., 2012).

Other models for Cdc42 polarization during symmetry breaking have also been proposed. An alternative positive-feedback pathway through actin-based delivery of Cdc42 on vesicles can function in cells overexpressing GTP-locked Cdc42 or when Bem1 is fused to an integral membrane protein, but the extent to which this pathway contributes to the initial polarization remains a matter of debate (Ayscough et al., 1997; Freisinger et al., 2013; Howell et al., 2009; Irazoqui et al., 2003; Layton et al., 2011; Marco et al., 2007; Orlando et al., 2011; Slaughter et al., 2013; Wedlich-Soldner et al., 2003; Wedlich-Soldner et al., 2004). However, actin-dependent processes such as endocytosis and vesicle trafficking appear to contribute to the maintenance or robustness

of Cdc42 polarization (Irazoqui et al., 2005; Jose et al., 2013; Wedlich-Soldner et al., 2004).

Many questions about the mechanism of Cdc42 polarization and activation are completely unresolved. First, how the bud site selection machinery is integrated with the symmetry breaking module in wild-type cells is poorly studied. For example, how Rsr1 contributes to Cdc42 polarization and activation is unclear (Smith et al., 2013; Wu et al., 2013). The nature of the negative feedback required for Bem1-dependent symmetry breaking remains a mystery – Cdc42-GAPs are obvious candidates but the exact mechanism remains to be elucidated. Whether additional proteins besides Rdi1 have the ability to promote nucleotide-selective membrane extraction of Cdc42 is also unknown. Finally, whether Cdc42 GTP-loading increases during polarization has never been determined experimentally (see Chapter 2). Understanding how these mechanisms are integrated in wild-type yeast cells will likely continue to occupy mathematical modelers and cell biologists for years to come.

1.3.3 Cdc42-dependent bud growth and septin assembly

Current models posit that once Cdc42 becomes activated at the presumptive bud site, it promotes actin polarization and exocytosis through local activation of effector proteins. The PAK kinases Cla4 and Ste20 bind directly to GTP-Cdc42 *via* CRIB domains, which relieves their intramolecular autoinhibition and allows them to phosphorylate substrates involved in actin polarization, septin assembly (see below in this section), mating, and mitotic exit (Benton et al., 1997; Cvrckova et al., 1995; Gulli et al., 2000; Hofken and Schiebel, 2002; Holly and Blumer, 1999; Leberer et al., 1992;

Moskow et al., 2000; Seshan et al., 2002; Versele and Thorner, 2004). Cdc42 also regulates the actin nucleators Bni1 and the Arp2/3 complex, apparently through indirect mechanisms rather than through direct binding (Chen et al., 2012; Lechler et al., 2001; Lechler et al., 2000), leading to polarization of actin cables and patches. Cdc42 also plays an actin-independent role in regulating the exocyst complex, which tethers post-Golgi vesicles for subsequent fusion with the plasma membrane, *via* direct interactions with the Sec3 and Exo70 subunits (Adamo et al., 2001; Roumanie et al., 2005; Wu et al., 2009; Zhang et al., 2001). These processes cooperate to promote bud growth.

Cdc42 is also essential for the organization of the septin cytoskeleton at the bud neck. Septins are small GTPases that assemble into rod-shaped hetero-oligomers, which can subsequently organize into higher-order structures such as filaments and rings (Oh and Bi, 2011). During G_1/S a cloud of septin subunits localize to the presumptive bud site, which transforms into a nascent ring that matures into a collar after bud emergence (Chen et al., 2011; Iwase et al., 2006). The septin collar is thought to function as a diffusion barrier between the mother and bud and also contributes to normal bud morphology (Barral et al., 2000; Gladfelter et al., 2005; Takizawa et al., 2000). Septins are also essential for cytokinesis; during late anaphase the septin collar splits into two rings that delimit the region of cytokinesis (Cid et al., 2001; Dobbelaere et al., 2003; Hartwell, 1971). During cytokinesis septins are likely to function both as a scaffold for protein recruitment and as a diffusion barrier to define a specialized membrane domain for cytokinesis (Dobbelaere and Barral, 2004; Oh and Bi, 2011; Wloka et al., 2011).

Cdc42 is required for the initial recruitment of septin subunits to a cloud at the presumptive bud site through the CRIB domain-containing effectors Gic1 and Gic2 (Cid

et al., 2001; Iwase et al., 2006). The proper organization of septins into a ring depends on the PAK kinase Cla4, which phosphorylates several septin subunits including Cdc10 and Cdc3 (Cvrckova et al., 1995; Longtine et al., 2000; Schmidt et al., 2003; Versele and Thorner, 2004; Weiss et al., 2000). Interestingly, mutations of Cdc42 that impair GTP hydrolysis or deletion of Cdc42-GAP proteins also lead to defects in septin ring organization (Caviston et al., 2003; Gladfelter et al., 2002; Smith et al., 2002).

Recent work has proposed a model where active Cdc42 recruits septins, which in turn inhibit Cdc42 in a negative feedback loop *via* Cdc42-GAPs (Okada et al., 2013). Modeling and experimental data suggest that Cdc42-dependent exocytosis generates a hole in the septin cloud, which locally relieves Cdc42 inhibition and allows the formation of a discrete zone of Cdc42 activity centered within the septin cloud. Subsequently, the boundaries of the cloud are refined into a ring (Okada et al., 2013). How the cloud matures into a ring and the septin ultrastructural rearrangements that accompany this process remain a mystery. The growth of the septin ring into a mature collar, as well as the splitting of the septin collar during cytokinesis are poorly understood at a structural level, although polarized microscopy studies indicate the latter step involves a 90° change in septin subunit orientation state (DeMay et al., 2011; Vrabioiu and Mitchison, 2006).

1.3.4 Role of other Rho GTPases in cell polarization in *S. cerevisiae*

The budding yeast genome encodes several additional Rho GTPases besides Cdc42. While loss of *CDC42* function leads to a complete failure to polarize the cell and form a bud, loss of the other Rho GTPases leads to more modest polarity or

morphological defects, suggesting that these Rho GTPases play a contributory role in cell polarization downstream of Cdc42.

Besides Cdc42, Rho1 is the only essential Rho GTPase in budding yeast in most strain backgrounds (Bi and Park, 2012). Rho1 regulates a number of effectors, including the Protein Kinase C homolog Pkc1, the formin Bni1, the exocyst subunit Sec3, and glucan synthase Fks1, to promote cell wall synthesis and actin organization (Dong et al., 2003; Guo et al., 2001; Kamada et al., 1996; Levin, 2011; Qadota et al., 1996). Rho1 is an essential upstream regulator of the Cell Wall Integrity (CWI) Mitogen-Activated Protein Kinase (MAPK) signaling network that promotes survival in the face of numerous environmental stresses (Levin, 2011). Rho1 also plays an essential role in cytokinesis, as described in Section 1.6 and in Chapter 3.

Actin polarization and the formation of tiny buds can occur in cells that lack Rho1 function, suggesting that Rho1 is required after Cdc42 initiates polarization of the cell (Yamochi et al., 1994). However, consistent with Rho1's essential role in the CWI pathway and in cell wall remodeling, these cells subsequently lyse and die. Consistent with these data, Rho1's function in actin organization may be most important under stress conditions and for cytokinesis (Dong et al., 2003; Guo et al., 2001; Helliwell et al., 1998; Mazzoni et al., 1993; Yamochi et al., 1994). The highly related paralog Rho2 is a poorly-studied Rho GTPase that is thought to play a redundant role to Rho1 (Bi and Park, 2012).

The other well-studied Rho GTPases in budding yeast are Rho3 and Rho4, which may play overlapping roles in polarization, as judged by the synthetic lethal interaction in *rho3Δ rho4Δ* double mutants (Matsui and Toh, 1992). Rho3 and Rho4 have been implicated in activation of formin proteins (Dong et al., 2003). Rho3 interacts with the

Exo70 subunit of the exocyst, and some *rho3* mutants accumulate vesicles in medium and large budded cells without obvious effects on actin, suggesting an actin-independent role for Rho3 in promoting exocytosis later in the cell cycle (Adamo et al., 1999; Wu et al., 2009). The function of Rho5 is mysterious, although it may be involved in downregulation of the CWI pathway or in other stress responses (Schmitz et al., 2002; Singh et al., 2008).

1.3.5 Coordination of Rho GTPase activity in budding yeast

A major outstanding question is how Cdc42 activity is coordinated with the activity of other Rho GTPases, especially Rho1. This is likely to be important because Cdc42 and Rho1 share several effector proteins, including Bni1 and Sec3, which could give rise to competition for binding (Guo et al., 2001; Zhang et al., 2001). Consistently, genetic experiments suggest the possibility of antagonism between Cdc42 and Rho1 (Gao et al., 2004). The paxillin homolog Pxl1 may be involved, as *PXL1* overexpression suppresses *cdc42* mutants but kills *rho1* mutants (Gao et al., 2004). We presently lack a detailed understanding of this potential crosstalk or how Pxl1 could relay information between Rho1 and Cdc42. However, by analogy to mammalian paxillin, which coordinates the activity of Rho GTPases at sites of cell adhesion, phospho-regulation of Pxl1 by PAK kinases and phosphatases could create specific docking sites for Rho-GEF and GAP complexes (Deakin and Turner, 2008).

During cytokinesis, the recently-discovered protein Gps1 promotes Rho1 localization to the bud neck during late stages of cytokinesis to promote septum formation but prevents Cdc42-dependent polarization from occurring at the previous site

of cell division, suggesting that it also coordinates Rho1 and Cdc42 (Meitinger et al., 2013). While it is known that Gps1 can interact with both Rho1 and Cdc42 without nucleotide specificity, further work is required to understand the mechanism by which Gps1 exerts its functions. Interestingly, a high-throughput screen for genetic interactions found a negative interaction in double *pxl1Δ gps1Δ* mutants, suggesting that coordination between Rho1 and Cdc42 could be critical for cell survival (Costanzo et al., 2010). Overall, the mechanisms that coordinate local and temporal activation of Rho GTPases in yeast are unclear, but are likely to be relevant for our understanding of complex processes such as cell motility and cytokinesis (Jordan and Canman, 2012; Machacek et al., 2009).

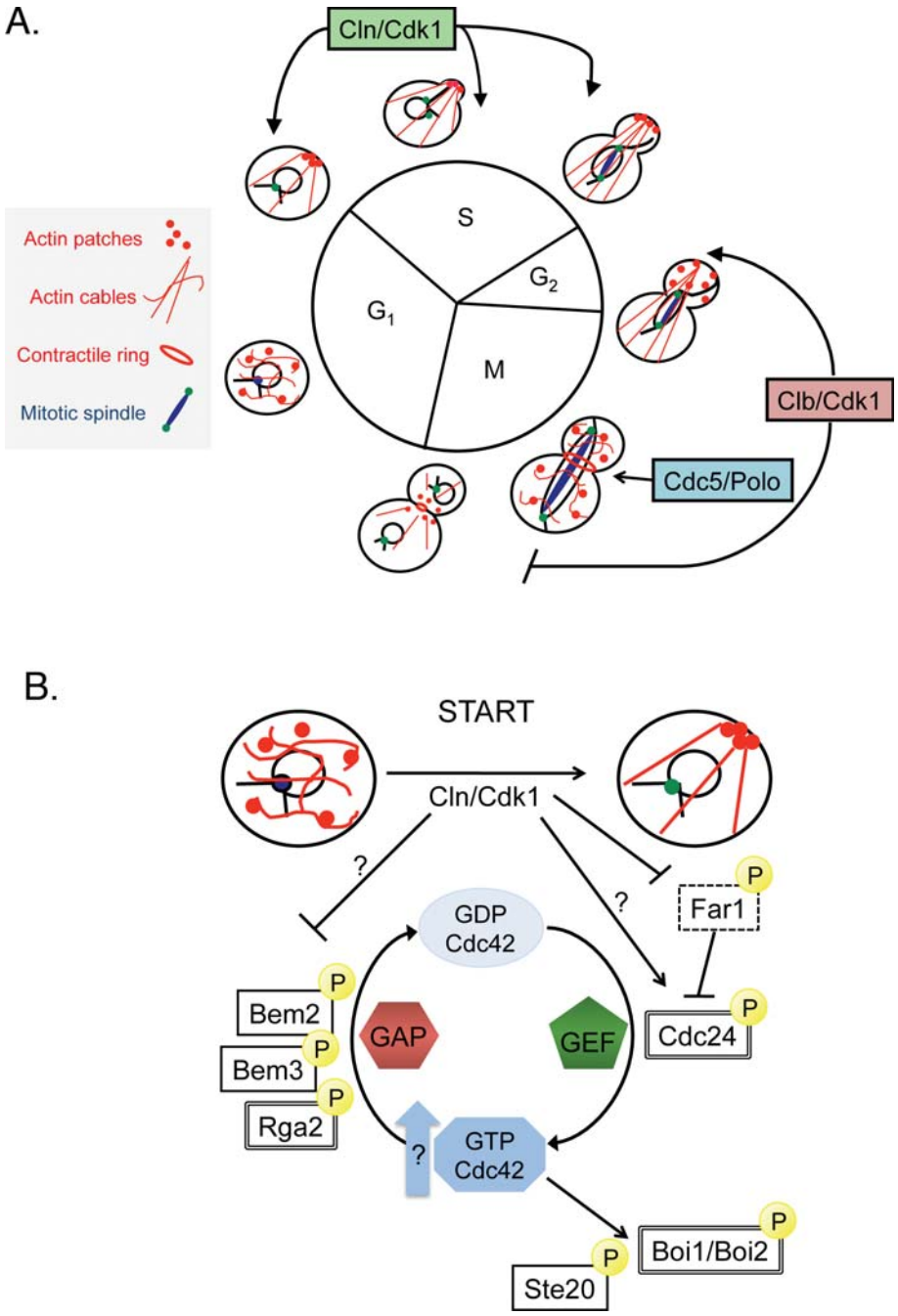


Figure 1-3: Actin polarization changes across the *S. cerevisiae* cell cycle

(A) Cartoon courtesy of Dr. Satoshi Yoshida. See Section 1.4 for details.

(B) Model for how Cln/Cdk1 promotes polarization at the commitment to a new cell cycle at START. Yellow circles indicate Cln/Cdk1-dependent phosphorylation *in vivo*. Proteins with double lines have been found in a complex (McCusker *et al.* 2007). Note that the exact function of any phosphorylation event shown is not understood.

1.4 Cell cycle regulation of cell polarization in budding yeast

As shown in Figure 1-3A, a number of actin reorganizations occur to promote proper bud growth and cell division as a budding yeast cell traverses through the cell cycle (Adams and Pringle, 1984; Kilmartin and Adams, 1984; Novick and Botstein, 1985). Early G_1 cells have an unpolarized actin cytoskeleton, with randomly distributed actin cables and patches. After commitment to a new cell cycle, which is termed START in budding yeast, the actin cytoskeleton becomes polarized, and approximately 10-15 minutes later bud emergence occurs (Lew and Reed, 1993). Actin polarization and cell growth remain tightly focused to the tip of the emerging bud until the apical-to-isotropic switch at the G_2/M transition, at which point growth occurs along the whole surface of the bud (Farkas et al., 1974; Lew and Reed, 1993). Late in mitosis during anaphase the actin cytoskeleton becomes briefly depolarized until mitotic exit, when actin patches and cables repolarize to the site of cell division at the bud neck (Bi and Park, 2012).

These rearrangements in actin polarization are under the control of the key driver of cell cycle progression, cyclin-dependent kinase (Cdc28, henceforth Cdk1), and its activating cyclin proteins (Fig 1-3A). Actin polarization at G_1/S requires the activation of Cdk1 by late G_1 cyclins (Cln1, 2, or 3), whereas depolarization during mitosis requires activation of Cdk1 by mitotic cyclins (Clb1 or Clb2). The repolarization to the bud neck during mitotic exit requires inactivation of Clb/Cdk1 activity (Lew and Reed, 1993). These observations suggest a link between the cell cycle machinery and Rho GTPases in controlling the actin rearrangements that occur during the cell division cycle.

Cdk1 activity is required for both initiation and maintenance of cell polarization (McCusker et al., 2007). Recent work has identified multiple substrates whose Cdk1-

dependent phosphorylation could be involved in polarization during G_1/S phase; as expected, many of these targets are regulators of Cdc42 (Fig 1-3B). In haploid cells, phosphorylation of Far1 by Cln/Cdk1 leads to its proteasome-dependent degradation, allowing the Cdc42-GEF Cdc24 to escape its sequestration in the nucleus and relocalize to the presumptive bud site (Gulli et al., 2000; Shimada et al., 2000). Cdc24 is also a direct substrate of Cdk1; however, the exact function of these modifications remains unclear (Moffat and Andrews, 2004; Wai et al., 2009). During polarization Cdc24 is in a complex with a Cdc42-GAP Rga2 and several other proteins, including Boi1 and Boi2; these proteins are phosphorylated by Cln/Cdk1 which at least in the case of Boi1 appears to promote its function (McCusker et al., 2007). Finally, the Cdc42-GAP proteins Bem2, Bem3, and Rga2 are phosphorylated in a Cln/Cdk1-dependent manner, which appears to inhibit their activities (Knaus et al., 2007; Sopko et al., 2007). Importantly, the exact function of Cdk1-dependent phosphorylation remains unclear due to the lack of clear phenotypes from the phospho-site mutants.

Overall, these data suggest that Cln/Cdk1 activity promotes normal localization (and perhaps activity) of the Cdc42-GEF Cdc24, while concurrently inhibiting the activity of Cdc42-GAP proteins, which is expected to have the combined effect of local Cdc42 activation at the G_1/S transition. Importantly, this hypothetical increase in Cdc42 activation has never been directly measured, but is a critical aspect of current models for cell polarization (see Chapter 2). By contrast, Rho1 GTP-loading is known to increase at this time in the cell cycle, at least in part due to Cln/Cdk1-dependent phosphorylation of the Rho1-GEF Tus1 (Kono et al., 2008). Thus, it is likely that Cln/Cdk1 promotes cell polarization and bud growth at G_1/S through activation of multiple Rho GTPases.

While we are beginning to understand how Cdk1 promotes cell polarization at G_1/S, we have less insight into how the cell cycle machinery controls later actin rearrangements, in particular the apical-to-isotropic switch at G_2/M and actin depolarization during mitosis. Deletion of the phospholipid flippase *LEM3* causes a delay in the apical-to-isotropic switch (Saito et al., 2007). Intriguingly, Lem3 promotes the accumulation of phosphatidylethanolamine on the inner leaflet of the plasma membrane, and this lipid stimulates the activities of two Cdc42-GAPs *in vitro*, suggesting that Lem3 may promote inactivation of Cdc42 *via* GAPs (Saito et al., 2007). Lem3's binding partner Dnf2 is an *in vitro* substrate of Clb2-Cdk1, suggesting that this module may be an important target of Cdk1 for the apical-to-isotropic switch (Ubersax et al., 2003, Saito et al., 2007). During mitosis, other important cell-cycle regulators such as Cdc5/Polo kinase are activated. Importantly, during late anaphase Cdc5/Polo promotes local and global activation of Rho1 to promote CAR assembly and cytokinesis (Yoshida et al., 2006). It remains unknown whether Cdc5/Polo regulates Cdc42, although it is tempting to speculate, as Cdc5 interacts with the Cdc42-GAP Bem3 (Yoshida et al., 2006, Chapter 2).

1.5 Function of Cdc42 during mitosis

In part due to the dearth of information on how Cdc42 activity changes later during the cell cycle, the function of Cdc42 during mitosis remains unclear. Cdc42 has an upstream role in regulating the Mitotic Exit Network (MEN) signaling pathway that controls Cdk1 inactivation *via* activation of the Cdc14 phosphatase during late anaphase (Hofken and Schiebel, 2002; Hofken and Schiebel, 2004; Monje-Casas and Amon, 2009; Stegmeier and Amon, 2004). Cdc14 is sequestered in the nucleolus prior to anaphase, but

is released in two waves that are controlled by the Cdc14 early anaphase release (FEAR) network in early anaphase and the MEN during late anaphase (Rock and Amon, 2009; Stegmeier and Amon, 2004). The MEN is a kinase cascade downstream of the spindle-pole body (SPB)-localized small GTPase Tem1. Current evidence supports a zone model where the MEN becomes active during late anaphase when the daughter-bound SPB escapes the mother compartment containing the MEN inhibitor Kin4 into the bud compartment containing the MEN activator Lte1 (Bardin et al., 2000; Chan and Amon, 2010). Lte1 functions by preventing Kin4 localization to the daughter SPB (Bertazzi et al., 2011; Falk et al., 2011).

The most clearly established target of Cdc42 involved in mitotic exit is the MEN activator Lte1, which requires Cdc42 and the PAK kinase Cla4 for phosphorylation and localization to the bud cortex, which initiates in S phase (Hofken and Schiebel, 2002; Seshan and Amon, 2005; Seshan et al., 2002). The PAK Ste20 appears to function in parallel, with potential inputs into the Cdc14 early anaphase release (FEAR) network through upstream activation of the high osmolarity glycerol (HOG) pathway, but the actual targets of Ste20 in mitotic exit are not defined (Hofken and Schiebel, 2002; Reiser et al., 2006; Rock and Amon, 2009). Finally, the Cdc42 effectors Gic1 and Gic2 have also been proposed to be involved in mitotic exit, possibly by inhibiting the interaction of the bipartite Tem1-GAP complex Bfa1/Bub2 with Tem1 (Hofken and Schiebel, 2004).

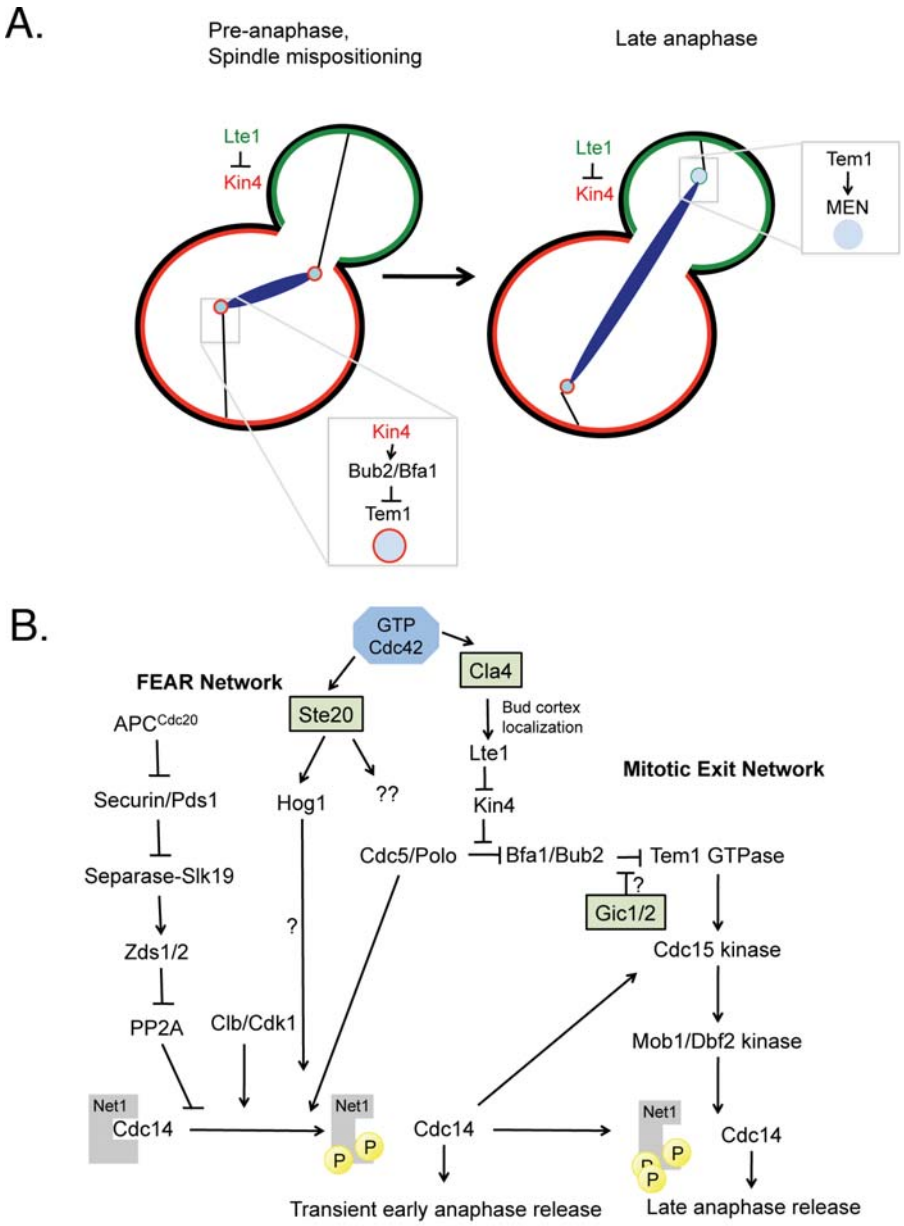


Figure 1-4: Role of Cdc42 in mitotic exit signaling networks.

(A) Spatial organization of the Mitotic Exit Network (MEN). In the mother compartment, Kin4 kinase inhibits Tem1 at spindle pole bodies through Bub2/Bfa1. When the anaphase spindle enters the bud, Lte1 can act to inhibit Kin4 binding to the daughter SPB, freeing Tem1 from inhibition.

(B) Wiring diagram of the Cdc14 early anaphase release (FEAR) and MEN (adapted from Rock and Amon, 2009). These networks promote release of the phosphatase Cdc14 from its sequestration in the nucleolus by promoting phosphorylation of the inhibitor Net1. Released Cdc14 promotes mitotic exit via dephosphorylation of Cdk1 targets. Green boxes show Cdc42 effectors. The exact roles of Ste20 and Gic1/2 are unclear.

These data suggest a possible function for Cdc42 during mitosis, but the active contribution of Cdc42 to MEN signaling during mitosis as opposed to simply determining the polarity axis required for asymmetric partitioning of MEN components remains unclear. Indeed, inactivation of Cdc42 using ts or degron mutants does not block completion of mitosis or cytokinesis, suggesting that Cdc42 activity is dispensible during mitosis (Iwase et al., 2006; Tolliday et al., 2002). As cells finish cytokinesis and enter G_1, local inhibition of Cdc42 by the GAP Rga1 and Gps1 is required to prevent rebudding in the next cell cycle into the old division site (Meitinger et al., 2013; Tong et al., 2007). Rebudding into the previous division site appears to be deleterious to cells, especially under conditions where septum formation is partially defective, as *gps1Δ* cells frequently undergo lysis after attempting to rebud through the old division site (Meitinger et al., 2013). Overall, these data indicate that Cdc42 activity may be under tight regulation during the M-to-G_1 transition.

In summary, we are only beginning to understand how the cell cycle machinery controls cell polarization at a mechanistic level. It is clear that Rho GTPase modules are critical targets of the cell cycle machinery, but many important questions remain, such as whether Cdc42 activity changes during the cell cycle, and which proteins are involved in this potential regulation (see Chapter 2). Furthermore, little is known about the function of Cdc42 during mitotic exit or how Cdc42 and Rho1 (or other Rho GTPases) are coordinated during the cell division cycle (see Chapter 3). These questions will be central topics of this study.

1.6 Control of cytokinesis by Rho GTPases

Cytokinesis is the process by which a mitotic cell that has completed chromosome segregation physically divides to produce two daughter cells. Broadly, in fungi and animals this process involves determination of a division site, formation of a contractile actin ring (CAR), contraction of the ring coupled with plasma membrane ingression, and finally abscission of the plasma membrane to resolve the daughter cells. This section provides a brief introduction to cytokinesis, with a focus on the regulation and function of Rho GTPases in yeast and animal cells. Additional details on mechanisms of cytokinesis in budding yeast that are relevant to this dissertation are provided in the introduction to Chapter 3. For reasons of clarity the unifying and conserved themes of cytokinesis in these systems will be highlighted, but it is important to keep in mind that different organisms have evolved different mechanisms to cope with their own unique requirements.

1.6.1 Rho1/RhoA activation at the division site is required for cytokinesis

Cytokinesis requires determination of a division plane that will bisect the segregated chromosome masses. This determination is one of the more divergent aspects of cytokinesis across different species. In budding yeast the division site is pre-determined by the site of budding, and the mitotic spindle must align along this axis in order for equal chromosome segregation to occur (Bi and Park, 2012). In fission yeast, the position of the nucleus in interphase defines the region of the cortex that will form the division site (Pollard and Wu, 2010; Rincon and Paoletti, 2012). In animal cells, it is thought that two mechanisms control the site of cleavage furrow formation; one

emanating from the central spindle, a region of microtubule overlap at the cell equator, and the other originating from the spindle asters (Green et al., 2012).

Despite the different mechanisms used to define the division site, work in both budding yeast and animal cells have shown that the positioning, formation, and function of the contractile actin ring (CAR) require Rho1/RhoA activation at the cell equator during late anaphase (Bement et al., 2005; Drechsel et al., 1997; Jantsch-Plunger et al., 2000; Jordan and Canman, 2012; Kishi et al., 1993; Tolliday et al., 2002; Yoshida et al., 2006). The cell cycle cue for Rho1/RhoA activation in both budding yeast and mammals is provided by Polo-like kinase. In budding yeast, Cdc5/Polo binds and phosphorylates the Rho1-GEFs Tus1 and Rom2, thereby targeting them to the bud neck, where they recruit and activate Rho1 itself (Yoshida et al., 2006). This GEF-dependent mechanism accounts for early Rho1 localization and activation during contractile ring assembly; during later stages of cytokinesis the membrane lipid phosphatidylinositol 4,5-bisphosphate and Gps1 maintain Rho1 at the bud neck to promote septum formation (Meitinger et al., 2013; Yoshida et al., 2009).

A conceptually similar module operates in mammals, where Plk1 phosphorylates the centralspindlin component Cyk-4/MgcRacGAP to create a binding site for the Rho-GEF Ect2, which recruits it to the central spindle (Burkard et al., 2009; Petronczki et al., 2007; Wolfe et al., 2009). Ect2 subsequently localizes to the underlying plasma membrane upon Cdk1 inactivation, which is required for cleavage furrow formation (Su et al., 2011). Ect2 recruitment is thought to control the local activation of RhoA at the cell equator that defines the site of contractile ring assembly and cleavage furrow formation (Bement et al., 2005; Kimura et al., 2000; Yoshizaki et al., 2003). In

metazoans additional Rho-GEFs such as GEF-H1 may play contributory roles in RhoA activation; it will be interesting to test whether these Rho-GEFs are also Plk1 substrates (Birkenfeld et al., 2007). A recently-discovered Rho-GAP, MP-GAP, appears to cooperate with a mysterious aster-derived signal to limit RhoA activation to the cell equator during cytokinesis (Zanin et al., 2013). Overall, these studies suggest that a conserved Polo kinase-RhoGEF axis controls local activation of Rho1/RhoA during cytokinesis.

1.6.2 Rho-dependent contractile actin ring assembly

Contractile actin ring assembly requires several downstream Rho effectors. Formins, which nucleate linear actin filaments, are conserved Rho1/RhoA effectors from yeasts to humans that are required for CAR assembly (Chang et al., 1997; Coffman et al., 2009; Coffman et al., 2013; Kovar et al., 2003; Tolliday et al., 2002; Vallen et al., 2000; Watanabe et al., 2010). In animal cells two RhoA effector kinases, Rho kinase (ROCK) and Citron kinase, stimulate myosin II contractility during cytokinesis *via* activating phosphorylation of the myosin regulatory light chain (Amano et al., 1996; Kosako et al., 2000; Yamashiro et al., 2003).

Despite our knowledge of several Rho effectors, the actual mechanism of contractile actin ring assembly remains mysterious. The best-elaborated model comes from the fission yeast *Schizosaccharomyces pombe.* In this system, the anillin-like protein Mid1 promotes formation of approximately 65 nodes at the cell equator that contain cytokinesis proteins including IQGAP, type II myosin, myosin light chains, and formins at the cell equator at G_2/M (Pollard and Wu, 2010; Wu et al., 2006). Live cell

imaging and mathematical modeling support a 'search-capture-pull-release' model where a broad band of interconnected nodes coalesce into an organized ring *via* iterative transient cycles of capture of formin-nucleated actin filaments from one node by myosin motors in a neighboring node followed by myosin motor-dependent contraction (Laporte et al., 2011; Vavylonis et al., 2008). Importantly, the MEN-related Septation Initation Network (SIN) signaling pathway controls a parallel pathway that redundantly promotes contractile ring assembly and maintenance (Bohnert et al., 2013, Hachet and Simanis, 2008; Huang et al., 2008).

The role of Rho1 or other Rho GTPases in controlling contractile ring assembly in fission yeast is less clear than in other systems. Notably, the formin Cdc12 may not require Rho-dependent release of autoinhibition as is common for other formins; instead phospho-regulation may play a major role in Cdc12 regulation (Bohnert et al., 2013; Yonetani et al., 2008). The Rho-GEF Gef2 cooperates with Plo1/Polo kinase to promote Mid1 localization to the cell equator and also contributes to contractile ring stability; Gef2 interacts with Rho1 *in vitro* but further work is required to determine its targets *in vivo* (Ye et al., 2012; Zhu et al., 2013). Overall, the 'search-capture-pull-release' model serves as a useful conceptual starting point to address Rho-dependent CAR assembly in higher eukaryotes.

1.6.3 CAR contraction and furrow ingression

After the CAR has been assembled at the division site it undergoes contraction, causing the underlying plasma membrane to ingress. For this step, the major Rho-dependent input is thought to be myosin contractility. A 'purse-string' model analogous

to muscle sarcomeres where myosin II-dependent sliding of actin filaments provides the force for contraction has been the most commonly assumed mechanism (Schroeder, 1973). An alternative model proposes that actin filament disassembly coupled with end-tracking cross-linkers can provide force even in the absence of myosin motor activity (Dickinson et al., 2004; Mogilner and Oster, 2003; Zumdieck et al., 2007). In this scenario myosin could be most important for actin filament cross-linking or alignment (Fang et al., 2010; Ma et al., 2012). Further, myosin II can promote turnover or disassembly of actin filaments (Guha et al., 2005; Mendes Pinto et al., 2012; Murthy and Wadsworth, 2005; Wilson et al., 2010). These models are not mutually exclusive, and it is possible that both operate to promote efficient CAR contraction and disassembly.

Another outstanding question is how the contracting CAR physically deforms the plasma membrane to promote cleavage furrow ingression. While several candidate proteins including anillin, F-BAR proteins, septins, and ezrin-radixin-moesin (ERM) family members can interact with membrane phospholipids as well with components of the contractile ring, so far the specific linkages that are required for cleavage furrow ingression are unclear (Bertin et al., 2010; Fehon et al., 2010; Frost et al., 2008; Heath and Insall, 2008; Liu et al., 2012; Roberts-Galbraith and Gould, 2010). It is possible that these proteins, which individually interact weakly with the plasma membrane, act cooperatively to ensure that the force from CAR contraction is transduced to the plasma membrane (Echard, 2012).

1.6.4 Does Rho function in abscission?

The last step in cytokinesis is the final scission of the plasma membrane to generate two daughter cells (Fededa and Gerlich, 2012). This step occurs after the contractile actin ring has undergone contraction and has largely disassembled. In budding yeast, abscission is not well understood in part due to the very small size of the bud neck. The exocyst component Sec3, which promotes tethering of Golgi-derived vesicles to the plasma membrane prior to fusion, is required for abscission (Dobbelaere and Barral, 2004; VerPlank and Li, 2005). Sec3 is a Rho1 effector protein, so in this sense Rho1 could promote the final abscission event (Guo et al., 2001). Rho1 and Sec3 could promote abscission through septum formation (Onishi et al., 2013).

In mammalian cells, after cleavage furrow ingression the contractile actin ring transitions into a midbody ring that directly apposes the midbody. This transition requires anillin, Citron kinase, and septins (Bassi et al., 2013; El Amine et al., 2013; Kechad et al., 2012; Watanabe et al., 2013). Next, a secondary ingression leads to further narrowing of the intercellular bridge between the two cells, which involves actin depolymerization *via* delivery of FIP3 endosomes, recruitment of the endosomal sorting complex required for transport-III (ESCRT-III) complex and regulators to the midbody, and spastin-dependent microtubule severing (Agromayor and Martin-Serrano, 2013). The final scission event that resolves the daughter cell membranes occurs in an ESCRT-III dependent manner at a position on either side of the midbody bulge (Guizetti et al., 2011).

Besides its essential role in the prerequisite step of furrow ingression, RhoA has been considered to be dispensable for the final events of abscission. However, several recent studies suggest that RhoA may play a greater role than currently appreciated. Interestingly, tension on the intercellular bridge delays ESCRT-III assembly and

abscission, so RhoA-dependent cortical contractility or cell spreading could control the timing of abscission (Lafaurie-Janvore et al., 2013). Additionally, local RhoA regulation at the midbody could also contribute to abscission. The Rho-GAP p50RhoGAP is delivered to the midbody by FIP3 endosomes and is required for actin depolymerization, suggesting that RhoA inactivation could be important for secondary ingression (Schiel et al., 2012). Furthermore, the RhoA-GEF LARG appears to be important for a late stage in abscission after midbody formation (Martz et al., 2013). Perhaps most strikingly RhoA and anillin localize both to the midbody but also to the presumptive abscission site that flanks the midbody (Hu et al., 2012).The functional significance of this recruitment as well as the other findings will require additional experiments to specifically inactivate RhoA after cleavage furrow ingression is complete.

1.6.5 Role of other Rho family GTPases in cytokinesis

In contrast to the conserved positive role of Rho1/RhoA in cytokinesis described above, the role of other Rho-family GTPases, including Rac1 and Cdc42, is less clear and the subject of ongoing debate. Rac1 is active at the cell poles but is inactivated at the cell equator during cytokinesis in mammalian cells (Yoshizaki et al., 2003). Expression of dominant-active GTP-locked Rac1 can block cytokinesis in several cell types, whereas Rac1 inactivation has little effect on successful cytokinesis (Jordan and Canman, 2012). Biochemical and genetic data suggest that Rac1 is a critical target of the Rho GTPase activating protein (GAP) Cyk-4's GAP activity, and that Rac1 inactivation may prevent inappropriate PAK1-dependent cell matrix adhesion at the division site or relaxation of cortical stiffness (Bastos et al., 2012; Canman et al., 2008; D'Avino et al., 2004).

However, Cyk-4 may instead (or in addition) act on RhoA to restrict its activation to the equatorial region (Loria et al., 2012; Miller and Bement, 2009). In sum, an accumulating body of evidence suggests that inappropriate Rac1 activation inhibits cytokinesis.

The role of Cdc42 in cytokinesis is not well defined, with both positive and negative roles proposed. Cdc42 is required for polar body extrusion during oogenesis in *Xenopus* and mice (Ma et al., 2006; Na and Zernicka-Goetz, 2006), but is dispensable for cytokinesis in budding yeast and most somatic animal cells (Canman et al., 2008; Iwase et al., 2006; Jantsch-Plunger et al., 2000; Tolliday et al., 2002). In fission yeast, a Cdc42-GEF (Gef1) and an interacting Bin/Amphiphysin/Rvs (BAR) domain protein (Hob3) recruit Cdc42 to the division site, and this recruitment may contribute to cytokinesis or septation (Rincon et al., 2007). Potentially at odds with these results, or suggesting fine tuned temporal regulation, the Cdc42 effector PAK kinase Pak1/Orb2 has been proposed to inhibit cytokinesis *via* phosphorylation of myosin regulatory light chain (Loo and Balasubramanian, 2008). In animal cells, overexpression of dominant-active GTP-locked Cdc42 can inhibit cytokinesis (Jordan and Canman, 2012). However, overexpression of GTP-locked GTPases can be difficult to interpret because of indirect effects on multiple GTPases via sequestration of Rho guanine nucleotide dissociation inhibitor (GDI) proteins (Boulter et al., 2010). Overall, the function of Cdc42 during cytokinesis remains to be defined (see Chapter 3).

1.7 Goals of this dissertation

I have three major goals in this study. The first is to determine whether Cdc42 activation changes across the cell cycle. This will contribute to a fuller understanding of

how Cdc42 activation is controlled during polarization at G_1/S, and will also define the activation state of Cdc42 at later stages in the cell cycle (Chapter 2). The second goal is to understand how the cell cycle machinery controls Cdc42 activation, especially during mitosis (Chapter 2). The final goal is to understand the function of Cdc42 during mitosis, especially during the last step of cell division, cytokinesis (Chapter 3).

1.8 References

Adamo, J.E., J.J. Moskow, A.S. Gladfelter, D. Viterbo, D.J. Lew, and P.J. Brennwald. 2001. Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. *J Cell Biol*. 155:581-592.

Adamo, J.E., G. Rossi, and P. Brennwald. 1999. The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. *Mol Biol Cell*. 10:4121-4133.

Adams, A.E., D.I. Johnson, R.M. Longnecker, B.F. Sloat, and J.R. Pringle. 1990. CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast Saccharomyces cerevisiae. *J Cell Biol*. 111:131-142.

Adams, A.E., and J.R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant Saccharomyces cerevisiae. *J Cell Biol*. 98:934-945.

Adamson, P., C.J. Marshall, A. Hall, and P.A. Tilbrook. 1992a. Post-translational modifications of p21rho proteins. *J Biol Chem*. 267:20033-20038.

Adamson, P., H.F. Paterson, and A. Hall. 1992b. Intracellular localization of the P21rho proteins. *J Cell Biol*. 119:617-627.

Agromayor, M., and J. Martin-Serrano. 2013. Knowing when to cut and run: mechanisms that control cytokinetic abscission. *Trends Cell Biol*. 23:433-441.

Amano, M., H. Mukai, Y. Ono, K. Chihara, T. Matsui, Y. Hamajima, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. *Science*. 271:648-650.

Arkowitz, R.A., and M. Bassilana. 2011. Polarized growth in fungi: symmetry breaking and hyphal formation. *Semin Cell Dev Biol*. 22:806-815.

Ayscough, K.R., J. Stryker, N. Pokala, M. Sanders, P. Crews, and D.G. Drubin. 1997. High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. *J Cell Biol*. 137:399-416.

Bardin, A.J., R. Visintin, and A. Amon. 2000. A mechanism for coupling exit from mitosis to partitioning of the nucleus. *Cell*. 102:21-31.

Barral, Y., V. Mermall, M.S. Mooseker, and M. Snyder. 2000. Compartmentalization of the cell cortex by septins is required for maintenance of cell polarity in yeast. *Mol Cell*. 5:841-851.

Bassi, Z.I., M. Audusseau, M.G. Riparbelli, G. Callaini, and P.P. D'Avino. 2013. Citron kinase controls a molecular network required for midbody formation in cytokinesis. *Proc Natl Acad Sci U S A*. 110:9782-9787.

Bastos, R.N., X. Penate, M. Bates, D. Hammond, and F.A. Barr. 2012. CYK4 inhibits Rac1-dependent PAK1 and ARHGEF7 effector pathways during cytokinesis. *J Cell Biol*. 198:865-880.

Bement, W.M., H.A. Benink, and G. von Dassow. 2005. A microtubule-dependent zone of active RhoA during cleavage plane specification. *J Cell Biol*. 170:91-101.

Bender, A., and J.R. Pringle. 1989. Multicopy suppression of the cdc24 budding defect in yeast by CDC42 and three newly identified genes including the ras-related gene RSR1. *Proc Natl Acad Sci U S A*. 86:9976-9980.

Benton, B.K., A. Tinkelenberg, I. Gonzalez, and F.R. Cross. 1997. Cla4p, a Saccharomyces cerevisiae Cdc42p-activated kinase involved in cytokinesis, is activated at mitosis. *Mol Cell Biol*. 17:5067-5076.

Bertazzi, D.T., B. Kurtulmus, and G. Pereira. 2011. The cortical protein Lte1 promotes mitotic exit by inhibiting the spindle position checkpoint kinase Kin4. *J Cell Biol*. 193:1033-1048.

Bertin, A., M.A. McMurray, L. Thai, G. Garcia, 3rd, V. Votin, P. Grob, T. Allyn, J. Thorner, and E. Nogales. 2010. Phosphatidylinositol-4,5-bisphosphate promotes budding yeast septin filament assembly and organization. *J Mol Biol*. 404:711-731.

Bi, E., and H.O. Park. 2012. Cell polarization and cytokinesis in budding yeast. *Genetics*. 191:347-387.

Birkenfeld, J., P. Nalbant, B.P. Bohl, O. Pertz, K.M. Hahn, and G.M. Bokoch. 2007. GEF-H1 modulates localized RhoA activation during cytokinesis under the control of mitotic kinases. *Dev Cell*. 12:699-712.

Bohnert, K.A., A.P. Grzegorzewska, A.H. Willet, C.W. Vander Kooi, D.R. Kovar, and K.L. Gould. 2013. SIN-dependent phosphoinhibition of formin multimerization controls fission yeast cytokinesis. *Genes Dev*. 27:2164-2177.

Bose, I., J.E. Irazoqui, J.J. Moskow, E.S. Bardes, T.R. Zyla, and D.J. Lew. 2001. Assembly of scaffold-mediated complexes containing Cdc42p, the exchange factor Cdc24p, and the effector Cla4p required for cell cycle-regulated phosphorylation of Cdc24p. *J Biol Chem*. 276:7176-7186.

Boulter, E., S. Estrach, R. Garcia-Mata, and C.C. Feral. 2012. Off the beaten paths: alternative and crosstalk regulation of Rho GTPases. *FASEB J*. 26:469-479.

Boulter, E., R. Garcia-Mata, C. Guilluy, A. Dubash, G. Rossi, P.J. Brennwald, and K. Burridge. 2010. Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. *Nat Cell Biol*. 12:477-483.

Burbelo, P.D., D. Drechsel, and A. Hall. 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem*. 270:29071-29074.

Burkard, M.E., C.L. Randall, S. Larochelle, C. Zhang, K.M. Shokat, R.P. Fisher, and P.V. Jallepalli. 2009. Chemical genetics reveals the requirement for Polo-like kinase 1 activity in positioning RhoA and triggering cytokinesis in human cells. *Proc Natl Acad Sci U S A*. 104:4383-4388.

Canman, J.C., L. Lewellyn, K. Laband, S.J. Smerdon, A. Desai, B. Bowerman, and K. Oegema. 2008. Inhibition of Rac by the GAP activity of centralspindlin is essential for cytokinesis. *Science*. 322:1543-1546.

Caviston, J.P., M. Longtine, J.R. Pringle, and E. Bi. 2003. The role of Cdc42p GTPase-activating proteins in assembly of the septin ring in yeast. *Mol Biol Cell*. 14:4051-4066.

Chan, L.Y., and A. Amon. 2010. Spindle position is coordinated with cell-cycle progression through establishment of mitotic exit-activating and -inhibitory zones. *Mol Cell*. 39:444-454.

Chang, F., D. Drubin, and P. Nurse. 1997. cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. *J Cell Biol*. 137:169-182.

Chant, J., and I. Herskowitz. 1991. Genetic control of bud site selection in yeast by a set of gene products that constitute a morphogenetic pathway. *Cell*. 65:1203-1212.

Chen, H., A.S. Howell, A. Robeson, and D.J. Lew. 2011. Dynamics of septin ring and collar formation in Saccharomyces cerevisiae. *Biol Chem*. 392:689-697.

Chen, H., C.C. Kuo, H. Kang, A.S. Howell, T.R. Zyla, M. Jin, and D.J. Lew. 2012. Cdc42p regulation of the yeast formin Bni1p mediated by the effector Gic2p. *Mol Biol Cell*. 23:3814-3826.

Cherfils, J., and M. Zeghouf. 2013. Regulation of small GTPases by GEFs, GAPs, and GDIs. *Physiol Rev*. 93:269-309.

Cid, V.J., L. Adamikova, M. Sanchez, M. Molina, and C. Nombela. 2001. Cell cycle control of septin ring dynamics in the budding yeast. *Microbiology*. 147:1437-1450.

Coffman, V.C., A.H. Nile, I.J. Lee, H. Liu, and J.Q. Wu. 2009. Roles of formin nodes and myosin motor activity in Mid1p-dependent contractile-ring assembly during fission yeast cytokinesis. *Mol Biol Cell*. 20:5195-5210.

Coffman, V.C., J.A. Sees, D.R. Kovar, and J.Q. Wu. 2013. The formins Cdc12 and For3 cooperate during contractile ring assembly in cytokinesis. *J Cell Biol*. 203:101-114.

Costanzo, M., A. Baryshnikova, J. Bellay, Y. Kim, E.D. Spear, C.S. Sevier, H. Ding, J.L. Koh, K. Toufighi, S. Mostafavi, J. Prinz, R.P. St Onge, B. VanderSluis, T. Makhnevych, F.J. Vizeacoumar, S. Alizadeh, S. Bahr, R.L. Brost, Y. Chen, M. Cokol, R. Deshpande, Z. Li, Z.Y. Lin, W. Liang, M. Marback, J. Paw, B.J. San Luis, E. Shuteriqi, A.H. Tong, N. van Dyk, I.M. Wallace, J.A. Whitney, M.T. Weirauch, G. Zhong, H. Zhu, W.A. Houry, M. Brudno, S. Ragibizadeh, B. Papp, C. Pal, F.P. Roth, G. Giaever, C. Nislow, O.G. Troyanskaya, H. Bussey, G.D. Bader, A.C. Gingras, Q.D. Morris, P.M. Kim, C.A. Kaiser, C.L. Myers, B.J. Andrews, and C. Boone. 2010. The genetic landscape of a cell. *Science*. 327:425-431.

Cullen, P.J., and G.F. Sprague, Jr. 2012. The regulation of filamentous growth in yeast. *Genetics*. 190:23-49.

Cvrckova, F., C. De Virgilio, E. Manser, J.R. Pringle, and K. Nasmyth. 1995. Ste20-like protein kinases are required for normal localization of cell growth and for cytokinesis in budding yeast. *Genes Dev*. 9:1817-1830.

D'Avino, P.P., M.S. Savoian, and D.M. Glover. 2004. Mutations in sticky lead to defective organization of the contractile ring during cytokinesis and are enhanced by Rho and suppressed by Rac. *J Cell Biol*. 166:61-71.

Deakin, N.O., and C.E. Turner. 2008. Paxillin comes of age. *J Cell Sci*. 121:2435-2444.

DeMay, B.S., X. Bai, L. Howard, P. Occhipinti, R.A. Meseroll, E.T. Spiliotis, R. Oldenbourg, and A.S. Gladfelter. 2011. Septin filaments exhibit a dynamic, paired organization that is conserved from yeast to mammals. *J Cell Biol*. 193:1065-1081.

Dickinson, R.B., L. Caro, and D.L. Purich. 2004. Force generation by cytoskeletal filament end-tracking proteins. *Biophys J*. 87:2838-2854.

Didsbury, J., R.F. Weber, G.M. Bokoch, T. Evans, and R. Snyderman. 1989. rac, a novel ras-related family of proteins that are botulinum toxin substrates. *J Biol Chem*. 264:16378-16382.

Dobbelaere, J., and Y. Barral. 2004. Spatial coordination of cytokinetic events by compartmentalization of the cell cortex. *Science*. 305:393-396.

Dobbelaere, J., M.S. Gentry, R.L. Hallberg, and Y. Barral. 2003. Phosphorylation-dependent regulation of septin dynamics during the cell cycle. *Dev Cell*. 4:345-357.

Dong, Y., D. Pruyne, and A. Bretscher. 2003. Formin-dependent actin assembly is regulated by distinct modes of Rho signaling in yeast. *J Cell Biol*. 161:1081-1092.

Drechsel, D.N., A.A. Hyman, A. Hall, and M. Glotzer. 1997. A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos. *Curr Biol*. 7:12-23.

Dumont, S., and T.J. Mitchison. 2009. Force and length in the mitotic spindle. *Curr Biol*. 19:R749-761.

Echard, A. 2012. Phosphoinositides and cytokinesis: the "PIP" of the iceberg. *Cytoskeleton (Hoboken)*. 69:893-912.

El Amine, N., A. Kechad, S. Jananji, and G.R. Hickson. 2013. Opposing actions of septins and Sticky on Anillin promote the transition from contractile to midbody ring. *J Cell Biol*. 203:487-504.

Etienne-Manneville, S., and A. Hall. 2002. Rho GTPases in cell biology. *Nature*. 420:629-635.

Evangelista, M., D. Pruyne, D.C. Amberg, C. Boone, and A. Bretscher. 2002. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nat Cell Biol*. 4:260-269.

Falk, J.E., L.Y. Chan, and A. Amon. 2011. Lte1 promotes mitotic exit by controlling the localization of the spindle position checkpoint kinase Kin4. *Proc Natl Acad Sci U S A*. 108:12584-12590.

Fang, X., J. Luo, R. Nishihama, C. Wloka, C. Dravis, M. Travaglia, M. Iwase, E.A. Vallen, and E. Bi. 2010. Biphasic targeting and cleavage furrow ingression directed by the tail of a myosin II. *J Cell Biol*. 191:1333-1350.

Farkas, V., J. Kovarik, A. Kosinova, and S. Bauer. 1974. Autoradiographic study of mannan incorporation into the growing cell walls of Saccharomyces cerevisiae. *J Bacteriol*. 117:265-269.

Fededa, J.P., and D.W. Gerlich. 2012. Molecular control of animal cell cytokinesis. *Nat Cell Biol*. 14:440-447.

Fehon, R.G., A.I. McClatchey, and A. Bretscher. 2010. Organizing the cell cortex: the role of ERM proteins. *Nat Rev Mol Cell Biol*. 11:276-287.

Freifelder, D. 1960. Bud position in Saccharomyces cerevisiae. *J Bacteriol*. 80:567-568.

Freisinger, T., B. Klunder, J. Johnson, N. Muller, G. Pichler, G. Beck, M. Costanzo, C. Boone, R.A. Cerione, E. Frey, and R. Wedlich-Soldner. 2013. Establishment of a robust single axis of cell polarity by coupling multiple positive feedback loops. *Nat Commun*. 4:1807.

Frost, A., R. Perera, A. Roux, K. Spasov, O. Destaing, E.H. Egelman, P. De Camilli, and V.M. Unger. 2008. Structural basis of membrane invagination by F-BAR domains. *Cell*. 132:807-817.

Gao, X.D., J.P. Caviston, S.E. Tcheperegine, and E. Bi. 2004. Pxl1p, a paxillin-like protein in Saccharomyces cerevisiae, may coordinate Cdc42p and Rho1p functions during polarized growth. *Mol Biol Cell*. 15:3977-3985.

Garcia-Mata, R., E. Boulter, and K. Burridge. 2011. The 'invisible hand': regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol*. 12:493-504.

Gladfelter, A.S., I. Bose, T.R. Zyla, E.S. Bardes, and D.J. Lew. 2002. Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p. *J Cell Biol*. 156:315-326.

Gladfelter, A.S., L. Kozubowski, T.R. Zyla, and D.J. Lew. 2005. Interplay between septin organization, cell cycle and cell shape in yeast. *J Cell Sci*. 118:1617-1628.

Goryachev, A.B., and A.V. Pokhilko. 2008. Dynamics of Cdc42 network embodies a Turing-type mechanism of yeast cell polarity. *FEBS Lett*. 582:1437-1443.

Green, R.A., E. Paluch, and K. Oegema. 2012. Cytokinesis in animal cells. *Annu Rev Cell Dev Biol*. 28:29-58.

Guha, M., M. Zhou, and Y.L. Wang. 2005. Cortical actin turnover during cytokinesis requires myosin II. *Curr Biol*. 15:732-736.

Guizetti, J., L. Schermelleh, J. Mantler, S. Maar, I. Poser, H. Leonhardt, T. Muller-Reichert, and D.W. Gerlich. 2011. Cortical constriction during abscission involves helices of ESCRT-III-dependent filaments. *Science*. 331:1616-1620.

Gulli, M.P., M. Jaquenoud, Y. Shimada, G. Niederhauser, P. Wiget, and M. Peter. 2000. Phosphorylation of the Cdc42 exchange factor Cdc24 by the PAK-like kinase Cla4 may regulate polarized growth in yeast. *Mol Cell*. 6:1155-1167.

Guo, W., F. Tamanoi, and P. Novick. 2001. Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat Cell Biol*. 3:353-360.

Hachet, O., and V. Simanis. 2008. Mid1p/anillin and the septation initiation network orchestrate contractile ring assembly for cytokinesis. *Genes Dev*. 22:3205-3216.

Hartwell, L.H. 1971. Genetic control of the cell division cycle in yeast. IV. Genes controlling bud emergence and cytokinesis. *Exp Cell Res*. 69:265-276.

Heath, R.J., and R.H. Insall. 2008. F-BAR domains: multifunctional regulators of membrane curvature. *J Cell Sci*. 121:1951-1954.

Helliwell, S.B., A. Schmidt, Y. Ohya, and M.N. Hall. 1998. The Rho1 effector Pkc1, but not Bni1, mediates signalling from Tor2 to the actin cytoskeleton. *Curr Biol*. 8:1211-1214.

Hicks, J.B., J.N. Strathern, and I. Herskowitz. 1977. Interconversion of Yeast Mating Types III. Action of the Homothallism (HO) Gene in Cells Homozygous for the Mating Type Locus. *Genetics*. 85:395-405.

Hofken, T., and E. Schiebel. 2002. A role for cell polarity proteins in mitotic exit. *EMBO J*. 21:4851-4862.

Hofken, T., and E. Schiebel. 2004. Novel regulation of mitotic exit by the Cdc42 effectors Gic1 and Gic2. *J Cell Biol*. 164:219-231.

Holly, S.P., and K.J. Blumer. 1999. PAK-family kinases regulate cell and actin polarization throughout the cell cycle of Saccharomyces cerevisiae. *J Cell Biol*. 147:845-856.

Howell, A.S., M. Jin, C.F. Wu, T.R. Zyla, T.C. Elston, and D.J. Lew. 2012. Negative feedback enhances robustness in the yeast polarity establishment circuit. *Cell*. 149:322-333.

Howell, A.S., N.S. Savage, S.A. Johnson, I. Bose, A.W. Wagner, T.R. Zyla, H.F. Nijhout, M.C. Reed, A.B. Goryachev, and D.J. Lew. 2009. Singularity in polarization: rewiring yeast cells to make two buds. *Cell*. 139:731-743.

Hu, C.K., M. Coughlin, and T.J. Mitchison. 2012. Midbody assembly and its regulation during cytokinesis. *Mol Biol Cell*. 23:1024-1034.

Huang, Y., H. Yan, and M.K. Balasubramanian. 2008. Assembly of normal actomyosin rings in the absence of Mid1p and cortical nodes in fission yeast. *J Cell Biol*. 183:979-988.

Irazoqui, J.E., A.S. Gladfelter, and D.J. Lew. 2003. Scaffold-mediated symmetry breaking by Cdc42p. *Nat Cell Biol*. 5:1062-1070.

Irazoqui, J.E., A.S. Howell, C.L. Theesfeld, and D.J. Lew. 2005. Opposing roles for actin in Cdc42p polarization. *Mol Biol Cell*. 16:1296-1304.

Iwase, M., J. Luo, S. Nagaraj, M. Longtine, H.B. Kim, B.K. Haarer, C. Caruso, Z. Tong, J.R. Pringle, and E. Bi. 2006. Role of a Cdc42p effector pathway in recruitment of the yeast septins to the presumptive bud site. *Mol Biol Cell*. 17:1110-1125.

Jantsch-Plunger, V., P. Gonczy, A. Romano, H. Schnabel, D. Hamill, R. Schnabel, A.A. Hyman, and M. Glotzer. 2000. CYK-4: A Rho family gtpase activating protein (GAP) required for central spindle formation and cytokinesis. *J Cell Biol*. 149:1391-1404.

Johnson, D.I., C.W. Jacobs, J.R. Pringle, L.C. Robinson, G.F. Carle, and M.V. Olson. 1987. Mapping of the Saccharomyces cerevisiae CDC3, CDC25, and CDC42 genes to chromosome XII by chromosome blotting and tetrad analysis. *Yeast*. 3:243-253.

Johnson, D.I., and J.R. Pringle. 1990. Molecular characterization of CDC42, a Saccharomyces cerevisiae gene involved in the development of cell polarity. *J Cell Biol*. 111:143-152.

Johnson, J.L., J.W. Erickson, and R.A. Cerione. 2009. New insights into how the Rho guanine nucleotide dissociation inhibitor regulates the interaction of Cdc42 with membranes. *J Biol Chem*. 284:23860-23871.

Johnson, J.M., M. Jin, and D.J. Lew. 2011. Symmetry breaking and the establishment of cell polarity in budding yeast. *Curr Opin Genet Dev*. 21:740-746.

Jordan, S.N., and J.C. Canman. 2012. Rho GTPases in animal cell cytokinesis: An occupation by the one percent. *Cytoskeleton (Hoboken)*.

Jose, M., S. Tollis, D. Nair, J.B. Sibarita, and D. McCusker. 2013. Robust polarity establishment occurs via an endocytosis-based cortical corralling mechanism. *J Cell Biol*. 200:407-418.

Kaksonen, M., Y. Sun, and D.G. Drubin. 2003. A pathway for association of receptors, adaptors, and actin during endocytic internalization. *Cell*. 115:475-487.

Kamada, Y., H. Qadota, C.P. Python, Y. Anraku, Y. Ohya, and D.E. Levin. 1996. Activation of yeast protein kinase C by Rho1 GTPase. *J Biol Chem*. 271:9193-9196.

Katayama, M., M. Kawata, Y. Yoshida, H. Horiuchi, T. Yamamoto, Y. Matsuura, and Y. Takai. 1991. The posttranslationally modified C-terminal structure of bovine aortic smooth muscle rhoA p21. *J Biol Chem*. 266:12639-12645.

Kechad, A., S. Jananji, Y. Ruella, and G.R. Hickson. 2012. Anillin acts as a bifunctional linker coordinating midbody ring biogenesis during cytokinesis. *Curr Biol*. 22:197-203.

Kilmartin, J.V., and A.E. Adams. 1984. Structural rearrangements of tubulin and actin during the cell cycle of the yeast Saccharomyces. *J Cell Biol*. 98:922-933.

Kim, Y.J., L. Francisco, G.C. Chen, E. Marcotte, and C.S. Chan. 1994. Control of cellular morphogenesis by the Ip12/Bem2 GTPase-activating protein: possible role of protein phosphorylation. *J Cell Biol*. 127:1381-1394.

Kimura, K., T. Tsuji, Y. Takada, T. Miki, and S. Narumiya. 2000. Accumulation of GTP-bound RhoA during cytokinesis and a critical role of ECT2 in this accumulation. *J Biol Chem*. 275:17233-17236.

Kishi, K., T. Sasaki, S. Kuroda, T. Itoh, and Y. Takai. 1993. Regulation of cytoplasmic division of Xenopus embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *J Cell Biol*. 120:1187-1195.

Knaus, M., M.P. Pelli-Gulli, F. van Drogen, S. Springer, M. Jaquenoud, and M. Peter. 2007. Phosphorylation of Bem2p and Bem3p may contribute to local activation of Cdc42p at bud emergence. *EMBO J*. 26:4501-4513.

Kono, K., S. Nogami, M. Abe, M. Nishizawa, S. Morishita, D. Pellman, and Y. Ohya. 2008. G1/S cyclin-dependent kinase regulates small GTPase Rho1p through phosphorylation of RhoGEF Tus1p in Saccharomyces cerevisiae. *Mol Biol Cell*. 19:1763-1771.

Kosako, H., T. Yoshida, F. Matsumura, T. Ishizaki, S. Narumiya, and M. Inagaki. 2000. Rho-kinase/ROCK is involved in cytokinesis through the phosphorylation of myosin light chain and not ezrin/radixin/moesin proteins at the cleavage furrow. *Oncogene*. 19:6059-6064.

Kovar, D.R., J.R. Kuhn, A.L. Tichy, and T.D. Pollard. 2003. The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin. *J Cell Biol*. 161:875-887.

Kozminski, K.G., L. Beven, E. Angerman, A.H. Tong, C. Boone, and H.O. Park. 2003. Interaction between a Ras and a Rho GTPase couples selection of a growth site to the development of cell polarity in yeast. *Mol Biol Cell*. 14:4958-4970.

Kozubowski, L., K. Saito, J.M. Johnson, A.S. Howell, T.R. Zyla, and D.J. Lew. 2008. Symmetry-breaking polarization driven by a Cdc42p GEF-PAK complex. *Curr Biol*. 18:1719-1726.

Kubler, E., and H. Riezman. 1993. Actin and fimbrin are required for the internalization step of endocytosis in yeast. *EMBO J*. 12:2855-2862.

Lafaurie-Janvore, J., P. Maiuri, I. Wang, M. Pinot, J.B. Manneville, T. Betz, M. Balland, and M. Piel. 2013. ESCRT-III assembly and cytokinetic abscission are induced by tension release in the intercellular bridge. *Science*. 339:1625-1629.

Laporte, D., V.C. Coffman, I.J. Lee, and J.Q. Wu. 2011. Assembly and architecture of precursor nodes during fission yeast cytokinesis. *J Cell Biol*. 192:1005-1021.

Layton, A.T., N.S. Savage, A.S. Howell, S.Y. Carroll, D.G. Drubin, and D.J. Lew. 2011. Modeling vesicle traffic reveals unexpected consequences for Cdc42p-mediated polarity establishment. *Curr Biol*. 21:184-194.

Leberer, E., D. Dignard, D. Harcus, D.Y. Thomas, and M. Whiteway. 1992. The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein beta gamma subunits to downstream signalling components. *EMBO J*. 11:4815-4824.

Lechler, T., G.A. Jonsdottir, S.K. Klee, D. Pellman, and R. Li. 2001. A two-tiered mechanism by which Cdc42 controls the localization and activation of an Arp2/3-activating motor complex in yeast. *J Cell Biol*. 155:261-270.

Lechler, T., A. Shevchenko, and R. Li. 2000. Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. *J Cell Biol*. 148:363-373.

Levin, D.E. 2011. Regulation of cell wall biogenesis in Saccharomyces cerevisiae: the cell wall integrity signaling pathway. *Genetics*. 189:1145-1175.

Lew, D.J., and S.I. Reed. 1993. Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J Cell Biol*. 120:1305-1320.

Liu, J., G.D. Fairn, D.F. Ceccarelli, F. Sicheri, and A. Wilde. 2012. Cleavage furrow organization requires PIP(2)-mediated recruitment of anillin. *Curr Biol*. 22:64-69.

Longtine, M.S., C.L. Theesfeld, J.N. McMillan, E. Weaver, J.R. Pringle, and D.J. Lew. 2000. Septin-dependent assembly of a cell cycle-regulatory module in Saccharomyces cerevisiae. *Mol Cell Biol*. 20:4049-4061.

Loo, T.H., and M. Balasubramanian. 2008. Schizosaccharomyces pombe Pak-related protein, Pak1p/Orb2p, phosphorylates myosin regulatory light chain to inhibit cytokinesis. *J Cell Biol*. 183:785-793.

Loria, A., K.M. Longhini, and M. Glotzer. 2012. The RhoGAP domain of CYK-4 has an essential role in RhoA activation. *Curr Biol*. 22:213-219.

Ma, C., H.A. Benink, D. Cheng, V. Montplaisir, L. Wang, Y. Xi, P.P. Zheng, W.M. Bement, and X.J. Liu. 2006. Cdc42 activation couples spindle positioning to first polar body formation in oocyte maturation. *Curr Biol*. 16:214-220.

Ma, X., M. Kovacs, M.A. Conti, A. Wang, Y. Zhang, J.R. Sellers, and R.S. Adelstein. 2012. Nonmuscle myosin II exerts tension but does not translocate actin in vertebrate cytokinesis. *Proc Natl Acad Sci U S A*. 109:4509-4514.

Machacek, M., L. Hodgson, C. Welch, H. Elliott, O. Pertz, P. Nalbant, A. Abell, G.L. Johnson, K.M. Hahn, and G. Danuser. 2009. Coordination of Rho GTPase activities during cell protrusion. *Nature*. 461:99-103.

Madaule, P., and R. Axel. 1985. A novel ras-related gene family. *Cell*. 41:31-40.

Marco, E., R. Wedlich-Soldner, R. Li, S.J. Altschuler, and L.F. Wu. 2007. Endocytosis optimizes the dynamic localization of membrane proteins that regulate cortical polarity. *Cell*. 129:411-422.

Martz, M.K., E. Grabocka, N. Beeharry, T.J. Yen, and P.B. Wedegaertner. 2013. Leukemia-associated RhoGEF (LARG) is a novel RhoGEF in cytokinesis and required for the proper completion of abscission. *Mol Biol Cell*. 24:2785-2794.

Masuda, T., K. Tanaka, H. Nonaka, W. Yamochi, A. Maeda, and Y. Takai. 1994. Molecular cloning and characterization of yeast rho GDP dissociation inhibitor. *J Biol Chem*. 269:19713-19718.

Matsui, Y., and E.A. Toh. 1992. Yeast RHO3 and RHO4 ras superfamily genes are necessary for bud growth, and their defect is suppressed by a high dose of bud formation genes CDC42 and BEM1. *Mol Cell Biol*. 12:5690-5699.

Matthews, H.K., U. Delabre, J.L. Rohn, J. Guck, P. Kunda, and B. Baum. 2012. Changes in Ect2 localization couple actomyosin-dependent cell shape changes to mitotic progression. *Dev Cell*. 23:371-383.

Mazzoni, C., P. Zarov, A. Rambourg, and C. Mann. 1993. The SLT2 (MPK1) MAP kinase homolog is involved in polarized cell growth in Saccharomyces cerevisiae. *J Cell Biol*. 123:1821-1833.

McCusker, D., C. Denison, S. Anderson, T.A. Egelhofer, J.R. Yates, 3rd, S.P. Gygi, and D.R. Kellogg. 2007. Cdk1 coordinates cell-surface growth with the cell cycle. *Nat Cell Biol*. 9:506-515.

Meitinger, F., H. Richter, S. Heisel, B. Hub, W. Seufert, and G. Pereira. 2013. A safeguard mechanism regulates Rho GTPases to coordinate cytokinesis with the establishment of cell polarity. *PLoS Biol*. 11:e1001495.

Mendes Pinto, I., B. Rubinstein, A. Kucharavy, J.R. Unruh, and R. Li. 2012. Actin depolymerization drives actomyosin ring contraction during budding yeast cytokinesis. *Dev Cell*. 22:1247-1260.

Miller, A.L., and W.M. Bement. 2009. Regulation of cytokinesis by Rho GTPase flux. *Nat Cell Biol*. 11:71-77.

Moffat, J., and B. Andrews. 2004. Late-G1 cyclin-CDK activity is essential for control of cell morphogenesis in budding yeast. *Nat Cell Biol*. 6:59-66.

Mogilner, A., and G. Oster. 2003. Force generation by actin polymerization II: the elastic ratchet and tethered filaments. *Biophys J*. 84:1591-1605.

Monje-Casas, F., and A. Amon. 2009. Cell polarity determinants establish asymmetry in MEN signaling. *Dev Cell*. 16:132-145.

Moon, S.Y., and Y. Zheng. 2003. Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol*. 13:13-22.

Moskow, J.J., A.S. Gladfelter, R.E. Lamson, P.M. Pryciak, and D.J. Lew. 2000. Role of Cdc42p in pheromone-stimulated signal transduction in Saccharomyces cerevisiae. *Mol Cell Biol*. 20:7559-7571.

Mulholland, J., D. Preuss, A. Moon, A. Wong, D. Drubin, and D. Botstein. 1994. Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. *J Cell Biol*. 125:381-391.

Munemitsu, S., M.A. Innis, R. Clark, F. McCormick, A. Ullrich, and P. Polakis. 1990. Molecular cloning and expression of a G25K cDNA, the human homolog of the yeast cell cycle gene CDC42. *Mol Cell Biol*. 10:5977-5982.

Murthy, K., and P. Wadsworth. 2005. Myosin-II-dependent localization and dynamics of F-actin during cytokinesis. *Curr Biol*. 15:724-731.

Na, J., and M. Zernicka-Goetz. 2006. Asymmetric positioning and organization of the meiotic spindle of mouse oocytes requires CDC42 function. *Curr Biol*. 16:1249-1254.

Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell*. 40:405-416.

Oh, Y., and E. Bi. 2011. Septin structure and function in yeast and beyond. *Trends Cell Biol*. 21:141-148.

Okada, S., M. Leda, J. Hanna, N.S. Savage, E. Bi, and A.B. Goryachev. 2013. Daughter cell identity emerges from the interplay of Cdc42, septins, and exocytosis. *Dev Cell*. 26:148-161.

Onishi, M., N. Ko, R. Nishihama, and J.R. Pringle. 2013. Distinct roles of Rho1, Cdc42, and Cyk3 in septum formation and abscission during yeast cytokinesis. *J Cell Biol*. 202:311-329.

Orlando, K., X. Sun, J. Zhang, T. Lu, L. Yokomizo, P. Wang, and W. Guo. 2011. Exo-endocytic trafficking and the septin-based diffusion barrier are required for the

maintenance of Cdc42p polarization during budding yeast asymmetric growth. *Mol Biol Cell*. 22:624-633.

Park, H.O., E. Bi, J.R. Pringle, and I. Herskowitz. 1997. Two active states of the Ras-related Bud1/Rsr1 protein bind to different effectors to determine yeast cell polarity. *Proc Natl Acad Sci U S A*. 94:4463-4468.

Park, H.O., P.J. Kang, and A.W. Rachfal. 2002. Localization of the Rsr1/Bud1 GTPase involved in selection of a proper growth site in yeast. *J Biol Chem*. 277:26721-26724.

Peterson, J., Y. Zheng, L. Bender, A. Myers, R. Cerione, and A. Bender. 1994. Interactions between the bud emergence proteins Bem1p and Bem2p and Rho-type GTPases in yeast. *J Cell Biol*. 127:1395-1406.

Petronczki, M., M. Glotzer, N. Kraut, and J.M. Peters. 2007. Polo-like kinase 1 triggers the initiation of cytokinesis in human cells by promoting recruitment of the RhoGEF Ect2 to the central spindle. *Dev Cell*. 12:713-725.

Polakis, P.G., R. Snyderman, and T. Evans. 1989a. Characterization of G25K, a GTP-binding protein containing a novel putative nucleotide binding domain. *Biochem Biophys Res Commun*. 160:25-32.

Polakis, P.G., R.F. Weber, B. Nevins, J.R. Didsbury, T. Evans, and R. Snyderman. 1989b. Identification of the ral and rac1 gene products, low molecular mass GTP-binding proteins from human platelets. *J Biol Chem*. 264:16383-16389.

Pollard, T.D., and J.Q. Wu. 2010. Understanding cytokinesis: lessons from fission yeast. *Nat Rev Mol Cell Biol*. 11:149-155.

Pruyne, D.W., D.H. Schott, and A. Bretscher. 1998. Tropomyosin-containing actin cables direct the Myo2p-dependent polarized delivery of secretory vesicles in budding yeast. *J Cell Biol*. 143:1931-1945.

Qadota, H., Y. Anraku, D. Botstein, and Y. Ohya. 1994. Conditional lethality of a yeast strain expressing human RHOA in place of RHO1. *Proc Natl Acad Sci U S A*. 91:9317-9321.

Qadota, H., C.P. Python, S.B. Inoue, M. Arisawa, Y. Anraku, Y. Zheng, T. Watanabe, D.E. Levin, and Y. Ohya. 1996. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. *Science*. 272:279-281.

Reiser, V., K.E. D'Aquino, L.S. Ee, and A. Amon. 2006. The stress-activated mitogen-activated protein kinase signaling cascade promotes exit from mitosis. *Mol Biol Cell*. 17:3136-3146.

Richman, T.J., M.M. Sawyer, and D.I. Johnson. 2002. Saccharomyces cerevisiae Cdc42p localizes to cellular membranes and clusters at sites of polarized growth. *Eukaryot Cell*. 1:458-468.

Ridley, A.J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 70:389-399.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. 70:401-410.

Rincon, S., P.M. Coll, and P. Perez. 2007. Spatial regulation of Cdc42 during cytokinesis. *Cell Cycle*. 6:1687-1691.

Rincon, S.A., and A. Paoletti. 2012. Mid1/anillin and the spatial regulation of cytokinesis in fission yeast. *Cytoskeleton (Hoboken)*. 69:764-777.

Roberts-Galbraith, R.H., and K.L. Gould. 2010. Setting the F-BAR: functions and regulation of the F-BAR protein family. *Cell Cycle*. 9:4091-4097.

Rock, J.M., and A. Amon. 2009. The FEAR network. *Curr Biol*. 19:R1063-1068.

Rossman, K.L., C.J. Der, and J. Sondek. 2005. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol*. 6:167-180.

Roumanie, O., H. Wu, J.N. Molk, G. Rossi, K. Bloom, and P. Brennwald. 2005. Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex. *J Cell Biol*. 170:583-594.

Sagot, I., S.K. Klee, and D. Pellman. 2002. Yeast formins regulate cell polarity by controlling the assembly of actin cables. *Nat Cell Biol*. 4:42-50.

Saito, K., K. Fujimura-Kamada, H. Hanamatsu, U. Kato, M. Umeda, K.G. Kozminski, and K. Tanaka. 2007. Transbilayer phospholipid flipping regulates Cdc42p signaling during polarized cell growth via Rga GTPase-activating proteins. *Dev Cell*. 13:743-751.

Schiel, J.A., G.C. Simon, C. Zaharris, J. Weisz, D. Castle, C.C. Wu, and R. Prekeris. 2012. FIP3-endosome-dependent formation of the secondary ingression mediates ESCRT-III recruitment during cytokinesis. *Nat Cell Biol*. 14:1068-1078.

Schmidt, M., A. Varma, T. Drgon, B. Bowers, and E. Cabib. 2003. Septins, under Cla4p regulation, and the chitin ring are required for neck integrity in budding yeast. *Mol Biol Cell*. 14:2128-2141.

Schmitz, H.P., S. Huppert, A. Lorberg, and J.J. Heinisch. 2002. Rho5p downregulates the yeast cell integrity pathway. *J Cell Sci*. 115:3139-3148.

Schroeder, T.E. 1973. Actin in dividing cells: contractile ring filaments bind heavy meromyosin. *Proc Natl Acad Sci U S A*. 70:1688-1692.

Seshan, A., and A. Amon. 2005. Ras and the Rho effector Cla4 collaborate to target and anchor Lte1 at the bud cortex. *Cell Cycle*. 4:940-946.

Seshan, A., A.J. Bardin, and A. Amon. 2002. Control of Lte1 localization by cell polarity determinants and Cdc14. *Curr Biol*. 12:2098-2110.

Shimada, Y., M.P. Gulli, and M. Peter. 2000. Nuclear sequestration of the exchange factor Cdc24 by Far1 regulates cell polarity during yeast mating. *Nat Cell Biol*. 2:117-124.

Shimada, Y., P. Wiget, M.P. Gulli, E. Bi, and M. Peter. 2004. The nucleotide exchange factor Cdc24p may be regulated by auto-inhibition. *EMBO J*. 23:1051-1062.

Singh, K., P.J. Kang, and H.O. Park. 2008. The Rho5 GTPase is necessary for oxidant-induced cell death in budding yeast. *Proc Natl Acad Sci U S A*. 105:1522-1527.

Slaughter, B.D., A. Das, J.W. Schwartz, B. Rubinstein, and R. Li. 2009. Dual modes of cdc42 recycling fine-tune polarized morphogenesis. *Dev Cell*. 17:823-835.

Slaughter, B.D., J.R. Unruh, A. Das, S.E. Smith, B. Rubinstein, and R. Li. 2013. Non-uniform membrane diffusion enables steady-state cell polarization via vesicular trafficking. *Nat Commun*. 4:1380.

Smith, G.R., S.A. Givan, P. Cullen, and G.F. Sprague, Jr. 2002. GTPase-activating proteins for Cdc42. *Eukaryot Cell*. 1:469-480.

Smith, S.E., B. Rubinstein, I. Mendes Pinto, B.D. Slaughter, J.R. Unruh, and R. Li. 2013. Independence of symmetry breaking on Bem1-mediated autocatalytic activation of Cdc42. *J Cell Biol*.

Sopko, R., D. Huang, J.C. Smith, D. Figeys, and B.J. Andrews. 2007. Activation of the Cdc42p GTPase by cyclin-dependent protein kinases in budding yeast. *EMBO J*. 26:4487-4500.

Stegmeier, F., and A. Amon. 2004. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. *Annu Rev Genet*. 38:203-232.

Strochlic, T.I., J. Viaud, U.E. Rennefahrt, T. Anastassiadis, and J.R. Peterson. 2010. Phosphoinositides are essential coactivators for p21-activated kinase 1. *Mol Cell*. 40:493-500.

Su, K.C., T. Takaki, and M. Petronczki. 2011. Targeting of the RhoGEF Ect2 to the equatorial membrane controls cleavage furrow formation during cytokinesis. *Dev Cell*. 21:1104-1115.

Takahashi, S., and P.M. Pryciak. 2007. Identification of novel membrane-binding domains in multiple yeast Cdc42 effectors. *Mol Biol Cell*. 18:4945-4956.

Takizawa, P.A., J.L. DeRisi, J.E. Wilhelm, and R.D. Vale. 2000. Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. *Science*. 290:341-344.

Tiedje, C., I. Sakwa, U. Just, and T. Hofken. 2008. The Rho GDI Rdi1 regulates Rho GTPases by distinct mechanisms. *Mol Biol Cell*. 19:2885-2896.

Tolliday, N., L. VerPlank, and R. Li. 2002. Rho1 directs formin-mediated actin ring assembly during budding yeast cytokinesis. *Curr Biol*. 12:1864-1870.

Tong, Z., X.D. Gao, A.S. Howell, I. Bose, D.J. Lew, and E. Bi. 2007. Adjacent positioning of cellular structures enabled by a Cdc42 GTPase-activating protein-mediated zone of inhibition. *J Cell Biol*. 179:1375-1384.

Ubersax, J.A., E.L. Woodbury, P.N. Quang, M. Paraz, J.D. Blethrow, K. Shah, K.M. Shokat, and D.O. Morgan. 2003. Targets of the cyclin-dependent kinase Cdk1. *Nature*. 425:859-864.

Vallen, E.A., J. Caviston, and E. Bi. 2000. Roles of Hof1p, Bni1p, Bnr1p, and myo1p in cytokinesis in Saccharomyces cerevisiae. *Mol Biol Cell*. 11:593-611.

Vavylonis, D., J.Q. Wu, S. Hao, B. O'Shaughnessy, and T.D. Pollard. 2008. Assembly mechanism of the contractile ring for cytokinesis by fission yeast. *Science*. 319:97-100.

VerPlank, L., and R. Li. 2005. Cell cycle-regulated trafficking of Chs2 controls actomyosin ring stability during cytokinesis. *Mol Biol Cell*. 16:2529-2543.

Versele, M., and J. Thorner. 2004. Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. *J Cell Biol*. 164:701-715.

Vrabioiu, A.M., and T.J. Mitchison. 2006. Structural insights into yeast septin organization from polarized fluorescence microscopy. *Nature*. 443:466-469.

Wai, S.C., S.A. Gerber, and R. Li. 2009. Multisite phosphorylation of the guanine nucleotide exchange factor Cdc24 during yeast cell polarization. *PLoS One*. 4:e6563.

Wang, T., and A. Bretscher. 1995. The rho-GAP encoded by BEM2 regulates cytoskeletal structure in budding yeast. *Mol Biol Cell*. 6:1011-1024.

Watanabe, S., T. De Zan, T. Ishizaki, and S. Narumiya. 2013. Citron kinase mediates transition from constriction to abscission through its coiled-coil domain. *J Cell Sci*. 126:1773-1784.

Watanabe, S., K. Okawa, T. Miki, S. Sakamoto, T. Morinaga, K. Segawa, T. Arakawa, M. Kinoshita, T. Ishizaki, and S. Narumiya. 2010. Rho and anillin-dependent control of mDia2 localization and function in cytokinesis. *Mol Biol Cell*. 21:3193-3204.

Wedlich-Soldner, R., S. Altschuler, L. Wu, and R. Li. 2003. Spontaneous cell polarization through actomyosin-based delivery of the Cdc42 GTPase. *Science*. 299:1231-1235.

Wedlich-Soldner, R., S.C. Wai, T. Schmidt, and R. Li. 2004. Robust cell polarity is a dynamic state established by coupling transport and GTPase signaling. *J Cell Biol*. 166:889-900.

Weiss, E.L., A.C. Bishop, K.M. Shokat, and D.G. Drubin. 2000. Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. *Nat Cell Biol*. 2:677-685.

Wilson, C.A., M.A. Tsuchida, G.M. Allen, E.L. Barnhart, K.T. Applegate, P.T. Yam, L. Ji, K. Keren, G. Danuser, and J.A. Theriot. 2010. Myosin II contributes to cell-scale actin network treadmilling through network disassembly. *Nature*. 465:373-377.

Wloka, C., R. Nishihama, M. Onishi, Y. Oh, J. Hanna, J.R. Pringle, M. Krauss, and E. Bi. 2011. Evidence that a septin diffusion barrier is dispensable for cytokinesis in budding yeast. *Biol Chem*. 392:813-829.

Wolfe, B.A., T. Takaki, M. Petronczki, and M. Glotzer. 2009. Polo-like kinase 1 directs assembly of the HsCyk-4 RhoGAP/Ect2 RhoGEF complex to initiate cleavage furrow formation. *PLoS Biol*. 7:e1000110.

Wu, C.F., N.S. Savage, and D.J. Lew. 2013. Interaction between bud-site selection and polarity-establishment machineries in budding yeast. *Philos Trans R Soc Lond B Biol Sci*. 368:20130006.

Wu, H., C. Turner, J. Gardner, B. Temple, and P. Brennwald. 2009. The Exo70 Subunit of the Exocyst Is an Effector for Both Cdc42 and Rho3 Function in Polarized Exocytosis. *Mol Biol Cell*.

Wu, J.Q., V. Sirotkin, D.R. Kovar, M. Lord, C.C. Beltzner, J.R. Kuhn, and T.D. Pollard. 2006. Assembly of the cytokinetic contractile ring from a broad band of nodes in fission yeast. *J Cell Biol*. 174:391-402.

Yamashiro, S., G. Totsukawa, Y. Yamakita, Y. Sasaki, P. Madaule, T. Ishizaki, S. Narumiya, and F. Matsumura. 2003. Citron kinase, a Rho-dependent kinase, induces di-phosphorylation of regulatory light chain of myosin II. *Mol Biol Cell*. 14:1745-1756.

Yamochi, W., K. Tanaka, H. Nonaka, A. Maeda, T. Musha, and Y. Takai. 1994. Growth site localization of Rho1 small GTP-binding protein and its involvement in bud formation in Saccharomyces cerevisiae. *J Cell Biol*. 125:1077-1093.

Ye, Y., I.J. Lee, K.W. Runge, and J.Q. Wu. 2012. Roles of putative Rho-GEF Gef2 in division-site positioning and contractile-ring function in fission yeast cytokinesis. *Mol Biol Cell*. 23:1181-1195.

Yonetani, A., R.J. Lustig, J.B. Moseley, T. Takeda, B.L. Goode, and F. Chang. 2008. Regulation and targeting of the fission yeast formin cdc12p in cytokinesis. *Mol Biol Cell*. 19:2208-2219.

Yoshida, S., S. Bartolini, and D. Pellman. 2009. Mechanisms for concentrating Rho1 during cytokinesis. *Genes Dev*. 23:810-823.

Yoshida, S., K. Kono, D.M. Lowery, S. Bartolini, M.B. Yaffe, Y. Ohya, and D. Pellman. 2006. Polo-like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. *Science*. 313:108-111.

Yoshizaki, H., Y. Ohba, K. Kurokawa, R.E. Itoh, T. Nakamura, N. Mochizuki, K. Nagashima, and M. Matsuda. 2003. Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. *J Cell Biol*. 162:223-232.

Zanin, E., A. Desai, I. Poser, Y. Toyoda, C. Andree, C. Moebius, M. Bickle, B. Conradt, A. Piekny, and K. Oegema. 2013. A conserved RhoGAP limits M phase contractility and coordinates with microtubule asters to confine RhoA during cytokinesis. *Dev Cell*. 26:496-510.

Zhang, X., E. Bi, P. Novick, L. Du, K.G. Kozminski, J.H. Lipschutz, and W. Guo. 2001. Cdc42 interacts with the exocyst and regulates polarized secretion. *J Biol Chem*. 276:46745-46750.

Zheng, Y., A. Bender, and R.A. Cerione. 1995. Interactions among proteins involved in bud-site selection and bud-site assembly in Saccharomyces cerevisiae. *J Biol Chem*. 270:626-630.

Zheng, Y., R. Cerione, and A. Bender. 1994. Control of the yeast bud-site assembly GTPase Cdc42. Catalysis of guanine nucleotide exchange by Cdc24 and stimulation of GTPase activity by Bem3. *J Biol Chem*. 269:2369-2372.

Zheng, Y., M.J. Hart, K. Shinjo, T. Evans, A. Bender, and R.A. Cerione. 1993. Biochemical comparisons of the Saccharomyces cerevisiae Bem2 and Bem3 proteins. Delineation of a limit Cdc42 GTPase-activating protein domain. *J Biol Chem*. 268:24629-24634.

Zhu, Y.H., Y. Ye, Z. Wu, and J.Q. Wu. 2013. Cooperation between Rho-GEF Gef2 and its binding partner Nod1 in the regulation of fission yeast cytokinesis. *Mol Biol Cell*. 24:3187-3204.

Ziman, M., D. Preuss, J. Mulholland, J.M. O'Brien, D. Botstein, and D.I. Johnson. 1993. Subcellular localization of Cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity. *Mol Biol Cell*. 4:1307-1316.

Zumdieck, A., K. Kruse, H. Bringmann, A.A. Hyman, and F. Julicher. 2007. Stress generation and filament turnover during actin ring constriction. *PLoS One*. 2:e696.

Chapter 2

Cell cycle regulation of Cdc42 activity in *S. cerevisiae*

2.1 Summary **52**

2.2 Introduction **52**

2.3 Results **55**
2.3.1 Development of an assay to measure Cdc42 activity in yeast cell lysates *55*
2.3.2 Cdc42 GTP-loading is low during early G_1 but rises prior to bud emergence *57*
2.3.3 Cdc42 GTP-loading rises in anaphase but drops during mitotic exit *57*
2.3.4 Local Cdc42 activity is suppressed at the bud neck during cytokinesis *59*
2.3.5 Cdc5/Polo kinase inhibits Cdc42 activity *61*

2.4 Discussion **64**
2.4.1 Coordinating mitotic exit with cytokinesis *64*
2.4.2 A connection between Cdc5/Polo kinase and Cdc42 *65*
2.4.3 Implications for changes in polarization during mitosis *68*
2.4.4 Conclusions *69*

2.5 Materials and Methods **69**
2.5.1 Strains, media, and molecular biology *69*
2.5.2 Biochemical methods *71*
2.5.3 Cdc42 activation assay (CRIB pulldown) *71*
2.5.4 Quantification of CRIB-tdTomato and Bem1-GFP signal *72*
2.5.4 Cell synchronization methods *73*

2.6 Acknowledgments **73**

2.7 References **73**

2.1 Summary

Current models of cell polarization by Cdc42 in budding yeast assume that GTP-loading of Cdc42 increases at the presumptive bud site during G_1/S. Surprisingly, this has never been directly measured. Additionally, we have little understanding of how Cdc42 activity changes later during the cell cycle, which has hampered progress in studying Cdc42's potential functions (or lack thereof) after the initial polarization of the cell.

In this chapter I present evidence that activation of Cdc42 oscillates across the cell cycle. Using a biochemical assay I found that total GTP-Cdc42 levels indeed peak slightly prior to bud emergence at G_1/S, as expected. I observed another unexpected peak of Cdc42 GTP-loading that coincided with anaphase. Interestingly, at the onset of mitotic exit Cdc42 GTP-loading dramatically decreased, remaining low throughout cytokinesis and early G_1. In collaboration with Chi-Fang Wu and Daniel Lew (Duke University), we found that local Cdc42 activation at the site of cytokinesis (the bud neck) is indeed much lower than at the site of polarization in G_1/S. In collaboration with Satoshi Yoshida (now at Brandeis University), we found that Cdc5/Polo kinase is one upstream cell cycle regulator that suppresses Cdc42 activation, likely though regulation of Cdc42-GAP proteins. The data presented in this chapter was published in the Journal of Cell Biology (Atkins et al., 2013).

2.2 Introduction

As described in Chapter 1, budding yeast has been used extensively as a model to study the mechanisms of Cdc42-dependent cell polarization (Bi and Park, 2012; Johnson et al., 2011). Surprisingly, although current models and indirect evidence suggest that

Cdc42 is activated during bud emergence, this has never been measured experimentally (Bi and Park, 2012; McCusker et al., 2007; Moffat and Andrews, 2004; Sopko et al., 2007). Likewise, although the Cdc42 effector PAK kinase Cla4 appears to undergo periodic changes in activation, peaking during mitosis (Benton et al., 1997; Tjandra et al., 1998), how Cdc42 activity changes during mitosis has not been defined.

Defining how Cdc42 activity changes across the cell cycle would be of value not only to test the major assumption of a large body of literature indicating that GTP-Cdc42 levels rise at the G_1/S transition but will also be useful in generating hypotheses to test the function of Cdc42 in later stages of the cell cycle, including roles in actin polarization rearrangements, mitotic exit, and cytokinesis. Budding yeast is an ideal model to address these questions, due to the availability of excellent synchronization procedures and a large collection of well-characterized cell cycle mutants. Because the cell cycle regulators and timing of Rho1/RhoA activation have been found to be largely conserved between fungi and higher eukaryotes, it is likely that cell cycle changes in Cdc42 activity in budding yeast could be relevant to understanding changes in the activity of Cdc42 or related Rho GTPases in other systems (Bement et al., 2005; Kimura et al., 2000; Kono et al., 2008; Yoshida et al., 2006; Yoshizaki et al., 2003).

As with other Rho GTPases, both the global amount of active GTP-bound Cdc42 and the localization of this fraction of Cdc42 are of interest. During polarization, it is possible that total Cdc42 GTP-loading in the cell increases along with clustering of Cdc42 at the presumptive bud site, or that a small but constant pool of GTP-Cdc42 undergoes changes in localization to concentrate at the bud site. A fluorescent biosensor for active Cdc42, Gic2-CRIB-tdTomato, shows a polarized localization throughout most

of the cell cycle and has proven useful for understanding control of local Cdc42 activation; however, this construct also contains a polybasic region that binds to phosphoinositides in the plasma membrane, and could potentially also interact with septins (Iwase et al., 2006; Takahashi and Pryciak, 2007; Tong et al., 2007). Thus, this marker might not be totally sensitive to changes in Cdc42 activation that could occur during the cell cycle. Therefore, I decided to directly measure GTP-Cdc42 levels using a biochemical assay that has been widely used in other cell types, utilizing the CRIB domain of a Cdc42 effector to selectively pull down GTP-Cdc42 from yeast cell lysates (Benard and Bokoch, 2002; Burbelo et al., 1995; Pellegrin and Mellor, 2008). Similar approaches have been used in budding yeast, but the signal of GTP-Cdc42 in unperturbed wild-type cells has been difficult to detect (Boulter et al., 2010; Knaus et al., 2007; Wai et al., 2009).

Here I refined a CRIB pulldown assay to determine how Cdc42 GTP-loading changes across the cell cycle in budding yeast. I found that Cdc42 GTP-loading peaks at G_1/S and during anaphase, but is low during cytokinesis, early G_1, and G_2. Imaging of the CRIB-tdTomato biosensor corroborated my biochemical data and indicated that local Cdc42 activity at the bud neck during cytokinesis is low compared to the presumptive bud site during polarization. We identified the Polo-like kinase homolog Cdc5 as one upstream cell cycle regulator that inhibits Cdc42 activity during mitotic exit, likely acting at least in part through the Cdc42-GAP proteins Bem2 and Bem3.

2.3 Results

2.3.1 Development of an assay to measure Cdc42 activity in yeast cell lysates

To determine how Cdc42 GTP-loading changes throughout the cell cycle, I first developed conditions to reliably monitor GTP-loading of Cdc42 from yeast cell lysates using a GST-CRIB pulldown assay. CRIB domains promote selective binding of Cdc42 effectors to GTP-Cdc42 (Burbelo et al., 1995). After testing several yeast Cdc42 effector CRIB constructs, I found that the CRIB domain of the well-characterized Cdc42 effector Ste20 could pull down Cdc42 from yeast cell extracts, as shown in Fig 2-1A (Ash et al., 2003; Burbelo et al., 1995; Lamson et al., 2002). To test the specificity of GST-Ste20-CRIB for GTP-Cdc42, I inactivated the sole Cdc42-specific GEF Cdc24 using a temperature-sensitive allele. As expected, GTP-Cdc42 levels were reduced approximately four-fold in lysates from *cdc24-4* cells grown at the restrictive temperature compared to lysates from isogenic wild-type controls (Fig 2-1A). Qualitatively similar results were obtained using an independent CRIB domain from human PAK1 (Fig 2-1B).

This assay also distinguished between overexpressed activated mutant versions of Cdc42 (i.e. GTP-locked $Cdc42^{Q61L}$) and dominant-negative GDP-locked $Cdc42^{T17N}$, as shown in Figure 2-1C. Figure 2-1D shows that the assay pulls down more of a mutant version of Cdc42 ($Cdc42^{G60D}$) that was previously shown to be hyperactive compared to wild-type Cdc42 (Caviston et al., 2002). Finally, the assay could also detect the decrease in GTP-Cdc42 levels caused by overexpression of the Cdc42-GAP Bem3 (Fig 2-1E). Together, these data indicate that the CRIB pulldown assay faithfully reports Cdc42 GTP-loading *in vivo*.

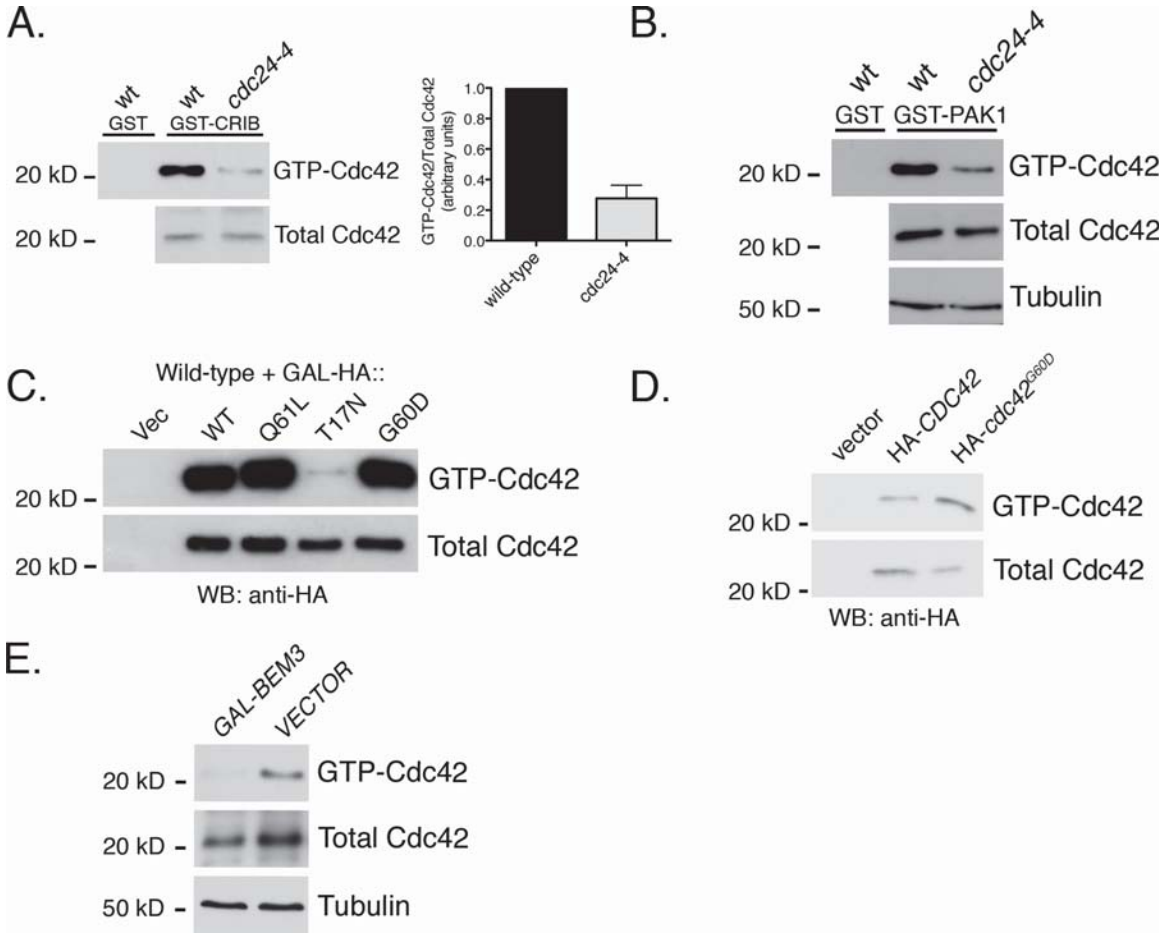


Figure 2-1: GST-CRIB pulldown detects active GTP-Cdc42 from yeast lysates

(A) Wild-type and *cdc24-4* ts cells were shifted to 37°C for 2.5 hr, and lysates were assayed for GTP-Cdc42 levels by GST-Ste20-CRIB binding. GST serves as a negative control. Graph: mean +/- SEM from 3 experiments, $p<0.01$ by unpaired two-tailed t test.

(B) Similar ability of GST-PAK1-CRIB to detect GTP-Cdc42 as for GST-Ste20-CRIB. Cells were grown as in (A) and lysates were assayed for GTP-Cdc42 levels by GST-PAK1-CRIB binding. Similar results were obtained in at least 3 experiments.

(C) GST-Ste20-CRIB binds to activated ($Cdc42^{Q61L}$, $Cdc42^{G60D}$) but not dominant-negative ($Cdc42^{T17N}$) Cdc42 mutants. Cells were transformed with the indicated plasmids, grown to early log phase, induced to express the Cd42 variants for 1 hr, and processed for Cdc42 GTP-loading.

(D GST-Ste20-CRIB pulls down more $Cdc42^{G60D}$ than wild-type Cdc42. Wild-type cells were transformed with the indicated plasmids, grown to early log phase, and processed for Cdc42 GTP-loading.

(E) Bem3 overexpression reduces Cdc42 GTP-loading. Cells were transformed with the indicated plasmids, grown to early log phase, shifted to media containing galactose for 3 hr, and processed for Cdc42 GTP-loading.

2.3.2 Cdc42 GTP-loading is low during early G_1 but rises prior to bud emergence

To examine Cdc42 activity in cells progressing from early G_1 through START, the time of actin polarization in budding yeast, I arrested cells in G_1 using the mating pheromone α-factor, released the cells into a synchronous cell cycle by washing out the mating pheromone, and measured Cdc42 GTP-loading at various time intervals following release from the G_1 block. Cells deleted for the secreted α-factor protease *BAR1* were used to facilitate synchronization of a large culture volume (Amon, 2002). I monitored cell cycle progression by determining the percentage of budded cells at each time point (Amon, 2002). Cdc42 GTP-loading peaked 30 min after release, which was approximately 15 min prior to bud emergence (Fig 2-2A). This correlates well with the known timing of Cdc42 and actin polarization at START (Lew and Reed, 1993; Ziman et al., 1993). A similar peak was observed in cells released from a metaphase block (see below). Furthermore, cells arrested in G_1 by depletion of all late G_1 cyclins (*cln1 cln2 cln3*-arrested cells) had lower Cdc42 activity than an asynchronously growing culture, independently supporting the idea that cells in early G_1 have low Cdc42 activity (Fig 2-2B). Overall these data suggest that GTP-Cdc42 levels increase coincident with cell polarization in late G_1.

2.3.3 Cdc42 GTP-loading rises in anaphase but drops during mitotic exit

To determine whether Cdc42 activity changes in cells progressing through mitosis and cytokinesis, I arrested cells in metaphase by repression of the essential Anaphase Promoting Complex/Cyclosome (APC/C) cofactor *CDC20*,

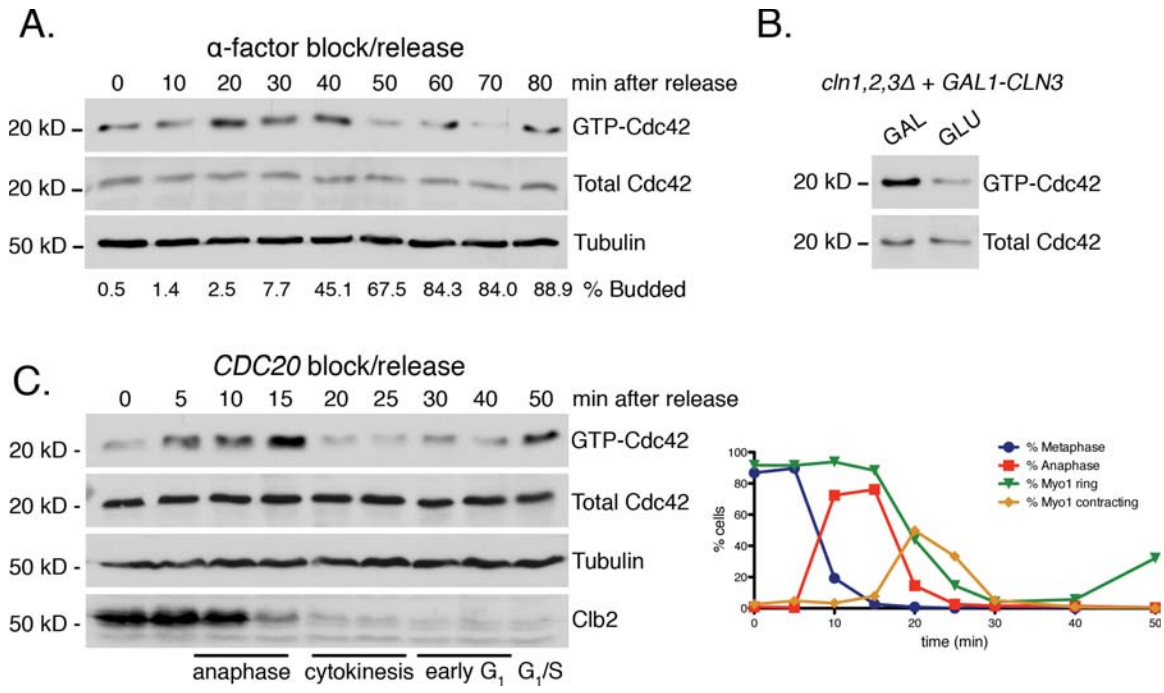


Figure 2-2: GTP-Cdc42 levels peak at G_1/S and anaphase but fall during mitotic exit

(A) Cdc42 GTP-loading peaks at G_1/S. *bar1Δ* cells were released from an α-factor block and Cdc42 GTP-loading was measured at the indicated timepoints.

(B) Cells arrested in early G_1 have lower Cdc42 GTP-loading than an asynchronous culture. *cln1,2,3Δ GAL1-CLN3* cells were split into media containing either galactose or glucose, where *CLN3* expression is repressed. The example is representative of 3 experiments.

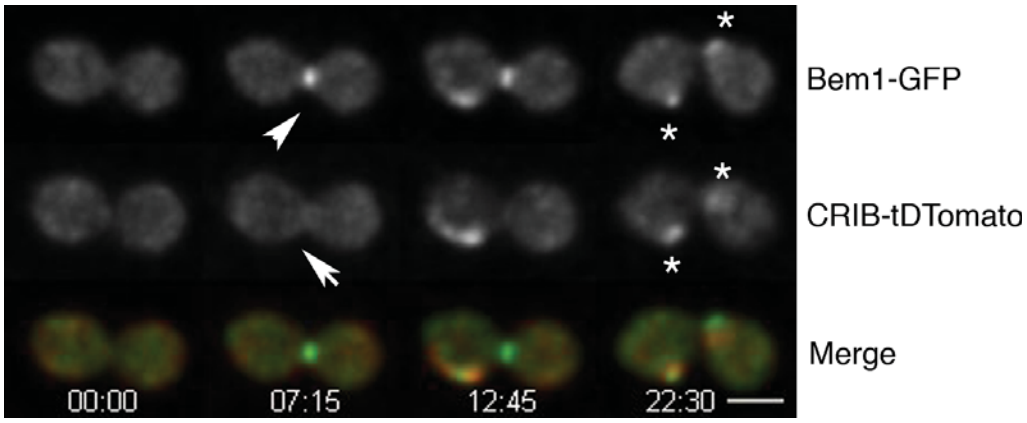
(C) Cdc42 GTP loading peaks at anaphase but drops at the onset of mitotic exit and cytokinesis. *GALL-CDC20 GFP-TUB1 MYO1-GFP* cells were arrested at metaphase and released. Samples were processed to measure GTP-Cdc42 activation and for microscopy to determine cell cycle stage. The example is representative of 3 experiments.

released the cells into a synchronous mitosis by re-induction of *CDC20*, and measured Cdc42 activity at intervals following the release. Mitotic progression was monitored by visualization of the mitotic spindle (GFP-Tub1) and cytokinesis was monitored by visualization of type II myosin (Myo1-GFP), which have distinct and non-overlapping localization patterns to the spindle and bud neck as previously described (VerPlank and Li, 2005). Cdc42 GTP-loading rose transiently coincident with anaphase (10-15 min after release), followed by a striking drop at the onset of mitotic exit and cytokinesis (20-25 min), as judged by the breakdown of anaphase spindles, contraction of the Myo1 ring, and degradation of cyclin B Clb2 (Fig 2-2C). Cdc42 GTP-loading remained low through early G_1 until cells entered into the next cell cycle (50 min after release).

2.3.4 Local Cdc42 activity is suppressed at the bud neck during cytokinesis

As a parallel approach to corroborate the biochemical data, we monitored the localization of a marker for active Cdc42, CRIB-tdTomato, and the polarity regulator Bem1 in live cells released from a hydroxyurea-induced S-phase arrest (Howell et al., 2012; Tong et al., 2007). Bem1, Cdc42, and the Cdc42-GEF Cdc24 are all known to localize to the bud neck during cytokinesis (Richman et al. 2002; Gulli et al. 2000), whereas other effectors such as Cla4 do not (Holly and Blumer, 1999). However, whether the bud neck pool of Cdc42 is active during cytokinesis is unclear. We compared the localization of the CRIB-tdTomato probe

A.



B.

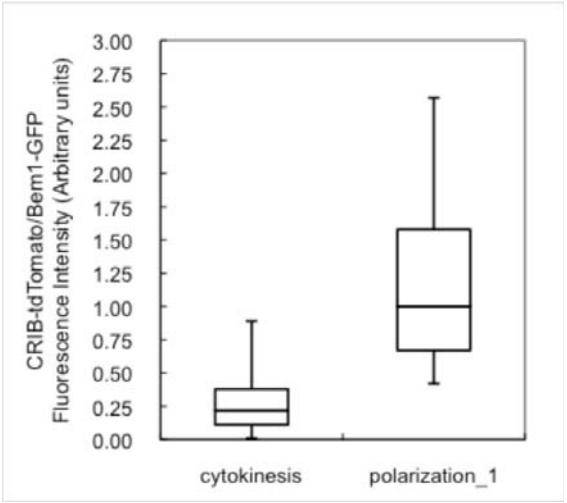


Figure 2-3: Local Cdc42 activity at the bud neck during cytokinesis is low compared to the site of polarization during G_1/S

(A) Time-lapse imaging of a *CRIB-tdTomato BEM1-GFP* cell undergoing mitotic exit. 00:00 (min:sec) is 1 hr after release from hydroxyurea (S-phase arrest). During cytokinesis little CRIB-tdTomato (arrow) localizes to the bud neck relative to Bem1-GFP (arrowhead); both localize to the subsequent bud sites (asterisks). Bar, 5 μm.

(B) Quantification of CRIB-tdTomato/Bem1-GFP intensity ratio during cytokinesis and polarization. Line is median, top and bottom edges indicate 25th and 75th quartiles, and whiskers are the highest and lowest measurements.

at the bud neck during cytokinesis to that at the polarization site in the next cell cycle. Bem1-GFP was included as an overall marker of polarization. Strikingly, in every cell relatively little CRIB-tdTomato localized to the bud neck during cytokinesis compared to Bem1. By contrast, both fluorescent proteins robustly localized at the new budding site (n=17, Fig 2-3A,B). A similar pattern was observed comparing CRIB-tdTomato to GFP-Cdc42 (i.e. active versus total Cdc42, data not shown), however, we focused on Bem1-GFP because GFP-Cdc42 is only partially functional.

These data indicate that although Cdc42 localizes to the bud neck following mitotic exit, Cdc42 is predominantly inactive at this site during cytokinesis and only becomes activated at the subsequent bud site when cells polarize during the next cell cycle (Meitinger et al., 2013b; Tong et al., 2007). We also infer that the bud neck recruitment of Bem1 during cytokinesis is largely independent of active Cdc42, unlike the case at polarization to the presumptive bud site during G_1/S (Butty et al., 2002). Together, our data support the idea that both total Cdc42 GTP-loading as well as local Cdc42 activation are cell cycle regulated, with global peaks around the time of bud emergence and anaphase, and troughs during G_2, mitotic exit/cytokinesis, and early G_1.

2.3.5 Cdc5/Polo kinase inhibits Cdc42 activity

Because Cdc5/Polo controls Rho1 activation during late anaphase, and because we found that the Cdc42 GTPase activating proteins (GAPs) Bem2 and Bem3 interact with the Polo-box domain of Cdc5 in a canonical manner for Cdc5 substrates (Fig 2-4A), we hypothesized that Cdc5/Polo could regulate Cdc42 reciprocally with its regulation of Rho1 (Elia et al., 2003; Yoshida et al., 2006). To determine if loss of Cdc5 affects the

activation of Cdc42, cells were arrested at the end of mitosis by conditional inactivation of Cdc15 (*cdc15-2* cells, which arrest with high Cdc5 kinase activity) (Cheng et al., 1998) or Cdc5 (*cdc5-2*) and GTP loading of Cdc42 was measured. For comparison, GTP-Cdc42 was also measured in control asynchronous cells or cells arrested in metaphase by conditional inactivation of Cdc20 (*cdc20-3*). At the mitotic exit block, cells that contain active Cdc5 (*cdc15-2*) had significantly lower levels of GTP-Cdc42 relative to cells lacking functional Cdc5 (*cdc5-2,* Fig 2-4B), suggesting that Cdc5/Polo suppresses Cdc42 activity during mitotic exit.

A N-terminal fragment of Bem3, Bem3(1-500), was sufficient to bind the Polo-box domain of Cdc5 (Fig 2-4C). Interestingly, this fragment of Bem3 was phosphorylated in a Cdc5-dependent manner (Fig 2-4D-E, detected by SDS-PAGE and Phos-tag gels), suggesting that Bem3 may be a Cdc5 substrate. We were unable to detect a mobility shift for Bem2, possibly because of its large (~250 kD) size (data not shown). Overall, these data suggest that Cdc42-GAP proteins could be direct substrates of Cdc5/Polo kinase during mitotic exit.

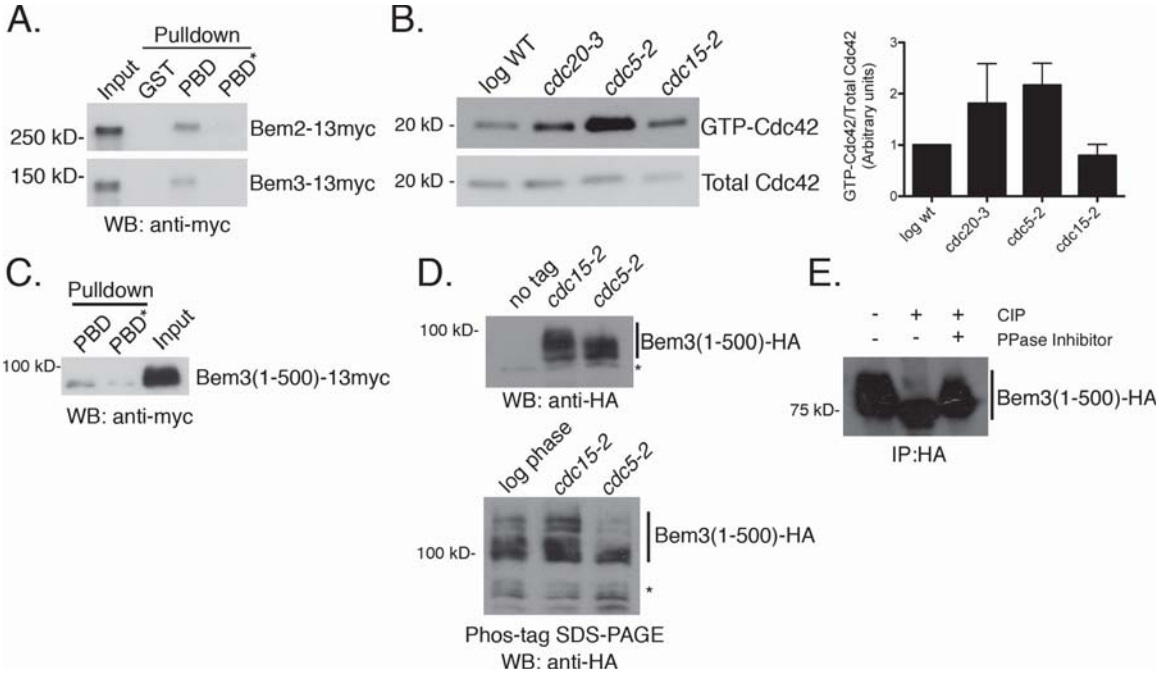


Figure 2-4: Cdc5/Polo kinase suppresses Cdc42 activity, likely through Cdc42-GAPs

(A) Bem2 and Bem3 interact with the Polo-box domain of Cdc5. Lysates from the indicated strains were incubated with GST, GST-PBD, or a control 'pincer' mutant PBD* (Elia et al., 2003). Bound GAPs were detected by Western blotting.

(B) *cdc5-2* mutants have elevated Cdc42-GTP loading compared to asynchronous controls or *cdc15-2* mutants. Log phase wild-type, *cdc20-3*, *cdc5-2,* and *cdc15-2* cells were shifted to 37°C for 3 hr and processed for Cdc42 activation. The example is representative of three experiments. Graph: mean +/- SEM from 3 experiments. The difference between *cdc5-2* and *cdc15-2* was statistically significant ($p<0.05$ by unpaired two-tailed t test).

(C) The N-terminal 500 amino acids of Bem3 is sufficient to bind the Polo-box domain of Cdc5. Lysate from a *Bem3(1-500)-13myc cdc15-2* strain (arrested 2.5 hr, 35°C) was incubated with purified PBD or PBD* as in Fig 2-4A.

(D) Bem3(1-500) undergoes a Cdc5/Polo-dependent mobility shift *in vivo.* The indicated strains were arrested as in Fig 2-4C; shown is a Western blot to detect altered mobility of Bem3(1-500)-3HA. In the lower panel Phos-tag SDS-PAGE was used for increased resolution of phosphorylated bands. Asterisk marks a non-specific band.

(E) The Cdc5-dependent mobility shift of Bem3(1-500) depends on phosphorylation. Bem3(1-500)-3HA was immunoprecipitated from *cdc15-2* cell lysates arrested in telophase as in Fig 2-4C and incubated with Calf intestinal phosphatase (CIP) and/or phosphatase inhibitors.

2.4 Discussion

2.4.1 Coordinating mitotic exit with cytokinesis

I identified two peaks of Cdc42 activation during the cell cycle in budding yeast during the G_1/S transition and anaphase, with troughs during G_2 and cytokinesis/early G_1. Although current models of cell polarization all assume that GTP-loading of Cdc42 increases during polarization, this has never been directly measured. My biochemical data provide a direct demonstration of this idea. These results are relevant to models of how Cdc42 becomes activated and polarized at the presumptive bud site. Rather than having a small but constant pool of active GTP-Cdc42 relocalize to the presumptive bud site, my data suggests that there is a burst of Cdc42 GTP-loading that accompanies Cdc42 clustering at the presumptive bud site. These biochemical data from cell populations are also consistent with a recent study that examined CRIB-tdTomato signal intensity at the polarization site starting from cytokinesis to bud emergence, where the signal progressively increased at the polarization site until septins began to be recruited (Okada et al., 2013).

The anaphase peak of Cdc42 GTP-loading was somewhat unexpected, but Cdc42 has been proposed to promote Mitotic Exit Network (MEN) signaling, which occurs in anaphase, through several effectors including Cla4 and Ste20 (Hofken and Schiebel, 2002; Hofken and Schiebel, 2004; Monje-Casas and Amon, 2009; Seshan et al., 2002). This peak of Cdc42 GTP-loading could therefore explain the peak of Cla4 kinase activity observed during mitosis (Benton et al., 1997; Tjandra et al., 1998). This potential function of Cdc42 during mitotic exit could require transient activation, which is

followed by the suppression that occurs during and is required for normal cytokinesis (shown in Chapter 3 of this dissertation)

The fact that Cdc42 promotes MEN signaling but inhibits cytokinesis (see Chapter 3) could reflect a function for Cdc42 in ordering these cell cycle events, which must occur sequentially in the yeast cell cycle. That is, Cdc42 could inhibit later steps in cytokinesis and septum formation (Chapter 3) until the cell is ready to perform them through sufficient Cdc14-dependent reversal of Cdk1 phosphorylation and activation of MEN kinases that promote cytokinesis (Meitinger et al., 2013a; Meitinger et al., 2011; Nishihama et al., 2009; Oh et al., 2012; Zhang et al., 2006). Alternatively, although Cdc42's role in exocytosis is thought to be most important early in the cell cycle during polarized bud growth (Adamo et al., 2001), the anaphase peak of Cdc42 activity could be involved in isotropic cell growth. Interestingly, cell growth rates appear to increase during anaphase (Goranov et al., 2009).

2.4.2 A connection between Cdc5/Polo kinase and Cdc42

Rho1/RhoA is known to undergo changes in GTP-loading and local activation in budding yeast and mammalian cells. In particular, Rho GTP-loading and local activation at the site of division peak in late anaphase/telophase (Bement et al., 2005; Kimura et al., 2000; Kono et al., 2008; Yoshida et al., 2006; Yoshizaki et al., 2003). Polo kinase is responsible for Rho1/RhoA activation during mitotic exit in both budding yeast and mammals (Petronczki et al., 2008; Yoshida et al., 2006). In this work, I show that Cdc42 activity also changes during mitosis, with a sharp drop at the onset of mitotic exit that persists through cytokinesis (Fig 2-2C). Importantly, while Cdc5/Polo mutants have

reduced Rho1 GTP-loading compared to controls, Cdc42 is hyperactive under the same conditions (Yoshida et al., 2006 and Fig 2-4B). Overall these data suggest that Polo kinase is an important cell cycle regulator that coordinates the activities of Rho GTPases during mitotic exit, which is functionally important for cytokinesis (Chapter 3).

A previous study in HeLa cells suggested that Cdc42 GTP-loading oscillated during a coarse synchronization, with a transient rise near metaphase and a decrease during telophase, although the relevance of these findings have been unclear due to the controversial nature of the function of Cdc42 during mitosis in mammalian cells (Narumiya and Yasuda, 2006; Oceguera-Yanez et al., 2005). The results from my synchronization studies in yeast are broadly in agreement with the results from HeLa cells, suggesting that a decrease in Cdc42 GTP-loading during mitotic exit is a conserved feature of cell division. Additionally, the activity of the related Rho GTPase Rac1 is suppressed at the cell equator in mammalian cells, which is important for cytokinesis in part through inhibition of inappropriate adhesion at the division site (Bastos et al., 2012; Canman et al., 2008; Yoshizaki et al., 2003). Therefore, testing the role of Polo kinase in regulation of Cdc42 and Rac1 activity during mitotic exit in mammalian cells will be an interesting topic of future research.

Intriguingly, loss of Polo kinase function in *Candida albicans* leads to highly polarized hyphal-like growth, suggesting that Polo kinase may also control polarization in other contexts beyond cell division (Bachewich et al., 2003). Although our findings suggest negative regulation of Cdc42 by Cdc5/Polo during mitotic exit, Polo kinases may impact on cell polarization and Cdc42 in other ways at different times during the cell division cycle. For example, the fission yeast homolog of Cdc5/Polo kinase, Plo1, is

required for the resumption of cell tip growth after stress in G_2-phase cells (Petersen and Hagan, 2005).

Details of the mechanism linking Cdc5/Polo to Cdc42 remain to be elucidated, but our results suggest that Cdc42-GAP regulation is likely to be an important element. A previous study found that the Cdc42-GAP Bem3 binds the Polo-box domain of Cdc5 like canonical Cdc5/Polo substrates (Yoshida et al., 2006). In this study, we found that Bem3 is phosphorylated in a Cdc5-dependent and Polo-box binding motif-dependent manner, suggesting that Bem3 is a Cdc5/Polo substrate *in vivo*. We also found that the major Cdc42-GAP Bem2 also binds the Polo-box domain, which suggests redundant control of Cdc42-GAPs by Cdc5/Polo.

Another known negative regulatory input for Cdc42 during mitotic exit is the Cdc42-GAP Rga1, which localizes to the division site and prevents budding from occurring into the old bud by local inactivation of Cdc42 (Tong et al., 2007). While we cannot exclude the possibility that Cdc5/Polo also regulates Rga1, we did not detect an interaction between Rga1 and the Cdc5 Polo box domain. Cdc5/Polo regulation of Cdc42-GAPs could increase their GAP activity, promote their local recruitment to the bud neck, or promote their stability during mitotic exit. Cdc5/Polo could also regulate Cdc42 at other levels, such as by inactivation of its GEF, Cdc24, or by regulation of the recently-discovered protein Gps1 that inhibits Cdc42-dependent polarization at the bud neck to promote normal bud site selection (Meitinger et al., 2013b).

Importantly, these findings indicate that negative regulation of Cdc42 during mitotic exit is integrated into the cell cycle circuitry that controls cytokinesis. Of course, mitotic exit is a time when Cdk1 activity drops while other cell cycle-dependent signaling

pathways, such as the Mitotic Exit Network and its downstream target Cdc14, as well as the Regulation of Ace2 and morphogenesis (RAM) network, become sequentially activated; it is tempting to speculate that these other pathways could also regulate Cdc42 activity at this stage in the cell cycle.

2.4.3 Implications for changes in polarization during mitosis

The actin cytoskeleton undergoes dramatic rearrangement during mitosis; actin cables and patches are depolarized due to Clb2/Cdk1 activation and subsequently reorganized under the regulation of Cdc5/Polo kinase and Rho1 to form the contractile actin ring at the division site (Lew and Reed, 1993; Tolliday et al., 2002; Yoshida et al., 2006). After contractile actin ring assembly, actin cables and patches repolarize towards the bud neck, enabling polarized secretion and endocytic recycling of proteins involved in septum formation and cell separation (Bi and Park, 2012). Because Cdc42 plays an essential role in polarization early in the cell cycle and localizes to the bud neck, it has widely been assumed that it may also control this repolarization during mitotic exit and thus perhaps contribute in an indirect way in promoting cytokinesis.

However, the following evidence suggests that Cdc42 is unlikely to be the relevant Rho GTPase that controls repolarization during mitotic exit: Cdc42 is inactivated upon the onset of mitotic exit (this chapter), cell division occurs mostly normally in the absence of Cdc42 (Iwase et al., 2006; Tolliday et al., 2002), and mutant versions of Cdc42 that impair exocyst regulation have defects in vesicle accumulation early, but not late, during the cell cycle (Adamo et al., 2001). Because Rho1 is activated at the bud neck during both early and late stages of cytokinesis (Kono et al., 2008; Meitinger et al.,

2013b; Onishi et al., 2013; Yoshida et al., 2006), and can control formin activation and exocyst function (Guo et al., 2001; Kohno et al., 1996; Tolliday et al., 2002), Rho1 seems likely to be the main regulator of actin repolarization during mitotic exit. Of course, it is possible that other Rho GTPases such as Rho3 and Rho4 also contribute to actin polarization and polarized exocytosis during cytokinesis, consistent with the fact that *rho3* mutants that are specifically defective for regulation of the exocyst display abnormal accumulation of Golgi-derived vesicles in large-budded cells (Adamo et al., 1999; Wu et al., 2009).

2.4.4 Conclusions

Overall, in this chapter I showed that Cdc42 activation undergoes periodic changes across the cell cycle, with peaks at G_1/S and anaphase, and troughs during G_2, mitotic exit/cytokinesis, and early G_1. In particular, Cdc42 activation at the bud neck, the site of cytokinesis in budding yeast, was strongly suppressed compared to at the polarization site at G_1/S. Cdc5/Polo kinase is an upstream negative regulator of Cdc42 GTP-loading that connects the cell cycle machinery to Cdc42 inactivation during mitotic exit, likely though the Cdc42-GAPs Bem2 and Bem3.

2.5 Materials and Methods

2.5.1 Strains, media, and molecular biology

Standard approaches were used for molecular biology and genetic manipulation of yeast (Sherman, 1991). All yeast strains are isogenic or congenic with the S288c-derived BY4741 (*MATa his3Δ leu2Δ met15Δ ura3Δ*). Gene deletion or modification was

confirmed by PCR. For a list of yeast strains and plasmids used in this chapter see Table 2-1 and Table 2-2.

Table 2-1: Yeast strains used in Chapter 2

Strain	Relevant Genotype	Source
PY3295	BY4741 *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MATa*	Pellman lab
PY7205	YEF473a S288c *his3 leu2 trp1 ura3 MATa*	E. Bi (Penn)
PY7206	YEF473a S288c *his3 leu2 trp1 ura3 cdc24-4 MATa*	E. Bi (Penn)
PY7207	BY4741 *cln1::natMX cln2::hygR GAL1-CLN3-kanR MATa*	This study
PY3665	BY4741 *bar1::kanR MATa*	Pellman lab
PY7208	BY4741 *GALL-CDC20-kanR GFP-TUB1-URA3 MYO1-GFP-HIS3 MATa*	This study
PY6573	BY4741 *GALL-CDC20-kanR GFP-TUB1-URA3 SHS1-GFP-HIS3 MATa*	This study
DLY 13157	YEF473a *BEM1-GFP-LEU2 Gic2(CRIB)-tdTomato-URA3 MATa/α*	Howell et al., 2012
PY7209	*cdc5-2::URA3* 5x backcross to BY4741 *MATa*	Pellman lab
PY5220	*cdc15-2* 5x backcross to BY4741 *MATa*	Pellman lab
PY6200	BY4741 *cdc20-3*::*kanR MATa*	C. Boone (U. Toronto)
SY1108	BY4741 *Bem3(1-500)-13myc-HIS3 cdc15-2 MATa*	This study
SY940	BY4741 *Bem3(1-500)-3HA-HIS3 cdc5-2::URA3 MATα*	This study
SY944	BY4741 *Bem3(1-500)-3HA-HIS3 cdc15-2 MATa*	This study
SY938	BY4741 *Bem3(1-500)-3HA MATa*	This study

Table 2-2 Plasmids used in Chapter 2

PB3050	pRS415 *GAL1-HA-CDC42 LEU2 CEN AmpR*	This study
PB3051	pRS415 *GAL1-HA-CDC42^{Q61L} LEU2 CEN AmpR*	This study
PB3052	pRS415 *GAL1-HA-CDC42^{T17N} LEU2 CEN AmpR*	This study
PB3053	pRS415 *GAL1-HA-CDC42^{G60D} LEU2 CEN AmpR*	This study
PB1622	pGEX-5X-1 *GST AmpR*	Pellman lab
PB3056	pGEX-5X-1 *GST-STE20(332-411) AmpR*	This study
PB2680	GST-PAK1 *AmpR*	Y. Zheng (Cincinnati)
pMK187	*GALS-BEM3-GFP CEN URA3 AmpR*	M. Peter (ETH Zurich)

2.5.2 Biochemical methods

Protein isolation and Western blotting were performed using standard approaches. Rat anti-alpha tubulin antibody (Accurate Chem), rabbit anti-Cdc42 antibody (y191, Santa Cruz), and rabbit anti-Clb2 antibody (y-180, Santa Cruz) were obtained from commercial sources. Densitometry was performed using ImageJ software (NIH).

For phosphatase treatment, cell lysates were prepared in ice-cold phospho-buffered saline (PBS) supplemented with phosphatase inhibitor cocktail (Roche). Bem3-3HA was immunoprecipitated from the lysates by addition of anti-HA antibody (12CA5) followed by protein A-sepharose beads (Amersham). The beads were split into three aliquots; one was mock treated, the second was treated with Calf intestinal alkaline phosphatase (CIAP, NEB), and the third was treated with CIAP and phosphatase inhibitor cocktail. After 30 min incubation at 37°C, samples were boiled and analyzed by Western blotting using the anti-HA antibody (12CA5).

2.5.3 Cdc42 activation assay (CRIB pulldown)

GST-CRIB was expressed in 200ml cultures grown to OD_{600} ~0.4 by addition of 1mM IPTG at 30°C for 3 h. Cell pellets were washed twice with Lysis Buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$) and stored at -80°C before purification. Pellets were typically stored for no longer than a month at -80°C. GST-CRIB was purified from *E. coli* by lysing cell pellets in 5ml Lysis Buffer A + 5 mM dithiothreitol (DTT), 0.2 mM phenylmethanesulfonylfluoride (PMSF), and 1% Triton X-100 (Triton X-100 was added after sonication to minimize foaming). Lysates were cleared by

centrifugation at 17,000 rcf for 20 min. The clarified lysates were incubated with pre-equilibrated glutathione-sepharose 4B (Amersham) for 1-2 h. The beads were then washed five times with Wash Buffer A (same as Lysis Buffer A but with 0.5% Triton X-100), and twice with Wash Buffer A + 10% glycerol. The beads were resuspended as a 1:1 slurry and snap frozen in small aliquots, and stored at -80°C until use (usually the next day).

The CRIB pulldown assay was adapted for use in yeast based on previous methods (Benard and Bokoch, 2002; Pellegrin and Mellor, 2008). Yeast lysates were prepared by bead beating in lysis buffer (50mM Tris-HCl pH 7.5, 500mM NaCl, 12mM $MgCl_2$, 1mM DTT), using a Mini BeadBeater-16 (BioSpec Products), followed by addition of 1% Nonidet P-40. Clarified lysates were incubated with purified GST-CRIB for 30min at 4°C. The beads were washed three times, boiled in SDS sample buffer, and subjected to Western blotting. Wild-type and *cdc24-4* control samples were always processed in parallel as a quality control for the beads.

2.5.4 Quantification of CRIB-tdTomato and Bem1-GFP signal

For quantification of CRIB-tdTomato and Bem1-GFP fluorescence intensity, cells were released from a hydroxyurea arrest for ~1 hr, and imaged using 30 z-planes per time point. Images were deconvolved, and for each cell in a movie, we picked two timepoints: (1) when the neck Bem1-GFP signal was brightest ("cytokinesis") and (2) when the subsequent polarized Bem1-GFP was brightest ("polarization"). Whichever daughter polarized first was used for the analysis. A region of interest (neck or polarity site) was

selected and the average intensity of the Gic2 CRIB-tdTomato probe was divided by the average intensity of the Bem1-GFP probe (after subtracting background in each case).

2.5.4 Cell synchronization methods

Synchronization experiments were performed essentially as described (Amon, 2002). For alpha factor block/release, 0.5 μg/ml of alpha factor (Zymo Research) was added to log phase cultures until >90% of cells were arrested. To release the cells from the arrest, the cells were washed and resuspended in media containing 50 μg/ml protease type XIV (Sigma). For the *GALL-CDC20* block/release, cultures grown to log phase in YEP-Raffinose/Galactose (2% each) media were transferred to YEP-Raffinose supplemented until >90% of cells were large-budded. To release the cells into the cell cycle, 2% galactose (v/v final) was added directly to the culture.

2.6 Acknowledgments

Chi-Fang Wu and Daniel Lew (Duke University, Durham, NC) generated the data in Fig 2-3. Satoshi Yoshida (Brandeis University, Waltham, MA) generated the data in Fig 2-4 (panels C-E). All other data was generated by the author. I thank C. Boone (U. Toronto), Y. Zheng (Cincinnati), and M. Peter (ETH Zurich) for their generous gifts of reagents used in this chapter. I also thank S. Yoshida, K. Kono, S. Buttery, X. Su, and other members of the Pellman lab for advice and useful discussions.

2.7 References

Adamo, J.E., J.J. Moskow, A.S. Gladfelter, D. Viterbo, D.J. Lew, and P.J. Brennwald. 2001. Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. *J Cell Biol*. 155:581-592.

Adamo, J.E., G. Rossi, and P. Brennwald. 1999. The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. *Mol Biol Cell*. 10:4121-4133.

Amon, A. 2002. Synchronization procedures. *Methods Enzymol*. 351:457-467.

Ash, J., C. Wu, R. Larocque, M. Jamal, W. Stevens, M. Osborne, D.Y. Thomas, and M. Whiteway. 2003. Genetic analysis of the interface between Cdc42p and the CRIB domain of Ste20p in Saccharomyces cerevisiae. *Genetics*. 163:9-20.

Atkins, B.D., S. Yoshida, K. Saito, C.F. Wu, D.J. Lew, and D. Pellman. 2013. Inhibition of Cdc42 during mitotic exit is required for cytokinesis. *J Cell Biol*. 202:231-240.

Bachewich, C., D.Y. Thomas, and M. Whiteway. 2003. Depletion of a polo-like kinase in Candida albicans activates cyclase-dependent hyphal-like growth. *Mol Biol Cell*. 14:2163-2180.

Bastos, R.N., X. Penate, M. Bates, D. Hammond, and F.A. Barr. 2012. CYK4 inhibits Rac1-dependent PAK1 and ARHGEF7 effector pathways during cytokinesis. *J Cell Biol*. 198:865-880.

Bement, W.M., H.A. Benink, and G. von Dassow. 2005. A microtubule-dependent zone of active RhoA during cleavage plane specification. *J Cell Biol*. 170:91-101.

Benard, V., and G.M. Bokoch. 2002. Assay of Cdc42, Rac, and Rho GTPase activation by affinity methods. *Methods Enzymol*. 345:349-359.

Benton, B.K., A. Tinkelenberg, I. Gonzalez, and F.R. Cross. 1997. Cla4p, a Saccharomyces cerevisiae Cdc42p-activated kinase involved in cytokinesis, is activated at mitosis. *Mol Cell Biol*. 17:5067-5076.

Bi, E., and H.O. Park. 2012. Cell polarization and cytokinesis in budding yeast. *Genetics*. 191:347-387.

Boulter, E., R. Garcia-Mata, C. Guilluy, A. Dubash, G. Rossi, P.J. Brennwald, and K. Burridge. 2010. Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. *Nat Cell Biol*. 12:477-483.

Burbelo, P.D., D. Drechsel, and A. Hall. 1995. A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. *J Biol Chem*. 270:29071-29074.

Butty, A.C., N. Perrinjaquet, A. Petit, M. Jaquenoud, J.E. Segall, K. Hofmann, C. Zwahlen, and M. Peter. 2002. A positive feedback loop stabilizes the guanine-nucleotide exchange factor Cdc24 at sites of polarization. *EMBO J*. 21:1565-1576.

Canman, J.C., L. Lewellyn, K. Laband, S.J. Smerdon, A. Desai, B. Bowerman, and K. Oegema. 2008. Inhibition of Rac by the GAP activity of centralspindlin is essential for cytokinesis. *Science*. 322:1543-1546.

Caviston, J.P., S.E. Tcheperegine, and E. Bi. 2002. Singularity in budding: a role for the evolutionarily conserved small GTPase Cdc42p. *Proc Natl Acad Sci U S A*. 99:12185-12190.

Cheng, L., L. Hunke, and C.F. Hardy. 1998. Cell cycle regulation of the Saccharomyces cerevisiae polo-like kinase cdc5p. *Mol Cell Biol*. 18:7360-7370.

Elia, A.E., P. Rellos, L.F. Haire, J.W. Chao, F.J. Ivins, K. Hoepker, D. Mohammad, L.C. Cantley, S.J. Smerdon, and M.B. Yaffe. 2003. The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell*. 115:83-95.

Goranov, A.I., M. Cook, M. Ricicova, G. Ben-Ari, C. Gonzalez, C. Hansen, M. Tyers, and A. Amon. 2009. The rate of cell growth is governed by cell cycle stage. *Genes Dev*. 23:1408-1422.

Guo, W., F. Tamanoi, and P. Novick. 2001. Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat Cell Biol*. 3:353-360.

Hofken, T., and E. Schiebel. 2002. A role for cell polarity proteins in mitotic exit. *EMBO J*. 21:4851-4862.

Hofken, T., and E. Schiebel. 2004. Novel regulation of mitotic exit by the Cdc42 effectors Gic1 and Gic2. *J Cell Biol*. 164:219-231.

Howell, A.S., M. Jin, C.F. Wu, T.R. Zyla, T.C. Elston, and D.J. Lew. 2012. Negative feedback enhances robustness in the yeast polarity establishment circuit. *Cell*. 149:322-333.

Iwase, M., J. Luo, S. Nagaraj, M. Longtine, H.B. Kim, B.K. Haarer, C. Caruso, Z. Tong, J.R. Pringle, and E. Bi. 2006. Role of a Cdc42p effector pathway in recruitment of the yeast septins to the presumptive bud site. *Mol Biol Cell*. 17:1110-1125.

Johnson, J.M., M. Jin, and D.J. Lew. 2011. Symmetry breaking and the establishment of cell polarity in budding yeast. *Curr Opin Genet Dev*. 21:740-746.

Kimura, K., T. Tsuji, Y. Takada, T. Miki, and S. Narumiya. 2000. Accumulation of GTP-bound RhoA during cytokinesis and a critical role of ECT2 in this accumulation. *J Biol Chem*. 275:17233-17236.

Knaus, M., M.P. Pelli-Gulli, F. van Drogen, S. Springer, M. Jaquenoud, and M. Peter. 2007. Phosphorylation of Bem2p and Bem3p may contribute to local activation of Cdc42p at bud emergence. *EMBO J*. 26:4501-4513.

Kohno, H., K. Tanaka, A. Mino, M. Umikawa, H. Imamura, T. Fujiwara, Y. Fujita, K. Hotta, H. Qadota, T. Watanabe, Y. Ohya, and Y. Takai. 1996. Bni1p implicated in cytoskeletal control is a putative target of Rho1p small GTP binding protein in Saccharomyces cerevisiae. *EMBO J.* 15:6060-6068.

Kono, K., S. Nogami, M. Abe, M. Nishizawa, S. Morishita, D. Pellman, and Y. Ohya. 2008. G1/S cyclin-dependent kinase regulates small GTPase Rho1p through phosphorylation of RhoGEF Tus1p in Saccharomyces cerevisiae. *Mol Biol Cell*. 19:1763-1771.

Lamson, R.E., M.J. Winters, and P.M. Pryciak. 2002. Cdc42 regulation of kinase activity and signaling by the yeast p21-activated kinase Ste20. *Mol Cell Biol*. 22:2939-2951.

Lew, D.J., and S.I. Reed. 1993. Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J Cell Biol*. 120:1305-1320.

McCusker, D., C. Denison, S. Anderson, T.A. Egelhofer, J.R. Yates, 3rd, S.P. Gygi, and D.R. Kellogg. 2007. Cdk1 coordinates cell-surface growth with the cell cycle. *Nat Cell Biol*. 9:506-515.

Meitinger, F., S. Palani, B. Hub, and G. Pereira. 2013a. Dual function of the NDR-kinase Dbf2 in the regulation of the F-BAR protein Hof1 during cytokinesis. *Mol Biol Cell*. 24:1290-1304.

Meitinger, F., B. Petrova, I.M. Lombardi, D.T. Bertazzi, B. Hub, H. Zentgraf, and G. Pereira. 2011. Targeted localization of Inn1, Cyk3 and Chs2 by the mitotic-exit network regulates cytokinesis in budding yeast. *J Cell Sci*. 123:1851-1861.

Meitinger, F., H. Richter, S. Heisel, B. Hub, W. Seufert, and G. Pereira. 2013b. A safeguard mechanism regulates Rho GTPases to coordinate cytokinesis with the establishment of cell polarity. *PLoS Biol*. 11:e1001495.

Moffat, J., and B. Andrews. 2004. Late-G1 cyclin-CDK activity is essential for control of cell morphogenesis in budding yeast. *Nat Cell Biol*. 6:59-66.

Monje-Casas, F., and A. Amon. 2009. Cell polarity determinants establish asymmetry in MEN signaling. *Dev Cell*. 16:132-145.

Narumiya, S., and S. Yasuda. 2006. Rho GTPases in animal cell mitosis. *Curr Opin Cell Biol*. 18:199-205.

Nishihama, R., J.H. Schreiter, M. Onishi, E.A. Vallen, J. Hanna, K. Moravcevic, M.F. Lippincott, H. Han, M.A. Lemmon, J.R. Pringle, and E. Bi. 2009. Role of Inn1 and its interactions with Hof1 and Cyk3 in promoting cleavage furrow and septum formation in S. cerevisiae. *J Cell Biol*. 185:995-1012.

Oceguera-Yanez, F., K. Kimura, S. Yasuda, C. Higashida, T. Kitamura, Y. Hiraoka, T. Haraguchi, and S. Narumiya. 2005. Ect2 and MgcRacGAP regulate the activation and function of Cdc42 in mitosis. *J Cell Biol*. 168:221-232.

Oh, Y., K.J. Chang, P. Orlean, C. Wloka, R. Deshaies, and E. Bi. 2012. Mitotic exit kinase Dbf2 directly phosphorylates chitin synthase Chs2 to regulate cytokinesis in budding yeast. *Mol Biol Cell*. 23:2445-2456.

Okada, S., M. Leda, J. Hanna, N.S. Savage, E. Bi, and A.B. Goryachev. 2013. Daughter cell identity emerges from the interplay of Cdc42, septins, and exocytosis. *Dev Cell*. 26:148-161.

Onishi, M., N. Ko, R. Nishihama, and J.R. Pringle. 2013. Distinct roles of Rho1, Cdc42, and Cyk3 in septum formation and abscission during yeast cytokinesis. *J Cell Biol*. 202:311-329.

Pellegrin, S., and H. Mellor. 2008. Rho GTPase activation assays. *Curr Protoc Cell Biol*. Chapter 14:Unit 14 18.

Petersen, J., and I.M. Hagan. 2005. Polo kinase links the stress pathway to cell cycle control and tip growth in fission yeast. *Nature*. 435:507-512.

Petronczki, M., P. Lenart, and J.M. Peters. 2008. Polo on the Rise-from Mitotic Entry to Cytokinesis with Plk1. *Dev Cell*. 14:646-659.

Seshan, A., A.J. Bardin, and A. Amon. 2002. Control of Lte1 localization by cell polarity determinants and Cdc14. *Curr Biol*. 12:2098-2110.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol*. 194:3-21.

Sopko, R., D. Huang, J.C. Smith, D. Figeys, and B.J. Andrews. 2007. Activation of the Cdc42p GTPase by cyclin-dependent protein kinases in budding yeast. *EMBO J*. 26:4487-4500.

Takahashi, S., and P.M. Pryciak. 2007. Identification of novel membrane-binding domains in multiple yeast Cdc42 effectors. *Mol Biol Cell*. 18:4945-4956.

Tjandra, H., J. Compton, and D. Kellogg. 1998. Control of mitotic events by the Cdc42 GTPase, the Clb2 cyclin and a member of the PAK kinase family. *Curr Biol*. 8:991-1000.

Tolliday, N., L. VerPlank, and R. Li. 2002. Rho1 directs formin-mediated actin ring assembly during budding yeast cytokinesis. *Curr Biol*. 12:1864-1870.

Tong, Z., X.D. Gao, A.S. Howell, I. Bose, D.J. Lew, and E. Bi. 2007. Adjacent positioning of cellular structures enabled by a Cdc42 GTPase-activating protein-mediated zone of inhibition. *J Cell Biol*. 179:1375-1384.

VerPlank, L., and R. Li. 2005. Cell cycle-regulated trafficking of Chs2 controls actomyosin ring stability during cytokinesis. *Mol Biol Cell*. 16:2529-2543.

Wai, S.C., S.A. Gerber, and R. Li. 2009. Multisite phosphorylation of the guanine nucleotide exchange factor Cdc24 during yeast cell polarization. *PLoS One*. 4:e6563.

Wu, H., C. Turner, J. Gardner, B. Temple, and P. Brennwald. 2009. The Exo70 Subunit of the Exocyst Is an Effector for Both Cdc42 and Rho3 Function in Polarized Exocytosis. *Mol Biol Cell*.

Yoshida, S., K. Kono, D.M. Lowery, S. Bartolini, M.B. Yaffe, Y. Ohya, and D. Pellman. 2006. Polo-like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. *Science*. 313:108-111.

Yoshizaki, H., Y. Ohba, K. Kurokawa, R.E. Itoh, T. Nakamura, N. Mochizuki, K. Nagashima, and M. Matsuda. 2003. Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. *J Cell Biol*. 162:223-232.

Zhang, G., R. Kashimshetty, K.E. Ng, H.B. Tan, and F.M. Yeong. 2006. Exit from mitosis triggers Chs2p transport from the endoplasmic reticulum to mother-daughter neck via the secretory pathway in budding yeast. *J Cell Biol*. 174:207-220.

Ziman, M., D. Preuss, J. Mulholland, J.M. O'Brien, D. Botstein, and D.I. Johnson. 1993. Subcellular localization of Cdc42p, a Saccharomyces cerevisiae GTP-binding protein involved in the control of cell polarity. *Mol Biol Cell*. 4:1307-1316.

Chapter 3:

Inhibition of Cdc42 during mitotic exit is required for cytokinesis

3.1 Summary **80**

3.2 Introduction **80**

3.3 Results **84**
3.3.1 Genetic evidence that Cdc42 inhibits cytokinesis *84*
3.3.2 Activation of Cdc42 during mitosis causes a cell separation defect *86*
3.3.3 bem2-ts cells are defective for septum formation *93*
3.3.4 Inefficient localization of Iqg1 and Inn1 in bem2-ts cells *101*
3.3.5 The Cdc42 effector Ste20 mediates Cdc42's inhibitory effect on cytokinesis *104*

3.4 Discussion **106**
3.4.1 Cdc42 is a novel inhibitor of cytokinesis in budding yeast *106*
3.4.2 Active Cdc42 interferes with septum formation *107*
3.4.3 PAK kinases are evolutionarily conserved negative regulators of cytokinesis *110*

3.5 Materials and methods **111**
3.5.1 Strains, media, and molecular biology *111*
3.5.2 Cell synchronization *113*
3.5.3 Isolation of bem2-ts mutants *114*
3.5.4 Microscopy and image analysis *114*

3.6 Acknowledgments **115**

3.7 References **116**

3.1 Summary

In Chapter 2, I presented evidence that Cdc42 activation is suppressed during mitotic exit, and that Cdc5/Polo is one upstream cell cycle regulator that inhibits Cdc42 activation. The finding that Cdc42 inhibition is wired into the budding yeast cell cycle suggests that it could have functional relevance. This chapter addresses the consequences of persistent Cdc42 activation during mitotic exit. I tested the novel hypothesis that Cdc42 may inhibit some aspect of cytokinesis. My genetic and cell biological data support this idea; cytokinesis mutants are very sensitive to Cdc42 activation, and inactivation of a Cdc42-GAP causes a cell separation defect. I found that cells with persistent Cdc42 activation during mitotic exit were defective for normal septum formation, and exhibited a partial defect in localization of two important cytokinesis regulators, Iqg1 and Inn1. Interestingly, many of these phenotypes depended on the Cdc42 effector PAK kinase Ste20, strongly supporting the idea that Cdc42 activation is the underlying cause, and that PAK kinases are conserved negative regulators of cytokinesis. I performed all the experiments in this chapter except for isolation of the *bem2-ts* allele, which was done by Koji Saito in Daniel Lew's laboratory; most of the data was published in the Journal of Cell Biology (Atkins et al., 2013).

3.2 Introduction

As introduced in Chapter 1, a number of rearrangements of the actin cytoskeleton and secretory system occur to facilitate cytokinesis, allowing the newly formed daughter cells to physically dissociate. Because cell polarization and cytokinesis make use of many of the same proteins to remodel actin and polarized secretion, mechanisms to properly

coordinate these processes in time and space seem likely to be important. Indeed, an emerging theme first shown in fission yeast is that normal cytokinesis requires inhibition of interphase polarity by the signaling networks that promote mitotic exit (Ray et al., 2010; Sanchez-Diaz et al., 2012). Interestingly, defects in cytokinesis can also affect polarization in the subsequent cell cycle (Bohnert and Gould, 2012). However, the relevant Rho GTPases involved that could impair cytokinesis have not been determined.

As described in Chapter 1, the Rho GTPase Rho1/RhoA is a highly conserved positive regulator of cytokinesis in both fungi and animal cells (Green et al., 2012). By contrast, the role of other Rho family GTPases such as Rac1 and Cdc42 during cytokinesis remains unclear and a matter of debate, in part due to complications from the typical experimental approaches used to manipulate Rho GTPase activity (Jordan and Canman, 2012). In this chapter, I will address the role of Cdc42 in cytokinesis in budding yeast. Budding yeast offers a number of advantages as a system to address this question. First, in budding yeast the Rac1 homologue has been lost during evolution, allowing studies on Cdc42 function to be free of potential redundancy (Boureux et al., 2007). Second, as discussed below, most budding yeast strains can survive in the absence of the contractile actin ring (CAR), enabling the interrogation of Cdc42 function on multiple aspects of cytokinesis. Finally, the genetic and cell biological tools available enable careful cell cycle synchronization and loss-of-function genetic analysis of components of the Cdc42 network**.**

Budding yeast cytokinesis has both similarities and differences with cytokinesis in metazoans (Bi and Park, 2012). The contractile actin ring (CAR) guides the centripetal deposition of a primary septum (PS) synthesized by the chitin synthase Chs2; CAR

contraction and PS formation are interdependent processes (Fang et al., 2010; Nishihama et al., 2009; Schmidt et al., 2002b; Shaw et al., 1991; VerPlank and Li, 2005). Subsequently, a secondary septum (SS) is deposited surrounding the PS (Bi and Park, 2012). Both CAR assembly and septum deposition are dependent upon the assembly of the septin scaffold, which splits into two rings during mitotic exit that delimit the area of cytokinesis (Dobbelaere et al., 2003; Hartwell, 1971). While a chitin-based septum is not found in higher eukaryotes, extracellular matrix deposition occurs during cytokinesis in animals and in some cases makes a functional contribution to the process (Hwang et al., 2003; Mizuguchi et al., 2003; Xu and Vogel, 2011).

In budding yeast, CAR assembly starts at G_1/S, when the type II myosin Myo1 localizes as a ring at the presumptive bud site in a septin-dependent fashion (Bi et al., 1998; Lippincott and Li, 1998). However, the final assembly of the CAR occurs in late anaphase in a similar manner as in higher eukaryotes, requiring Rho1 activation, the myosin light chain Mlc1, the IQGAP homologue Iqg1, Myo1, and the formin Bni1 (Bi and Park, 2012). By comparison to fission yeast, little is known about how the CAR is formed, or the structural orientation of the proteins involved, although the localization hierarchies of key cytokinesis proteins are conserved (Fang et al., 2010; Laporte et al., 2011; Pollard and Wu, 2010). Interestingly, the motor domain of Myo1 is dispensable for cytokinesis, and motor domain truncation mutants undergo relatively normal CAR contraction and primary septum formation (Fang et al., 2010; Lord et al., 2005). However, loss of the CAR causes defects in PS orientation, consistent with the idea that its main function is to serve as a guide during centripetal primary septum deposition (Fang et al., 2010). Finally, the CAR is disassembled near the end of cytokinesis in a manner that

depends on the myosin regulatory light chain Mlc2, Myo1 motor activity, and APC/C-mediated degradation of Iqg1 (Fang et al., 2010; Ko et al., 2007; Luo et al., 2004; Tully et al., 2009).

Primary septum formation is catalyzed by the chitin synthase II Chs2, a transmembrane protein that is delivered to the bud neck at mitotic exit *via* polarized exocytosis (Chuang and Schekman, 1996; Shaw et al., 1991; VerPlank and Li, 2005; Zhang et al., 2006). Chs2 likely requires activation at the bud neck, which appears to involve the proteins Inn1 and Cyk3 (Devrekanli et al., 2012; Jendretzki et al., 2009; Korinek et al., 2000; Meitinger et al., 2011; Nishihama et al., 2009). Iqg1 also contributes to PS formation, likely functioning upstream of Inn1 (Epp and Chant, 1997; Ko et al., 2007; Lippincott and Li, 1998; Meitinger et al., 2013; Nishihama et al., 2009). Recent EM data suggest that a tiny gap remains in the center of the primary septum of most wild-type cells, which may be filled in by deposition of the secondary septum, which requires Rho1 and the glucan synthase Fks1 (Cabib and Arroyo, 2013; Meitinger et al., 2013; Onishi et al., 2013; Yoshida et al., 2009).

In most strain backgrounds the CAR is not essential for cytokinesis but the cells survive because of septum formation genes (Bi et al., 1998; Vallen et al., 2000). However, cells cannot survive simultaneous loss of the CAR and defects in septum formation. In situations where PS formation is defective because of the absence of a CAR or Chs2, a thick, misorganized remedial septum is formed as a backup survival mechanism (Schmidt et al., 2002b). The unique genetic architecture of cytokinesis in budding yeast enabled us to interrogate Cdc42's effect on aspects of cytokinesis and septum formation that do or do not require the CAR.

Here I take both genetic and cell biological approaches to test the effect of Cdc42 activation on cytokinesis in the budding yeast *Saccharomyces cerevisiae*. I found that persistent Cdc42 activation during cytokinesis causes defects in septum formation and cell separation, likely due to inefficient localization of two important septum formation proteins, the IQGAP homolog Iqg1 and Inn1, to the bud neck. The PAK kinase Ste20, a well-characterized Cdc42 effector protein, mediates Cdc42's inhibitory effect on cytokinesis. Overall these findings show that inhibition of Cdc42 activity during mitotic exit by Cdc5/Polo is functionally critical for normal cell division.

3.3 Results

3.3.1 Genetic evidence that Cdc42 inhibits cytokinesis

Cdc42 is dispensable for CAR assembly and cytokinesis (Iwase et al., 2006; Tolliday et al., 2002). As total GTP-loading and bud neck Cdc42 activity was suppressed at the onset of cytokinesis (Chapter 2), we wondered whether inactivation of Cdc42 might be important for normal cytokinesis. To test this idea I generated a series of centromeric plasmids to express Cdc42 or mutant variants; these constructs included a N-terminal haemagglutinin (HA) tag, which did not appear to affect Cdc42 functionality, as the construct complemented *cdc42Δ* even at 37°C (Fig 3-1A). Consistent with the hypothesis that Cdc42 inhibits cytokinesis, cytokinesis mutants but not wild-type strains were extremely sensitive to *CDC42* overexpression (Fig 3-1B). As a positive control I also overexpressed Cdc42 in cells deleted for the Cdc42-GAPs *BEM2* or *BEM3*. As expected for Cdc42-GAP proteins, *bem2Δ* cells were very sensitive to *CDC42* dosage, and *bem3Δ* cells were mildly sensitive (Fig 3-1B).

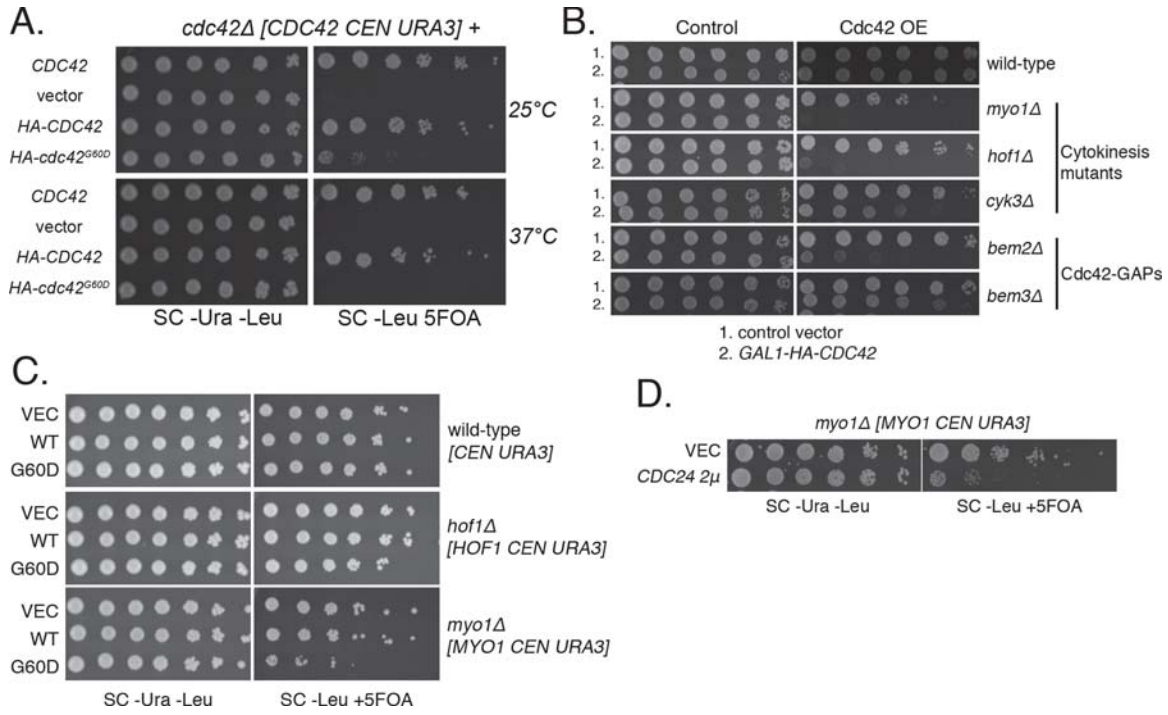


Figure 3-1: Genetic evidence that Cdc42 inhibits cytokinesis

(A) HA-Cdc42 complements *cdc42Δ* at both 25°C and 37°C. Five-fold dilutions of cells transformed with the indicated plasmids were grown on the indicated media (3 days). Complementation of *cdc42Δ* by HA-Cdc42 is detected on 5-FOA media as a counterselection for the *CDC42 CEN URA3*-containing plasmid.

(B) Cytokinesis mutants are sensitive to *CDC42* dosage. Cells transformed with vector or *GAL1-HA-CDC42* plasmids were spotted in 5-fold dilutions on the indicated media (3 days, 25°C).

(C) *myo1Δ* cells are sensitive to expression of $cdc42^{G60D}$ from the *CDC42* promoter on a CEN plasmid (1-3 copies per cell). Cells were grown on the indicated media (3 days, 25°C). Synthetic lethality is detected on 5-FOA media as a counterselection for the indicated *URA3*-containing plasmids: VEC, control vector; WT, *CDC42*; G60D, $cdc42^{G60D}$

(D) *myo1Δ* cells are sensitive to overexpression of the Cdc42-GEF Cdc24. *myo1Δ* cells transformed with a control (VEC) or *CDC24 2μ* plasmids were grown on plates as in Fig 3B.

More intriguingly, cells deleted for the type II myosin *MYO1* were very sensitive to a recessive but biochemically hyperactive allele of *CDC42, cdc42*^{G60D}, expressed from its native promoter from a centromeric plasmid (Caviston et al., 2002) (Fig 3-1C). This genetic interaction was specific because both wild-type cells and *hof1Δ* cells, defective for CAR-independent cytokinesis (Vallen et al., 2000), did not show significant sensitivity to *cdc42*^{G60D} expression (Fig 3-1C). Moreover, *myo1Δ* cells were also sensitive to overexpression of the Cdc42-GEF Cdc24 (Fig 3-1D). Finally, I confirmed a previously reported synthetic lethal interaction between *MYO1* and the Cdc42-GAP *BEM2* in our strains (data not shown; Wang and Bretscher, 1995). Overall, these genetic data suggest that active Cdc42 interferes with cytokinesis, especially in cells that lack a contractile ring and rely on septum formation for survival.

3.3.2 Activation of Cdc42 during mitosis causes a cell separation defect

Septins are essential for cytokinesis in budding yeast and Cdc42 hyperactivation can impair septin assembly (Gladfelter et al., 2002). It was therefore possible that the synthetic genetic interaction observed when Cdc42 is activated in *myo1Δ* cells could be an indirect consequence of abnormal septin assembly early in the cell cycle, manifesting later as a cytokinesis defect. To directly test whether Cdc42 activation affects cytokinesis after bud formation and septin assembly is complete, I synchronized cells in metaphase by conditional depletion of the APC/C coactivator Cdc20, hyperactivated Cdc42 using two independent approaches, and monitored cell cycle progression after release from the metaphase block.

First, I conditionally expressed the dominant GTP-locked *CDC42*^{Q61L} mutant in metaphase-arrested cultures. *CDC42*^{Q61L} expression was limited to 1 hr, a time frame that did not lead to obvious morphological defects or significant effects on cell viability (Irazoqui et al., 2003). I then released the cells into the cell cycle and followed cell cycle progression and bud morphology. Mitotic progression was monitored by visualization of the mitotic spindle (GFP-Tub1) and septins were monitored by visualization of the non-essential septin subunit Shs1-GFP (Iwase et al., 2006). These proteins exhibit non-overlapping localization to the spindle and bud neck respectively, allowing unambiguous scoring. Strikingly, many of the cells expressing *CDC42*^{Q61L} remained connected after mitotic exit (Fig 3-2A). Other cell cycle events such as the appearance of elongated anaphase spindles and septin ring splitting occurred on schedule, indicating that *CDC42*^{Q61L} does not significantly alter mitotic exit timing (Fig 3-2A,B). The connected cells in the *CDC42*^{Q61L} -expressing strain were resolved by treatment with zymolyase, demonstrating that the cells were defective in cell separation (Fig 3-2C). This experiment was consistent with the idea that increased GTP-Cdc42 levels could inhibit completion of cell division.

Next, I used an orthogonal approach to activate Cdc42 during mitosis utilizing a novel temperature-sensitive allele of the gene encoding the Cdc42-GAP *BEM2*. This was done because even moderate expression of GTP-locked *CDC42*^{Q61L} is lethal to cells and because overexpression of one Rho GTPase can have indirect effects on multiple GTPases by titrating Rho-GDI (Boulter et al., 2010; Irazoqui et al., 2003). Bem2 appears

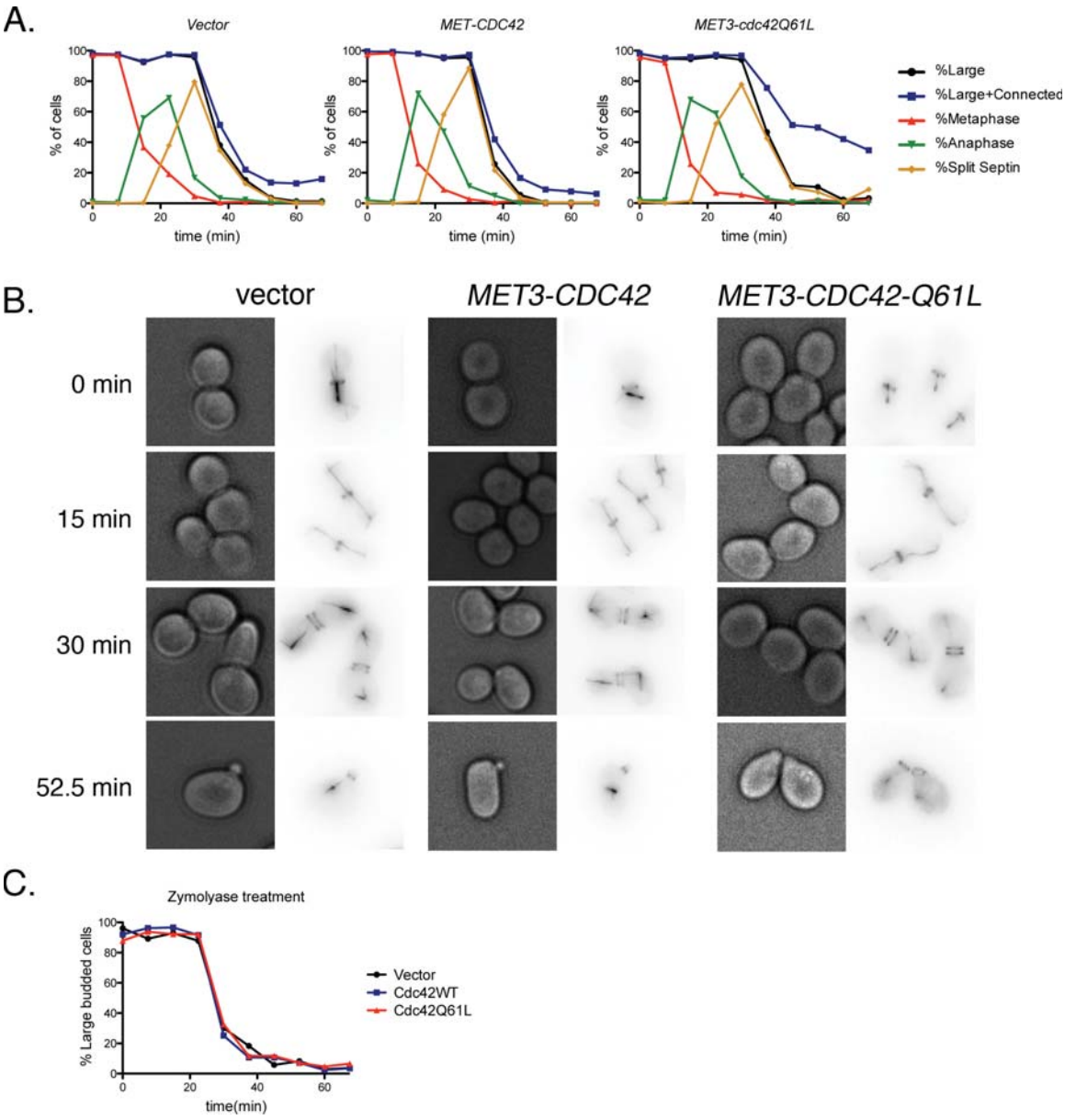


Figure 3-2: *CDC42*^{Q61L} expression causes a cell separation defect

(A) Expression of GTP-locked Cdc42 at metaphase causes a cell separation defect. *GALL-CDC20 GFP-TUB1 SHS1-GFP* cells were transformed with the indicated plasmids, arrested in metaphase for ~3 hr by depletion of Cdc20, shifted to media lacking methionine for 1 hr to express Cdc42 or the indicated variants, and then released into the cell cycle by addition of galactose. Cells were sampled at the indicated time points, fixed, and scored by fluorescence microscopy. The percentage of cells displaying the indicated bud, spindle, or septin morphology is plotted. %Large indicates the percentage of cells that were large budded. Data are representative of two experiments.
(B) No obvious septin rearrangement defect in cells expressing *CDC42*^{Q61L}. Cells from (A) were imaged with bright field (left panels) and for GFP-Tub1/Shs1-GFP (right panels).
(C) Cells expressing GTP-locked Cdc42 have a cell separation defect. Samples from an experiment in (A) were treated with zymolyase. The percentage of cells that were large budded or connected was scored at each time point.

to be the major Cdc42-GAP, at least in terms of regulating global Cdc42 activity (Knaus et al., 2007; Marquitz et al., 2002; Okada et al., 2013). Importantly, metaphase-arrested *bem2-ts* cells had a modest (~1.5-fold) increase in GTP-Cdc42 that is comparable in magnitude to downregulation of Cdc42 that occurs during cytokinesis and is also comparable to the degree of inhibition of Cdc42 by Cdc5 (Fig 3-3A).

Similar to expression of $CDC42^{Q61L}$, I observed a strong cell separation defect in synchronized *bem2-ts* cultures released from a mitotic *CDC20* arrest; approximately four-fold more cells remained connected after mitotic exit by comparison with control cells (Fig 3-3B). Other aspects of cell cycle timing, such as anaphase, as monitored by GFP-Tub1, or the initiation of septin ring splitting, as monitored by Shs1-mCherry, occurred on schedule. The *bem2-ts* cells had a strong defect in axial budding in the next cell cycle; approximately 90% of *bem2-ts* cells rebudded in a bipolar fashion, compared to 5-10% of wild-type cells (Fig 3-3C and data not shown). Because previous studies have found that *bem2* mutants have defects in bud site selection (Kim et al., 1994), this shows that *BEM2* was inactivated in my experimental regimen. The connected *bem2-ts* cells were resolved by treatment with zymolyase, indicating that the cells were defective for cell separation (Fig 3-3D). Importantly, cell separation was normal in *bem2-ts* cells grown at the permissive temperature (25°C, Fig 3-3E). The fact that either conditional expression of GTP-locked Cdc42 or conditional inactivation of a Cdc42-GAP causes a similar defect in cell separation argues that increased Cdc42 activation is the basis for this phenotype.

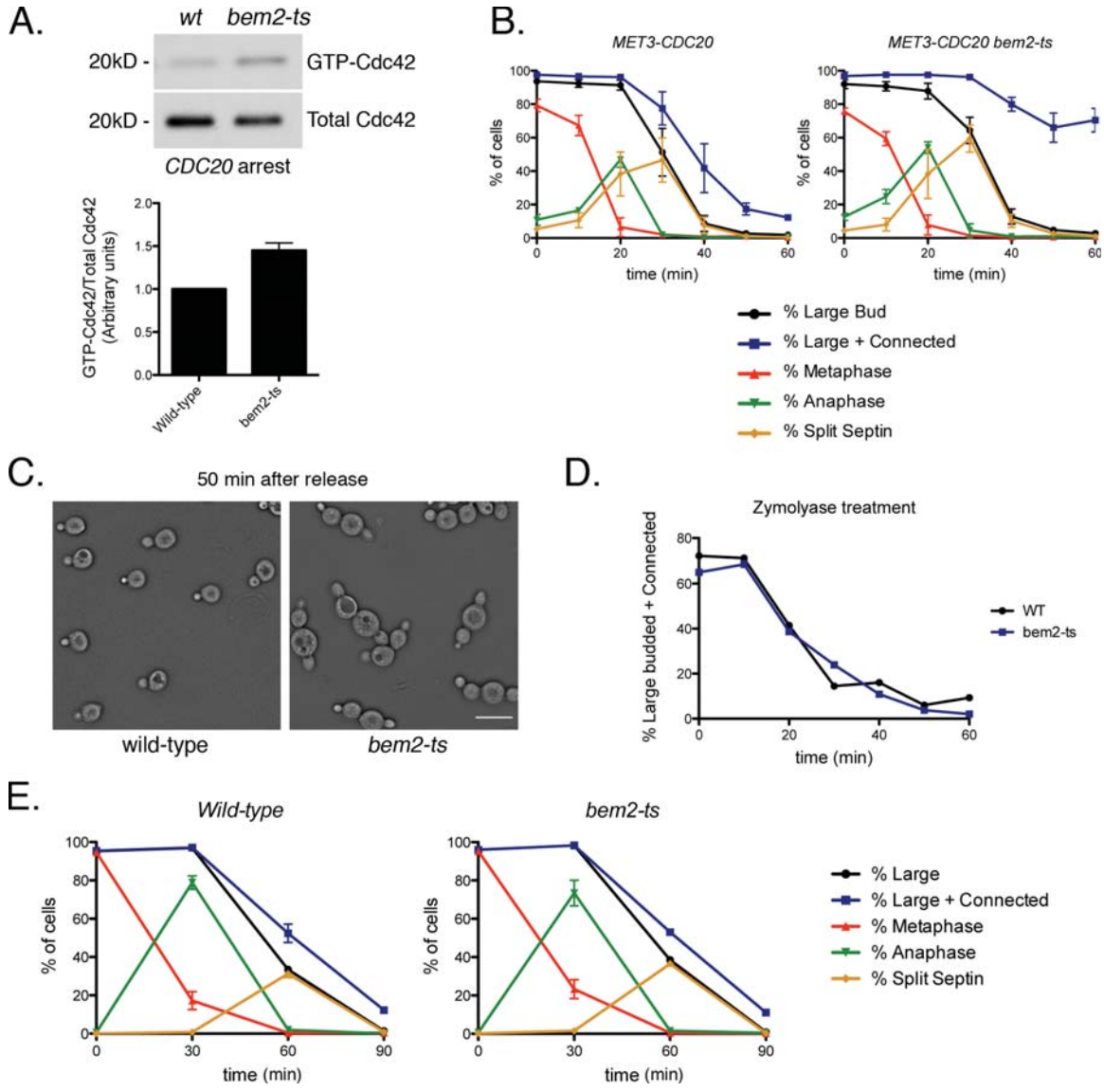


Figure 3-3: Inactivation of the Cdc42-GAP Bem2 causes a cell separation defect

(A) *bem2-ts* cells have elevated Cdc42 GTP-loading. Wild type and *bem2-ts* cells were arrested in metaphase by *CDC20* depletion, shifted to the restrictive temperature (37°C, 1.5 hr), and processed for Cdc42 activation. Graph is mean +/- SEM from 3 independent experiments, $p<0.01$ by unpaired two-tailed t-test.

(B) *bem2-ts* cells remain connected after cytokinesis. Cells were arrested in metaphase, shifted to 37°C for 1 hr, and released at 37°C. The percentage of cells with the indicated morphology is plotted as mean +/- SEM from 3 experiments. Connected indicates two adjacent cell bodies showing evidence of rebudding or repolarization.

(C) Bright field image of cells 50 min after release; bar, 15μm.

(D) *bem2-ts* cells have a cell separation defect. Cells from Fig 3-3B were treated with zymolyase to digest the cell wall, and the percentage of large-budded or connected cells was scored.

(E) *bem2-ts* cells undergo normal cell separation at the permissive temperature. Cells were arrested in metaphase for ~3 hr by depletion of Cdc20, then released into the cell cycle at 25°C, and processed and scored as in Fig 3-3B.

I also tested the effect of Cdc42 activation in other genetic contexts. Because deletion of *BEM2* is synthetic lethal with deletion of the type II myosin *MYO1*, I examined *myo1Δ bem2-ts* double mutants undergoing mitotic exit and cytokinesis. As expected, *myo1Δ* cells were defective for cell separation after mitotic exit to a degree comparable to *bem2-ts* mutants, and *myo1Δ bem2-ts* cells had a nearly complete failure in cell separation (Fig 3-4A). However, more than 90% of daughter cells rebudded in a bipolar fashion after mitotic exit, and the percentage of large budded or connected cells after zymolyase treatment was indistinguishable from single mutants, suggesting that the daughter cells were able to partition their cytoplasms (data not shown).

The Cdc42-GAP Rga1 inhibits Cdc42 activity within the old division site to promote normal axial bud site selection in the next cell cycle (Tong *et al.*, 2007). *rga1Δ* cells exhibited a cell separation defect comparable in magnitude to *bem2-ts* cells, and *rga1Δ bem2-ts* double mutants were only moderately if at all worse than the single mutants (Fig 3-4B). Interestingly, approximately 8-12% of *rga1Δ bem2-ts* double mutants exhibited a multi-budded phenotype, suggestive of a cytokinesis defect. However, after visualizing the plasma membrane using GFP-Ras2, I unexpectedly observed a pattern where one cell appeared to have lost GFP fluorescence (Fig 3-4C). I hypothesized that one of the cells lost viability due to cell lysis during or after cytokinesis. Consistently, adding 1M sorbitol as an osmotic support suppressed this abnormal multi-budded phenotype, reducing the percentage of cells with the phenotype to approximately 2-6%.

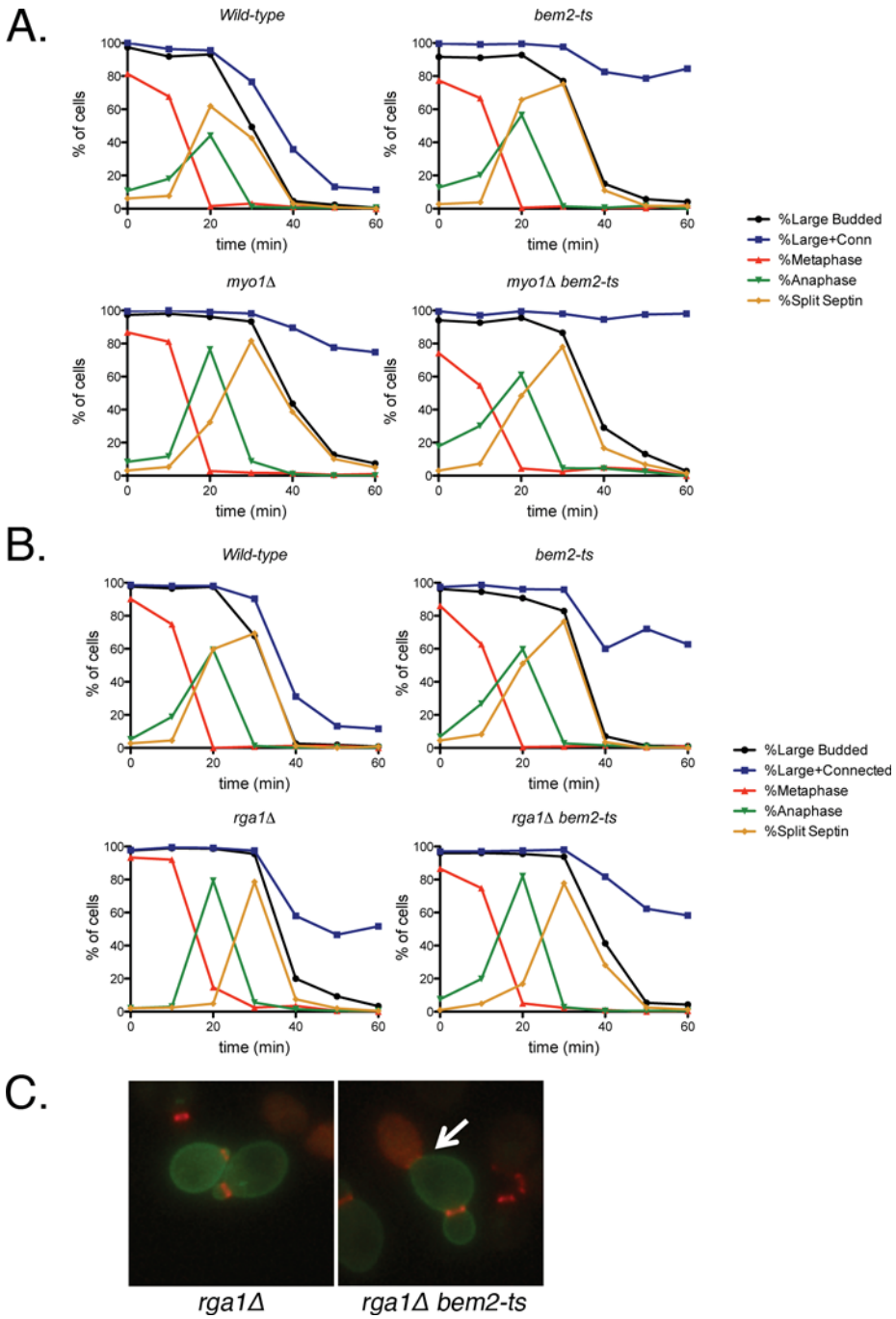


Figure 3-4: *bem2-ts* inactivation in sensitized genetic backgrounds

(A) *bem2-ts myo1Δ* cells fail cell separation. Cells of the indicated genotype were arrested, released, and scored as in Fig 3-3B.
(B) *bem2-ts rga1Δ* cells are defective for cell separation. Cells of the indicated genotype were arrested, released, and scored as in Fig 3-3B.
(C) Example of abnormal multi-budded cell with apparent loss of viability of one daughter cell in *bem2-ts rga1Δ* cells (arrow). Green: GFP-Ras2/GFP-Tubulin. Red: Shs1-mCherry.

The exact basis for this phenotype remains unclear, but a potentially similar phenomenon was reported recently in cells lacking the protein Gps1 where cells attempt to rebud into the previous division site (i.e. the bud neck) and subsequently lyse (Meitinger et al., 2013). Because *RGA1* functions in parallel to *GPS1* to prevent rebudding at the previous division site, it is likely that a minority of *rga1Δ bem2-ts* cells exhibit the same behavior (Meitinger et al., 2013; Tong et al., 2007). These data underscore the importance of proper regulation of Cdc42 activity during mitotic exit.

3.3.3 *bem2-ts* cells are defective for septum formation

The cell separation defect of *bem2-ts* cells could be explained by several non-exclusive hypotheses. First, a partial defect in septum formation could manifest as a cell separation defect if the septum is incorrectly formed and refractory to digestion by chitinase and other cell wall hydrolases. Aberrant septa have been observed in *bem2* mutants (Cid et al., 1998). Second, misregulation of the Regulation of Ace2 and Morphogenesis (RAM) signaling network could cause a defect in daughter-cell specific expression of genes involved in cell separation (Weiss, 2012). Finally, disruption of Cdc42-dependent exocytosis might cause a defect in delivery of chitinase or other cell wall hydrolases to their site of action at the bud neck (Weiss, 2012).

To distinguish among these possibilities, I examined the distribution of chitin in synchronized *bem2-ts* cells using Calcofluor-White (CFW) staining. I observed a strikingly aberrant accumulation of chitin at the bud necks of many connected *bem2-ts* cells (Fig 3-5A). This suggests that there may be a defect during cytokinesis or septum formation in *bem2-ts* cells that leads to formation of aberrant septa. Localization of the

Figure 3-5: *bem2-ts* cells have altered chitin deposition but not chitin synthase or Ace2 localization

(A) Abnormal chitin deposition at the bud neck in *bem2-ts* cells. Cells sampled 45min after release from a *CDC20* arrest were stained with calcofluor white (CFW) to visualize chitin. Bar, 10μm

(B) Chs2-GFP localizes normally in *bem2-ts* cells undergoing mitotic exit. *MET3-CDC20 GFP-TUB1 SHS1-mCherry CHS2-GFP* +/- *bem2-ts* cells were synchronized as in Fig 3-3B. Graph shows percentage of cells with the indicated spindle or septin morphology; '%Chs2 Neck' indicates the percentage of cells with Chs2-GFP localized to the bud neck. Right, representative image of cells from 20 min after release. Bar, 5μm. Example is representative of two independent experiments.

(C) Chs3-GFP localizes normally in *bem2-ts* cells undergoing mitotic exit. Similar strains as in (B) except with Chs3-GFP. Example is representative of two independent experiments.

(D) Ace2-GFP localizes to the nucleus in an asymmetric fashion in *bem2-ts* cells undergoing mitotic exit. Similar strains as in (B) except with Ace2-GFP. '%Ace2 Nuclear' indicates the percentage of cells displaying asymmetric nuclear localization of Ace2. Right; montage of representative cells (a single focal plane is shown for clarity), Scale bar, 5μm. Example is representative of two independent experiments.

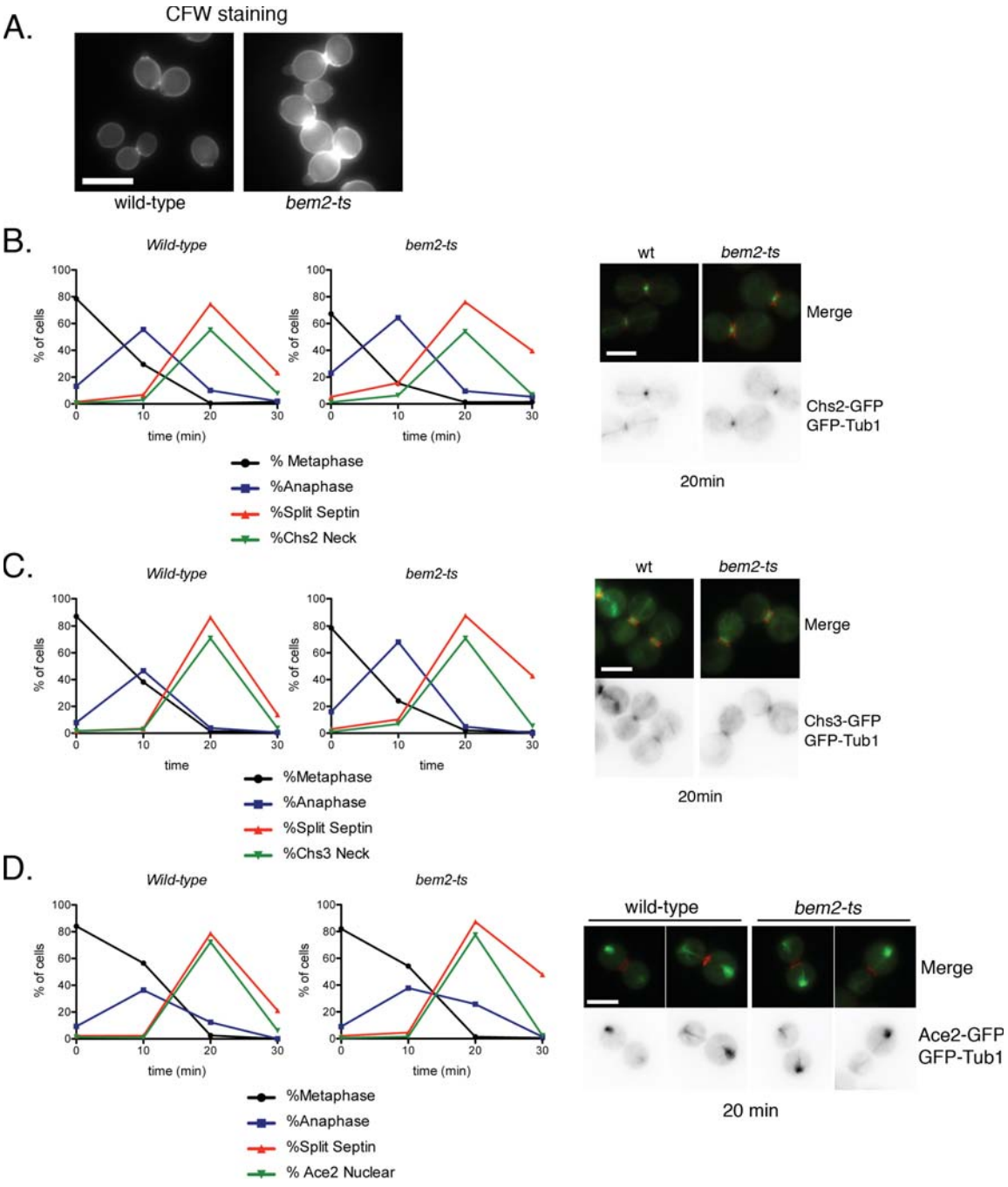


Figure 3-5 (continued): *bem2-ts* cells have altered chitin deposition but not chitin synthase or Ace2 localization

trans-membrane chitin synthases Chs2 and Chs3 was normal in *bem2-ts* cells, suggesting that polarized vesicular trafficking of these proteins from internal membrane stores to the bud neck was unaffected (Figs 3-5B,C). Furthermore, the RAM target Ace2 properly localized to the nucleus in an asymmetric fashion in *bem2-ts* cells undergoing mitotic exit, suggesting that this signaling network was functional and unlikely to be the cause of the observed cell separation defect (Fig 3-5D). These findings prompted me to study the process of cytokinesis and septation in *bem2-ts* cells in more detail.

Early work implicated Bem2 as a Rho1-GAP protein due to its ability to promote GTP hydrolysis by Rho1 *in vitro* (Peterson et al., 1994). To test whether the cell separation defect observed in *bem2-ts* mutants was due to hyperactivation of Rho1, I took two parallel approaches. First, I examined the Rho1 and Pkc1-dependent phosphorylation of Mpk1 as a readout of Rho1 activity in synchronized *bem2-ts* cells at 37°C. In wild-type cells Mpk1 phosphorylation peaked 10 min after the release from a *MET3-CDC20* arrest, which in other experiments coincided with anaphase (Fig 3-6A). Consistent with earlier work (Schmidt et al., 2002a), there was no obvious difference in Mpk1 phosphorylation in *bem2-ts* cells undergoing anaphase or mitotic exit, although the Mpk1 phosphorylation in the metaphase arrest (0 min) was reproducibly reduced in *bem2-ts* mutants (Fig 3-6A). Overall, these data suggest that during mitotic exit Mpk1 phosphorylation is largely unaffected in *bem2-ts* mutants.

Next I examined the localization of a marker for active Rho1 (Denis and Cyert, 2005; Kono et al., 2012) in cells released from a metaphase arrest. Mitotic progression was monitored by visualization of the mitotic spindle (GFP-Tub1) and septins were monitored by visualization of Shs1 (Shs1-mCherry). As expected based on previous

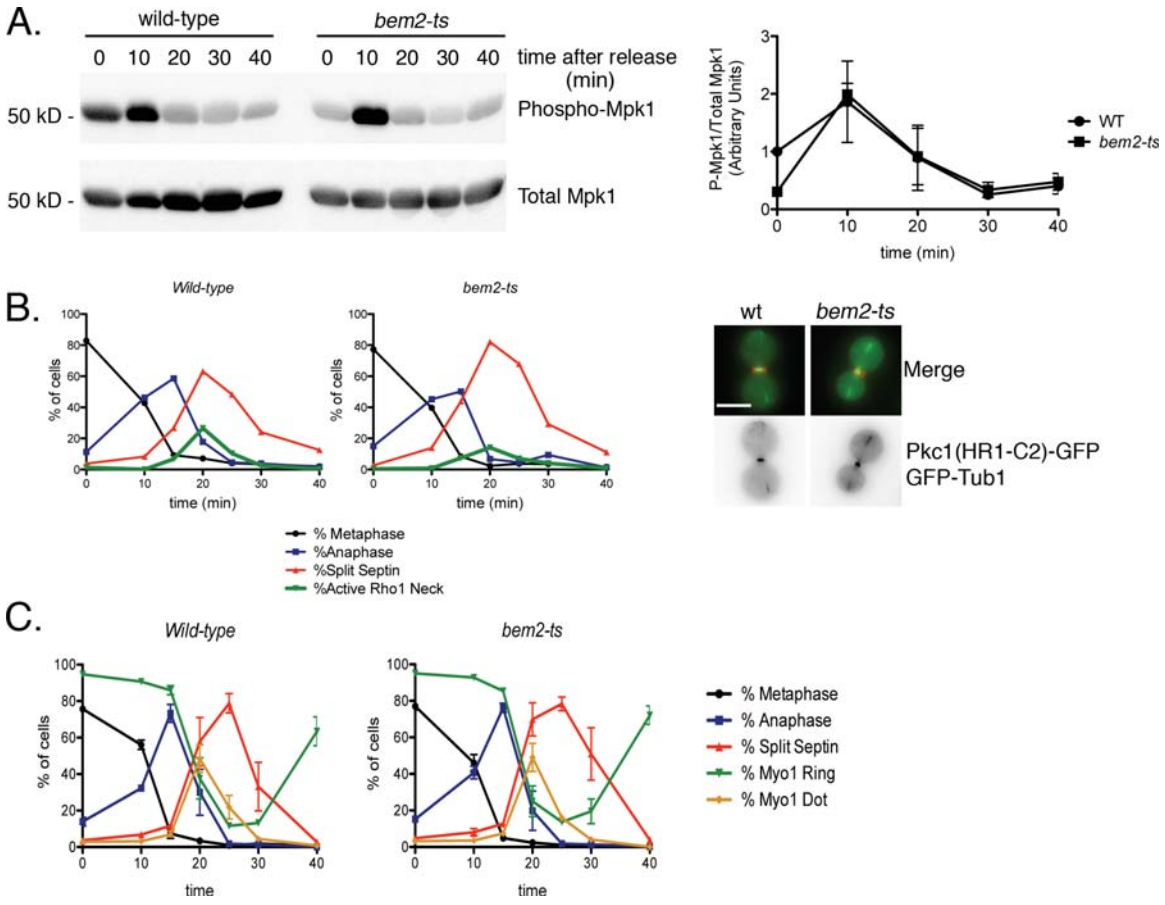


Figure 3-6: Rho1 activation and Myo1 contraction appear normal in *bem2-ts* cells

(A) Mpk1 phosphorylation is unaffected in synchronized *bem2-ts* cells. Cells were synchronized as in Fig 3-3B, and samples taken from the indicated time points were processed for Western blotting. Graph: mean +/- SEM from 2 independent experiments.

(B) The percentage of cells displaying bud neck localization of Pkc1(HR1-C2) is moderately reduced in *bem2-ts* cells. Cells expressing Pkc1(HR1-C2)-GFP were synchronized as in Fig 3-3B. '% Active Rho1 Neck' indicates the percentage of cells showing bud neck localization of Pkc1(HR1-C2)-GFP. The data is representative of two independent experiments. Right panel; example *GFP-TUB1 SHS1-mCherry +/- bem2-ts* cells expressing Pkc1(HR1-C2)-GFP are shown. Bar, 5μm.

(C) Myo1 contraction appears normal in *bem2-ts* cells. *MET3-CDC20 GFP-TUB1 SHS1-mCherry MYO1-GFP* strains with or without *bem2-ts* were synchronized as in Fig 3-3B. Graph: mean +/- SEM from three independent experiments; 'Myo1 ring' indicates a ring-like Myo1 localization while 'Myo1 Dot' indicates a contracted appearance (cytokinesis).

work, this marker localized transiently to the bud neck during the time of cytokinesis in wild-type cells (20 min after release from *CDC20* arrest, Fig 3-6B). The percentage of cells showing bud neck localization of this marker was not elevated (indeed, it was mildly reduced) in *bem2-ts* cells (Fig 3-6B), suggesting that loss of Bem2 does not result in excessive Rho1 activation.

The type II myosin Myo1 localizes as a ring at the bud neck from the G_1/S transition until initiation of cytokinesis, when it undergoes contraction and finally disassembly (Bi et al., 1998; Lippincott and Li, 1998). I observed Myo1-GFP localization in cells released from a *CDC20* arrest. Mitotic progression was monitored by visualization of the mitotic spindle (GFP-Tub1) and septins were monitored by visualization of Shs1 (Shs1-mCherry). Myo1-GFP, if present at the bud neck, was scored as either being a ring or contracted 'dot'. Myo1 underwent apparent contraction followed by disassembly with normal kinetics during mitotic exit in synchronized *bem2-ts* cells compared to wild-type, arguing that Myo1 function is unlikely to be impaired by Cdc42 activation during cytokinesis (Fig 3-6C). This finding is consistent with the conclusion from our genetic experiments suggesting that Cdc42 predominantly inhibits the CAR-independent mechanisms that promote cytokinesis.

Our observations above suggested that a defect in CAR-independent septum formation in *bem2-ts* cells was likely responsible for the cell separation defect of these cells. To directly test this idea, I examined the ultrastructure of septa in wild type and *bem2-ts* cells using transmission electron microscopy. To increase the number of cells undergoing cytokinesis, I pooled samples from 20 min and 30 min after the release from a metaphase arrest.

Wild-type cells displayed well-organized trilaminar septa, with an electron-lucent primary septum sandwiched by secondary septa (Fig 3-7A, [n=34/36]), as previously described (Cabib et al., 1974). Interestingly, approximately 31% of the *bem2-ts* cells had misorganized primary septa [n=11/35] that were misaligned, bifurcated, and/or contained extra primary septa (Fig 3-7B). In some cases, the adjacent secondary septa appeared to be thickened or enlarged (e.g. Fig 3-7Biii). These data indicate that septum formation is indeed partially defective in *bem2-ts* cells. Connected *bem2-ts* cells 50 min after the release from the arrest exhibited similar structures at the connection between the two daughter cells, suggesting that these defective septa could be difficult to remodel to achieve normal cell separation (data not shown).

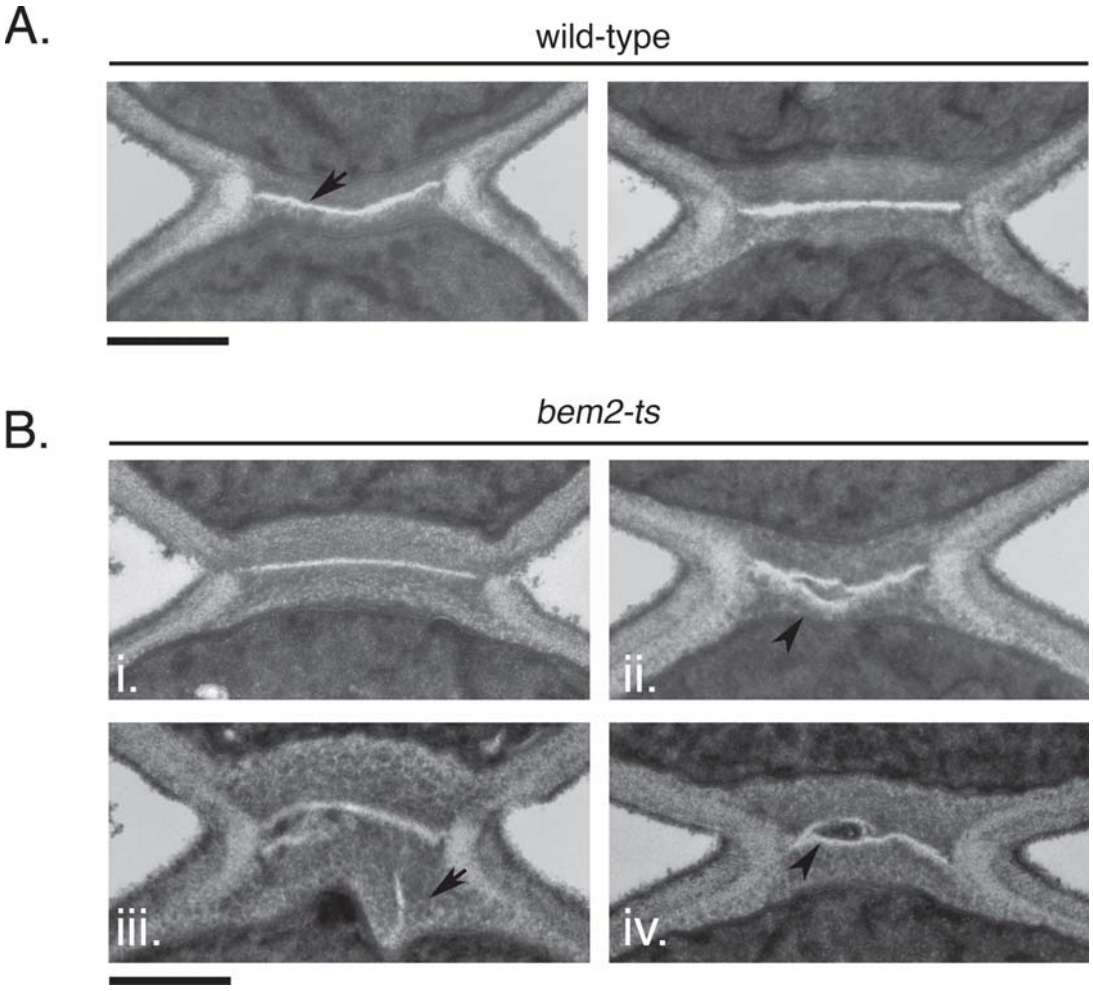


Figure 3-7: *bem2-ts* cells have a defect in septum formation

(A) Wild-type cells have trilaminar septa, with a primary septum (white line marked by arrowhead) sandwiched by secondary septal material. Scale bar indicates 500nm

(B) *bem2-ts* cells have aberrant septa. Approximately 31% of *bem2-ts* cells exhibited misaligned, multiple or abnormal primary septa in the section examined. The primary septum often appeared bifurcated near the center of the bud neck (arrowheads in panels ii and iv). Occasionally ectopic primary septa structures were observed (arrowhead in panel iii). Scale bar, 500nm.

3.3.4 Inefficient localization of Iqg1 and Inn1 in *bem2-ts* cells

Like the chitin synthases Chs2 and Chs3, the F-BAR protein Hof1 localized normally in *bem2-ts* cells (data not shown). However, the IQGAP homologue Iqg1, a protein involved in both CAR formation and septum formation (Fang et al., 2010; Osman and Cerione, 1998; Shannon and Li, 1999), and Inn1, a protein involved in primary septum formation (Devrekanli et al., 2012; Meitinger et al., 2011; Nishihama et al., 2009; Sanchez-Diaz et al., 2008), were inefficiently localized to the bud neck in synchronized *bem2-ts* cells undergoing mitotic exit compared to wild-type controls. These strains also contain GFP-Tub1 and Shs1-mCherry to monitor cell cycle progression. For Iqg1, the initial localization as a ring at the bud neck during anaphase appeared normal in *bem2-ts* cells, but the protein was localized to bud neck of approximately two-fold fewer cells during the cytokinesis peak of 20 min after release, when Iqg1 localization appears as a contracted 'dot' at the bud neck (Fig 3-8A). The defect in Iqg1 localization was even more apparent after measurement of the fluorescence intensity of Iqg1 at the bud neck in *IQG1-GFP SHS1-mCherry* strains 20 min after release from the *CDC20* block (a ~ 20-fold effect, Fig 3-8C). A similar pattern was observed for Inn1-GFP in *bem2-ts* cells (Fig 3-8B,D), although the defect in localization was more modest. This suggests that aberrant Cdc42 activation during mitotic exit leads to a failure to maintain normal amounts of two key proteins required for septation.

Figure 3-8: Inefficient localization of Iqg1 and Inn1 to the bud neck in *bem2-ts* cells

(A) Iqg1 localizes inefficiently to the bud neck during cytokinesis in *bem2-ts* cells. *MET3-CDC20 GFP-TUB1 SHS1-mCherry IQG1-GFP* were synchronized as in Fig 3-3B. *IQG1-GFP*, if present at the bud neck, was scored as either being a ring or contracted 'dot'. The percentage of cells with the indicated localization pattern is plotted as mean +/-SEM from 3 experiments. Right: representative images of cells. Arrowheads show Iqg1 bud neck localization. Bar, 5μm.

(B) Inn1 localizes inefficiently to the bud neck during cytokinesis in *bem2-ts* cells. Similar strains as in (A) but with *INN1-GFP*. % Inn1 Neck represents percentage of cells with Inn1-GFP at the bud neck. Right; representative images of cells from the indicated time points. Arrowheads show Inn1 bud neck localization; bar, 5 μm.

(C) Fluorescence intensity of Iqg1-GFP at the bud neck is reduced in *bem2*-ts cells. Scatter plot of Iqg1-GFP and Shs1-mCherry background-corrected fluorescence intensity at the bud neck in *bem2-ts* cells 20 min after release from *CDC20* arrest in *SHS1-mCherry IQG1-GFP* cells. Note that negative values after background subtraction indicate either increased cytoplasmic background or completion of cytokinesis with a corresponding loss of background signal in the bud neck area. Line indicates mean, error bars represent SEM, n>60 cells. The difference for Iqg1-GFP intensity between WT and *bem2-ts* was statistically significant ($p < 0.0001$ by unpaired two-tailed t test), whereas the difference for Shs1-mCherry was not.

(D) Fluorescence intensity of Inn1-GFP at the bud neck is reduced in *bem2*-ts cells. Inn1-GFP fluorescence intensity at the bud neck was measured as in Fig 4D, n>50 cells. The difference for Inn1-GFP was significant ($p < 0.05$ by unpaired two-tailed t test).

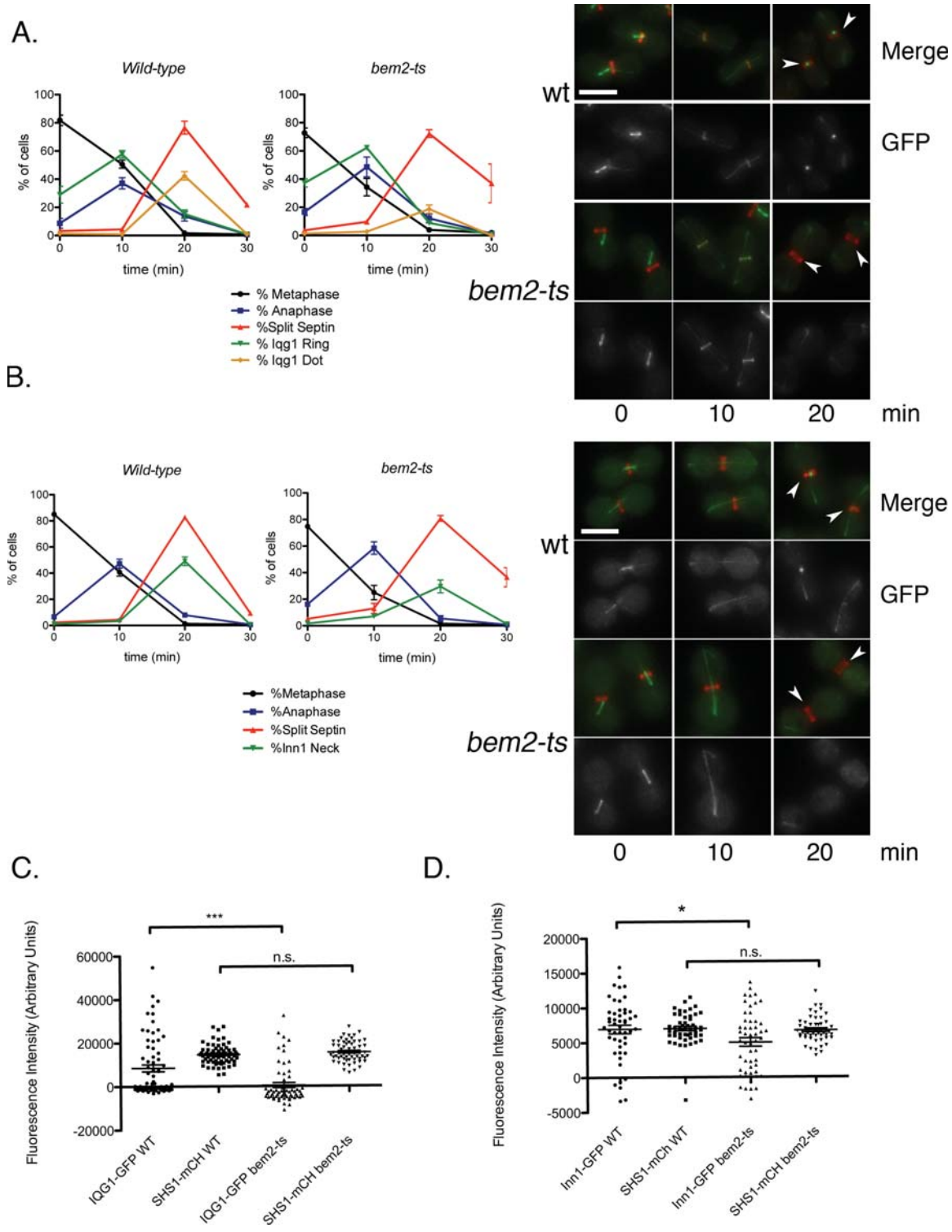


Figure 3-8 (Continued): Inefficient localization of Iqg1 and Inn1 to the bud neck in *bem2-ts* cells

To more directly test the significance of Iqg1 and Inn1 mislocalization during cytokinesis in *bem2-ts* cells, I determined whether overexpression of these genes could rescue the cell separation defect of this strain. Indeed, overexpression of Iqg1 could suppress the cell separation defect observed in synchronized *bem2-ts* cells to nearly wild-type levels (Fig 3-10A). Overexpression of Iqg1 has previously been shown to be sufficient for bud neck localization (Epp and Chant, 1997). Overall these data suggest that aberrant Cdc42 activation during mitotic exit leads to inefficient localization of key components of the septum formation machinery, aberrant septum formation, and ultimately a cell separation defect.

3.3.5 The Cdc42 effector Ste20 mediates Cdc42's inhibitory effect on cytokinesis

How does Cdc42 activation during cytokinesis affect the localization of Iqg1 and Inn1? Neither protein has recognizable CRIB domains for binding GTP-Cdc42. Like other family members, Iqg1 is missing critical residues from its Rho-GAP domain, and is inactive as a Cdc42-GAP (Shannon, 2012). Cdc42 and Iqg1 can interact by two-hybrid analysis, but fragments of Iqg1 bind very weakly to Cdc42, and the significance of these interactions is not known (Osman and Cerione, 1998; Shannon and Li, 1999). As a first step towards identifying the signaling mechanism linking Cdc42 to Iqg1 and Inn1, I determined whether known

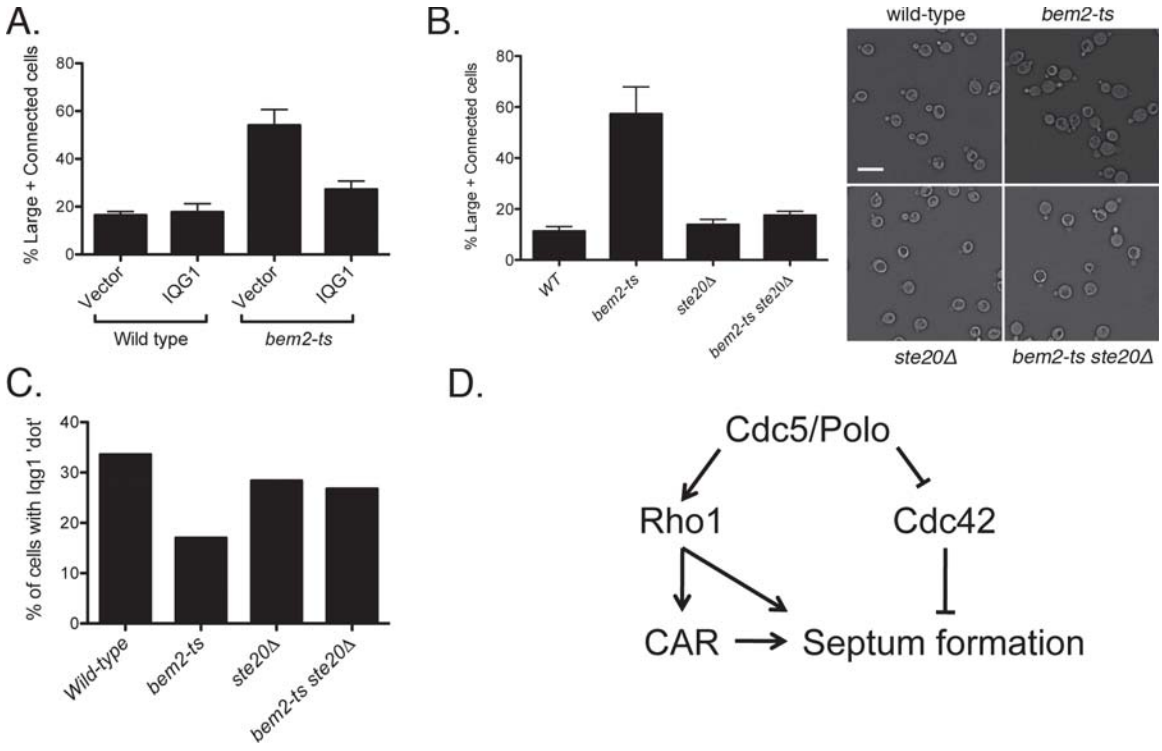


Figure 3-9: Ste20 and Iqg1 mediate the inhibitory effect of active Cdc42 on cytokinesis and cell separation

(A) Iqg1 overexpression suppresses the cell separation defect of *bem2-ts* cells. Cells transformed with *2μ IQG1* or a control vector were synchronized as in Fig 3-3B, and the percentage of large budded or connected cells was determined 50 min after release. Graph: mean +/- SEM from 3 experiments using independent 2μ-containing isolates

(B) Deletion of the PAK kinase *STE20* suppresses the cell separation defect of *bem2-ts* cells. Cells were synchronized and scored as in (A). Graph: mean +/- SEM for 3 experiments; bar, 10 μm

(C) Deletion of *STE20* partially rescues the localization defect of Iqg1-GFP to the bud neck during cytokinesis. Percentage of cells with contracting Iqg1-GFP 'dot' 20 min after release from *CDC20* arrest. Data are representative of 2 experiments.

(D) Model for how Cdc5/Polo controls Rho GTPases to control cytokinesis.

Cdc42-effectors are required to mediate the effect of persistent Cdc42 activation on cytokinesis.

I focused on the Cdc42-effector PAK kinases because of their known role in mitotic exit and cytokinesis. Deletion of the PAK kinase *CLA4* caused a cytokinesis and cell separation defect as well as abnormal septin organization, as previously reported (Dobbelaere et al., 2003; Longtine et al., 2000; Versele and Thorner, 2004). The cell separation defect was stronger than *bem2-ts* cells and did not appear additive (data not shown). By contrast, deletion of the gene for the other major PAK kinase, *STE20*, significantly reversed the cell separation defect of *bem2-ts* cells (Fig 3-9B), suggesting that excess GTP-Cdc42 may act through Ste20 to impair cytokinesis and cell separation. Moreover, loss of Ste20 in *bem2-ts* cells largely restored Iqg1 localization to the bud neck as a contracted 'dot' during cytokinesis (Fig 3-10C). Overall these data indicate that Ste20 mediates the inhibitory effect of Cdc42 on cytokinesis.

3.4 Discussion

3.4.1 Cdc42 is a novel inhibitor of cytokinesis in budding yeast

Cytokinesis requires fine-tuned coordination between small GTPases, which regulate the cortical actin cytoskeleton, and the cell cycle machinery. Previous work from the Pellman laboratory found one major link between these two signaling mechanisms, where the budding yeast Polo kinase ortholog Cdc5 promotes the activation of Rho1 to promote cytokinesis (Yoshida et al., 2009; Yoshida et al., 2006). Subsequent work by many groups uncovered a broadly similar regulatory mechanism in mammalian cells (Petronczki et al., 2008). In Chapter 2 we identified a major limb of this pathway that

operates in parallel: Cdc5/Polo kinase promotes the inactivation of Cdc42 during cytokinesis.

Inhibition of Cdc42 is critical for normal septum formation (Fig 3-10D). Persistent Cdc42 activity during mitotic exit prevents efficient localization of the IQGAP ortholog Iqg1 as well as Inn1 to the division site during cytokinesis. The Cdc42 effector Ste20 mediates these negative effects of Cdc42 to a large extent. These results highlight the importance of Polo kinase as a regulator of small GTPases and the cortical cytoskeleton. They are also consistent with an emerging general theme in eukaryotes that Rho family GTPases such as Rac1 and Cdc42, and cell polarization in general, need to be inhibited during cytokinesis (Bastos et al., 2012; Canman et al., 2008; Ray et al., 2010; Sanchez-Diaz et al., 2012).

3.4.2 Active Cdc42 interferes with septum formation

My results, especially in light of earlier indirect genetic data (Caviston et al., 2002; Gandhi et al., 2006; Gao et al., 2004), suggest that Cdc42 interferes with proper septum formation. Several lines of evidence support this conclusion. Cells lacking the budding yeast type II myosin Myo1, and thus lacking contractile rings, were very sensitive to genetic manipulations resulting in Cdc42 activation. Conversely, cells compromised for septum formation without impairing CAR assembly were less sensitive to Cdc42 activation. A caveat for this result, as well as for previous genetic experiments, is that inappropriate Cdc42 activation might impair normal septin assembly early in the cell cycle and then later cause cytokinesis defects indirectly. Such septin defects could be present but not strong enough to produce visibly abnormal septin structures. I addressed

this concern by synchronizing cells in metaphase, after septin assembly is complete, when maintanence of the septin collar are no longer requires Cdc42 (Gladfelter et al., 2002). I then activated Cdc42 either through expression of GTP-locked Cdc42 or inactivation of the GAP Bem2. These experiments demonstrate that Cdc42 activation interferes with normal cytokinesis, independent of Cdc42's role in septin assembly. These data agree with recent work from the Pringle lab showing that overexpression of Cdc42-GAP proteins can suppress mutants defective in primary septum formation (Onishi et al., 2013).

Several lines of evidence support the idea that Bem2's effect on cytokinesis is due to activation of Cdc42 and not other Rho-family GTPases. *In vitro*, a fragment of Bem2 has GAP activity for both Cdc42 and Rho1 (Marquitz et al., 2002; Peterson et al., 1994). Phenotypic analysis and biochemical pulldown experiments strongly suggest that Bem2 is the major Cdc42-GAP, at least in terms of total Cdc42 activity (Knaus et al., 2007; Marquitz et al., 2002; Wang and Bretscher, 1995). Consistently, the growth of *bem2*Δ cells is markedly impaired by increased *CDC42* dosage. This contrasts with Rho1 overexpression, which is reported to partially suppress *bem2* mutants (Kim et al., 1994). Moreover, examining Mpk1 phosphorylation and the localization of a Rho1 biosensor, I saw no evidence for Rho1 hyperactivation in *bem2* cells. Finally, the fact that deletion of the well-established Cdc42-effector Ste20 suppresses the cell separation defect of *bem2-ts* cells provides strong support for the conclusion that the effects of Bem2 inactivation are primarily mediated by Cdc42 activation.

My data indicate that Cdc42 activation interferes with the bud neck localization of specific proteins involved in septum formation—Iqg1 and Inn1—during cytokinesis, with

downstream consequences of aberrant chitin accumulation and abnormal septum formation. This likely explains the cell separation defect of cells with elevated Cdc42 activity because cells with abnormal septa are refractory to cell wall hydrolases that mediate cell separation (Weiss, 2012). Overexpression of Iqg1 could suppress the cell separation phenotype of *bem2-ts* cells, further supporting the conclusion that a defect in cytokinesis gives rise to a cell separation defect and that inefficient Iqg1 localization contributes to the defect. Although Inn1 localization is under complex controls (Meitinger et al., 2011; Nishihama et al., 2009), in some strain backgrounds Inn1 localization depends on Iqg1p (Sanchez-Diaz et al., 2008). Thus, inappropriate activation of Cdc42 during mitotic exit could affect Inn1 indirectly via effects on Iqg1 localization.

I found that *bem2-ts* cells often had bifurcated or misaligned primary septa (Fig 3-7B). Additionally, I also observed ectopic or multiple primary septa in some cases. Interestingly, Iqg1 mutants lacking the calponin-homology domain have a reminiscent phenotype, with both defects in primary septum orientation and the presence of additional primary septal structures (Fang et al., 2010). Therefore, the partial mislocalization of Iqg1 during cytokinesis in *bem2-ts* cells (Fig 3-8A,C) could explain the defect in primary septum formation. Since secondary septum deposition has been suggested to contribute to the final closure of the bud neck, it is also possible that Cdc42 could affect the coordination between primary and secondary septum formation (Onishi et al., 2013).

Another function for inhibition of Cdc42 during the M-to-G_1 transition is to 'clear the slate' to prevent rebudding into the previous division site, the bud neck (Meitinger et al., 2013; Tong et al., 2007). Notably, cells lacking *GPS1* undergo defective secondary septum formation due to reduced Rho1 localization and also often exhibit

rebudding of one of the daughter cells into the bud neck due to inappropriate Cdc42-dependent polarization at this site, which leads to cell lysis (Meitinger et al., 2013). I observed a similar phenomenon in approximately 10% of *rga1Δ bem2-ts* cells (Fig 3-4B,C and data not shown). Since *rga1Δ* cells are also biased to rebud into the previous division site (Tong et al., 2007), I hypothesize that bud emergence through abnormally-formed septa presents a formidable challenge for the active remodeling that is required to maintain cell wall integrity (Levin, 2011). These data emphasize the importance of proper inhibition of Cdc42 during mitotic exit not only for cytokinesis but also for bud site selection and cell viability.

3.4.3 PAK kinases are evolutionarily conserved negative regulators of cytokinesis

Inappropriate Cdc42 signaling during mitotic exit impairs cytokinesis *via* the Cdc42-effector PAK kinase Ste20. Loss of Ste20 significantly restored both normal cell separation and the localization of Iqg1 to the bud neck of *bem2-ts* cells. Defining how Ste20 regulates Iqg1 will be an interesting topic for future research. The regulatory effect could be mediated by direct phosphorylation of Iqg1 or by other more indirect mechanisms. Although Ste20 is clearly a major target of this regulation, other Cdc42 effectors, such as Gic1 and Gic2, could also be in the regulatory circuit. Because of the previously described interaction between Cdc42 and Iqg1, we do not exclude the possibility of direct effects of Cdc42 on Iqg1 (Osman and Cerione, 1998; Shannon and Li, 1999). Finally, I cannot absolutely exclude a role for the other PAK kinase, Cla4. Although deletion of *CLA4* did not bypass the effect of Cdc42 hyperactivation, Cla4 has an important positive role in septin formation that could have obscured a negative role

late in the cell cycle. My data, along with previous work in *S. pombe* and mammalian cells, suggest that negative regulation of cytokinesis by PAK kinases is a conserved process (Bastos et al., 2012; Loo and Balasubramanian, 2008).

3.5 Materials and methods

3.5.1 Strains, media, and molecular biology

Standard approaches were used for molecular biology and genetic manipulation of yeast (Sherman, 1991). All yeast strains are isogenic or congenic with the S288c-derived BY4741 (*MATa his3Δ leu2Δ met15Δ ura3Δ*). Gene deletion or modification was confirmed by PCR. GST-CRIB pulldowns were performed as in Chapter 2. For a list of yeast strains and plasmids see Table 3-1 and Table 3-2.

Table 3-1: Yeast strains used in Chapter 3

Strain	Relevant Genotype	Source
PY3295	BY4741 *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 MATa*	Pellman perms
PY7205	YEF473a S288c *his3 leu2 trp1 ura3 MATa*	E. Bi (U. Penn)
PY7206	YEF473a S288c *his3 leu2 trp1 ura3 cdc24-4 MATa*	E. Bi (U. Penn)
PY6573	BY4741 *GALL-CDC20-kanR GFP-TUB1-URA3 SHS1-GFP-HIS3 MATa*	This study
PY6545	BY4741 *hof1::his3MX6 [HOF1 CEN URA3] MATa*	This study
PY5453	BY4741 *myo1::his3MX6 [MYO1 CEN URA3] MATa*	This study
PY5039	BY4741 *bem2::kanR MATa*	Pellman perms
PY5032	BY4741 *cyk3::kanR MATa*	Pellman perms
PY5033	BY4741 *bem3::kanR MATa*	Pellman perms
PY7210	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR MATa*	This study
PY7211	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR bem2-ts(#84)-HIS3 MATa*	This study
PY7212	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR MYO1-GFP-kanR MATa*	This study
PY7213	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR MYO1-GFP-kanR bem2-ts(#84)-HIS3 MATa*	This study

Table 3-1 (continued): Yeast strains used in Chapter 3

PY7214	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR IQG1-GFP-kanR MATa*	This study
PY7215	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR IQG1-GFP-kanR bem2-ts(#84)-HIS3 MATa*	This study
PY7216	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR INN1-GFP-kanR MATa*	This study
PY7217	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR INN1-GFP-kanR bem2-ts(#84)-HIS3 MATa*	This study
PY7218	BY4741 *MET3-CDC20-TRP1 SHS1-mCherry-hygR IQG1-GFP-kanR MATa*	This study
PY7219	BY4741 *MET3-CDC20-TRP1 SHS1-mCherry-hygR IQG1-GFP-kanR bem2-ts(#84)-LEU2 MATa*	This study
PY7220	BY4741 *MET3-CDC20-TRP1 SHS1-mCherry-hygR INN1-GFP-kanR MATa*	This study
PY7221	BY4741 *MET3-CDC20-TRP1 SHS1-mCherry-hygR INN1-GFP-kanR bem2-ts(#84)-LEU2 MATa*	This study
PY7222	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR ste20::kanR MATa*	This study
PY7223	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR bem2-ts(#84)-HIS3 ste20::kanR MATa*	This study
PY7224	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR IQG1-kanR ste20::URA3 MATa*	This study
PY7225	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR IQG1-kanR bem2-ts(#84)-HIS3 ste20::URA3 MATa*	This study
PY7226	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR CHS2-GFP-kanR MATa*	This study
PY7227	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR CHS2-GFP-kanR bem2-ts(#84)-HIS3 MATa*	This study
PY7228	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR CHS3-GFP-kanR MATa*	This study
PY7229	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR CHS3-GFP-kanR bem2-ts(#84)-HIS3 MATa*	This study
PY7230	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR ACE2-GFP-kanR MATa*	This study
PY7231	BY4741 *MET3-CDC20-TRP1 GFP-TUB1-LEU2 SHS1-mCherry-hygR ACE2-GFP-kanR bem2-ts(#84)-HIS3 MATa*	This study

Table 3-2: Plasmids used in Chapter 3

Plasmid	Relevant features	Source
PB70	pRS316 *URA3 CEN AmpR*	Pellman perms
PB72	pRS415 *LEU2 CEN AmpR*	Pellman perms
PB158	*2μ URA3*	Pellman perms
PB3050	pRS415 *GAL1-HA-CDC42 LEU2 CEN AmpR*	This study
PB3054	pRS415 *HA-CDC42 LEU2 CEN AmpR*	This study
PB3055	pRS415 *HA-cdc42^{G60D} AmpR*	This study
PB3056	pGEX-5X-1 *GST-STE20(332-411) AmpR*	This study
PB3057	pRS415 *MET3-HA-CDC42 CEN LEU2 AmpR*	This study
PB3058	pRS415 *MET3-HA-CDC42^{Q61L} CEN LEU2 AmpR*	This study
YEp351-*CDC24*	*CDC24 LEU2 2μ AmpR*	D. Johnson (1)
pAJ044	*IQG1 2μ URA3*	J. Heinisch (2)
E735	pRS316-HOF1 *HOF1 CEN URA3 AmpR*	E. Bi (3)
pVD63	*PKC1*(HR1-C2)-GFP *URA3 CEN AmpR*	M. Cyert (5)
PB3063	pRS316 MYO1 CEN URA3 *AmpR*	This study
PB3064	*pMET3-CDC20-TRP1 AmpR*	F. Uhlmann (7)
PB3065	pDLB3138 *bem2-ts(#84)::LEU2* Integrative *AmpR*	This study
PB3066	*bem2-ts(#84)::HIS3* Integrative *AmpR*	This study
pEL45	*ste20::URA3* Integrative	M. Whiteway (8)

Source:

1) Richman *et al.,* 1999, *J. Biol. Chem.* 274:16861–16870.
2) Jendretzki *et al.,* 2009, *Mol Genet Genomics* 282:437–451.
3) Vallen *et al.,* 2000, *MBC* 11:593–611.
4) Tong *et al.,* 2007, *J. Cell Biol. 179:*1375–1384.
5) Denis & Cyert, 2005 *Eukaryotic Cell* 4:36–45.
6) Knaus *et al.,* 2006, *EMBO J* 26:4501–4513.
7) Uhlmann *et al.*, 2000, *Cell* 103:375–386.
8) Leberer *et al.,* 1992, *EMBO J* 11:4815–4824.

3.5.2 Cell synchronization

Synchronization experiments were performed essentially as described (Amon, 2002). For *MET3-CDC20* block/release, cultures grown to log phase in SD-Met media were transferred to YEPD supplemented with 2 mM methionine until >90% of cells were large-budded. For temperature shift experiments, cells were transferred to fresh pre-

warmed media. To release the cells into the cell cycle, the cells were washed with 3 culture volumes of SD-Met and resuspended in SD-Met.

3.5.3 Isolation of *bem2-ts* mutants

Temperature-sensitive *bem2* mutants were isolated by a mutagenic PCR and gap-repair strategy similar to that used for *cdc42* mutants (Moskow et al., 2000). In brief, mutants were generated using error-prone PCR of the Bem2 GAP domain, and recombined into a low-copy plasmid by gap repair (homologous recombination in yeast). Mutants were screened for those that could complement the synthetic lethality of *cla4*Δ *bem2*Δ and *rga1*Δ *bem2*Δ strains at 24°C but not 37°C. The tight *bem2-84* mutant was sequenced and found to carry two amino acid substitutions (K1986R and I2041N) in the GAP domain.

3.5.4 Microscopy and image analysis

Cells were imaged using an automated Zeiss AX10 microscope equipped with a 63x 1.4 NA Plan-Apochromat objective at room temperature. Images were acquired with a CoolSnap HQ (Roper Scientific) camera. The microscope and camera were controlled by Slidebook (Intelligent Imaging Innovations) software, which was also used for image analysis. Photoshop (Adobe) was used for assembling figures. Cells were fixed by addition of 3.7% formaldehyde directly to the culture for 5-10 min, followed by washing three times with phosphate-buffered saline. Fixed cells were vortexed for 90 sec before imaging. Cells were imaged at 0.3 μm intervals in the Z-plane to characterize localization of proteins. For scoring cell morphology, cells were considered connected if two

equivalent-sized cell bodies were directly adjacent and showed evidence of repolarization or rebudding. More than 200 cells were scored for each time point for each synchronization experiment. Zymolyase treatment and Calcofluor White staining were performed as described (Pringle, 1991; Tolliday et al., 2002). Briefly, for zymolyase treatment, cells were fixed for 1 hr with 3.7% formaldehyde, washed three times with PBS, twice with KS buffer (1.2M sorbitol 50mM $KHPO_4$), and then resuspended in KS containing 0.2 mg/ml zymolyase (Zymo Research) and 2 mM DTT until >90% of cells lost their refractive appearance. To visualize chitin, cells were fixed with 3.7% formaldehyde, washed three times with PBS, incubated with 1 mg/ml calcofluor white (Fluorescent brightener 28, Sigma) for 5 min, and then washed three times with PBS before imaging. For fluorescence microscopy images, unless otherwise noted a maximum-intensity projection of a Z-stack is shown.

For transmission electron microscopy cells were released from a metaphase arrest, and samples from 20 and 30 min after release were pooled and fixed with 2.5% glutaraldehyde for 1 hr, washed twice with water, resuspended in 4% potassium permanganate, washed twice with water, and incubated with 7% uranylacetate for 30 min, followed by dehydration and embedding, as described previously (Meitinger et al., 2011).

3.6 Acknowledgments

The *bem2-ts* allele was isolated by Koji Saito while in Daniel Lew's laboratory (Duke University). I thank Franz Meitinger and Gislene Pereira (DKFZ, Heidelberg) for TEM advice and protocols, and Elizabeth Benecchi (HMS EM Core) for TEM expertise and technical assistance. I appreciate the advice and discussions of past and current

members of the Pellman laboratory, including S.Yoshida, K. Kono, S. Buttery, X. Su, and K. Crasta. I am also grateful to E. Bi, F. Uhlmann, J. Heinisch, Y. Zheng, S. Valentini, M. Peter, and G. Fink for reagents and M. Onishi and J. Pringle (Stanford) for communicating results prior to publication.

3.7 References

Amon, A. 2002. Synchronization procedures. *Methods Enzymol*. 351:457-467.

Atkins, B.D., S. Yoshida, K. Saito, C.F. Wu, D.J. Lew, and D. Pellman. 2013. Inhibition of Cdc42 during mitotic exit is required for cytokinesis. *J Cell Biol*. 202:231-240.

Bastos, R.N., X. Penate, M. Bates, D. Hammond, and F.A. Barr. 2012. CYK4 inhibits Rac1-dependent PAK1 and ARHGEF7 effector pathways during cytokinesis. *J Cell Biol*. 198:865-880.

Bi, E., P. Maddox, D.J. Lew, E.D. Salmon, J.N. McMillan, E. Yeh, and J.R. Pringle. 1998. Involvement of an actomyosin contractile ring in Saccharomyces cerevisiae cytokinesis. *J Cell Biol*. 142:1301-1312.

Bi, E., and H.O. Park. 2012. Cell polarization and cytokinesis in budding yeast. *Genetics*. 191:347-387.

Bohnert, K.A., and K.L. Gould. 2012. Cytokinesis-based constraints on polarized cell growth in fission yeast. *PLoS Genet*. 8:e1003004.

Boulter, E., R. Garcia-Mata, C. Guilluy, A. Dubash, G. Rossi, P.J. Brennwald, and K. Burridge. 2010. Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. *Nat Cell Biol*. 12:477-483.

Boureux, A., E. Vignal, S. Faure, and P. Fort. 2007. Evolution of the Rho family of ras-like GTPases in eukaryotes. *Mol Biol Evol*. 24:203-216.

Cabib, E., and J. Arroyo. 2013. How carbohydrates sculpt cells: chemical control of morphogenesis in the yeast cell wall. *Nature reviews. Microbiology*. 11:648-655.

Cabib, E., R. Ulane, and B. Bowers. 1974. A molecular model for morphogenesis: the primary septum of yeast. *Curr Top Cell Regul*. 8:1-32.

Canman, J.C., L. Lewellyn, K. Laband, S.J. Smerdon, A. Desai, B. Bowerman, and K. Oegema. 2008. Inhibition of Rac by the GAP activity of centralspindlin is essential for cytokinesis. *Science*. 322:1543-1546.

Caviston, J.P., S.E. Tcheperegine, and E. Bi. 2002. Singularity in budding: a role for the evolutionarily conserved small GTPase Cdc42p. *Proc Natl Acad Sci U S A*. 99:12185-12190.

Chuang, J.S., and R.W. Schekman. 1996. Differential trafficking and timed localization of two chitin synthase proteins, Chs2p and Chs3p. *J Cell Biol*. 135:597-610.

Cid, V.J., R. Cenamor, M. Sanchez, and C. Nombela. 1998. A mutation in the Rho1-GAP-encoding gene BEM2 of Saccharomyces cerevisiae affects morphogenesis and cell wall functionality. *Microbiology*. 144 (Pt 1):25-36.

Denis, V., and M.S. Cyert. 2005. Molecular analysis reveals localization of Saccharomyces cerevisiae protein kinase C to sites of polarized growth and Pkc1p targeting to the nucleus and mitotic spindle. *Eukaryot Cell*. 4:36-45.

Devrekanli, A., M. Foltman, C. Roncero, A. Sanchez-Diaz, and K. Labib. 2012. Inn1 and Cyk3 regulate chitin synthase during cytokinesis in budding yeasts. *J Cell Sci*. 125:5453-5466.

Dobbelaere, J., M.S. Gentry, R.L. Hallberg, and Y. Barral. 2003. Phosphorylation-dependent regulation of septin dynamics during the cell cycle. *Dev Cell*. 4:345-357.

Epp, J.A., and J. Chant. 1997. An IQGAP-related protein controls actin-ring formation and cytokinesis in yeast. *Curr Biol*. 7:921-929.

Fang, X., J. Luo, R. Nishihama, C. Wloka, C. Dravis, M. Travaglia, M. Iwase, E.A. Vallen, and E. Bi. 2010. Biphasic targeting and cleavage furrow ingression directed by the tail of a myosin II. *J Cell Biol*. 191:1333-1350.

Gandhi, M., B.L. Goode, and C.S. Chan. 2006. Four novel suppressors of gic1 gic2 and their roles in cytokinesis and polarized cell growth in Saccharomyces cerevisiae. *Genetics*. 174:665-678.

Gao, X.D., J.P. Caviston, S.E. Tcheperegine, and E. Bi. 2004. Pxl1p, a paxillin-like protein in Saccharomyces cerevisiae, may coordinate Cdc42p and Rho1p functions during polarized growth. *Mol Biol Cell*. 15:3977-3985.

Gladfelter, A.S., I. Bose, T.R. Zyla, E.S. Bardes, and D.J. Lew. 2002. Septin ring assembly involves cycles of GTP loading and hydrolysis by Cdc42p. *J Cell Biol*. 156:315-326.

Green, R.A., E. Paluch, and K. Oegema. 2012. Cytokinesis in animal cells. *Annu Rev Cell Dev Biol*. 28:29-58.

Hartwell, L.H. 1971. Genetic control of the cell division cycle in yeast. IV. Genes controlling bud emergence and cytokinesis. *Exp Cell Res*. 69:265-276.

Hwang, H.Y., S.K. Olson, J.D. Esko, and H.R. Horvitz. 2003. Caenorhabditis elegans early embryogenesis and vulval morphogenesis require chondroitin biosynthesis. *Nature*. 423:439-443.

Irazoqui, J.E., A.S. Gladfelter, and D.J. Lew. 2003. Scaffold-mediated symmetry breaking by Cdc42p. *Nat Cell Biol*. 5:1062-1070.

Iwase, M., J. Luo, S. Nagaraj, M. Longtine, H.B. Kim, B.K. Haarer, C. Caruso, Z. Tong, J.R. Pringle, and E. Bi. 2006. Role of a Cdc42p effector pathway in recruitment of the yeast septins to the presumptive bud site. *Mol Biol Cell*. 17:1110-1125.

Jendretzki, A., I. Ciklic, R. Rodicio, H.P. Schmitz, and J.J. Heinisch. 2009. Cyk3 acts in actomyosin ring independent cytokinesis by recruiting Inn1 to the yeast bud neck. *Mol Genet Genomics*. 282:437-451.

Jordan, S.N., and J.C. Canman. 2012. Rho GTPases in animal cell cytokinesis: An occupation by the one percent. *Cytoskeleton (Hoboken)*.

Kim, Y.J., L. Francisco, G.C. Chen, E. Marcotte, and C.S. Chan. 1994. Control of cellular morphogenesis by the Ip12/Bem2 GTPase-activating protein: possible role of protein phosphorylation. *J Cell Biol*. 127:1381-1394.

Knaus, M., M.P. Pelli-Gulli, F. van Drogen, S. Springer, M. Jaquenoud, and M. Peter. 2007. Phosphorylation of Bem2p and Bem3p may contribute to local activation of Cdc42p at bud emergence. *EMBO J*. 26:4501-4513.

Ko, N., R. Nishihama, G.H. Tully, D. Ostapenko, M.J. Solomon, D.O. Morgan, and J.R. Pringle. 2007. Identification of yeast IQGAP (Iqg1p) as an anaphase-promoting-complex substrate and its role in actomyosin-ring-independent cytokinesis. *Mol Biol Cell*. 18:5139-5153.

Kono, K., Y. Saeki, S. Yoshida, K. Tanaka, and D. Pellman. 2012. Proteasomal degradation resolves competition between cell polarization and cellular wound healing. *Cell*. 150:151-164.

Korinek, W.S., E. Bi, J.A. Epp, L. Wang, J. Ho, and J. Chant. 2000. Cyk3, a novel SH3-domain protein, affects cytokinesis in yeast. *Curr Biol*. 10:947-950.

Laporte, D., V.C. Coffman, I.J. Lee, and J.Q. Wu. 2011. Assembly and architecture of precursor nodes during fission yeast cytokinesis. *J Cell Biol*. 192:1005-1021.

Levin, D.E. 2011. Regulation of cell wall biogenesis in Saccharomyces cerevisiae: the cell wall integrity signaling pathway. *Genetics*. 189:1145-1175.

Lippincott, J., and R. Li. 1998. Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *J Cell Biol*. 140:355-366.

Longtine, M.S., C.L. Theesfeld, J.N. McMillan, E. Weaver, J.R. Pringle, and D.J. Lew. 2000. Septin-dependent assembly of a cell cycle-regulatory module in Saccharomyces cerevisiae. *Mol Cell Biol*. 20:4049-4061.

Loo, T.H., and M. Balasubramanian. 2008. Schizosaccharomyces pombe Pak-related protein, Pak1p/Orb2p, phosphorylates myosin regulatory light chain to inhibit cytokinesis. *J Cell Biol*. 183:785-793.

Lord, M., E. Laves, and T.D. Pollard. 2005. Cytokinesis depends on the motor domains of myosin-II in fission yeast but not in budding yeast. *Mol Biol Cell*. 16:5346-5355.

Luo, J., E.A. Vallen, C. Dravis, S.E. Tcheperegine, B. Drees, and E. Bi. 2004. Identification and functional analysis of the essential and regulatory light chains of the only type II myosin Myo1p in Saccharomyces cerevisiae. *J Cell Biol*. 165:843-855.

Marquitz, A.R., J.C. Harrison, I. Bose, T.R. Zyla, J.N. McMillan, and D.J. Lew. 2002. The Rho-GAP Bem2p plays a GAP-independent role in the morphogenesis checkpoint. *EMBO J*. 21:4012-4025.

Meitinger, F., B. Petrova, I.M. Lombardi, D.T. Bertazzi, B. Hub, H. Zentgraf, and G. Pereira. 2011. Targeted localization of Inn1, Cyk3 and Chs2 by the mitotic-exit network regulates cytokinesis in budding yeast. *J Cell Sci*. 123:1851-1861.

Meitinger, F., H. Richter, S. Heisel, B. Hub, W. Seufert, and G. Pereira. 2013. A safeguard mechanism regulates Rho GTPases to coordinate cytokinesis with the establishment of cell polarity. *PLoS Biol*. 11:e1001495.

Mizuguchi, S., T. Uyama, H. Kitagawa, K.H. Nomura, K. Dejima, K. Gengyo-Ando, S. Mitani, K. Sugahara, and K. Nomura. 2003. Chondroitin proteoglycans are involved in cell division of Caenorhabditis elegans. *Nature*. 423:443-448.

Moskow, J.J., A.S. Gladfelter, R.E. Lamson, P.M. Pryciak, and D.J. Lew. 2000. Role of Cdc42p in pheromone-stimulated signal transduction in Saccharomyces cerevisiae. *Mol Cell Biol*. 20:7559-7571.

Nishihama, R., J.H. Schreiter, M. Onishi, E.A. Vallen, J. Hanna, K. Moravcevic, M.F. Lippincott, H. Han, M.A. Lemmon, J.R. Pringle, and E. Bi. 2009. Role of Inn1 and its interactions with Hof1 and Cyk3 in promoting cleavage furrow and septum formation in S. cerevisiae. *J Cell Biol*. 185:995-1012.

Okada, S., M. Leda, J. Hanna, N.S. Savage, E. Bi, and A.B. Goryachev. 2013. Daughter cell identity emerges from the interplay of Cdc42, septins, and exocytosis. *Dev Cell*. 26:148-161.

Onishi, M., N. Ko, R. Nishihama, and J.R. Pringle. 2013. Distinct roles of Rho1, Cdc42, and Cyk3 in septum formation and abscission during yeast cytokinesis. *J Cell Biol*. 202:311-329.

Osman, M.A., and R.A. Cerione. 1998. Iqg1p, a yeast homologue of the mammalian IQGAPs, mediates cdc42p effects on the actin cytoskeleton. *J Cell Biol*. 142:443-455.

Peterson, J., Y. Zheng, L. Bender, A. Myers, R. Cerione, and A. Bender. 1994. Interactions between the bud emergence proteins Bem1p and Bem2p and Rho-type GTPases in yeast. *J Cell Biol*. 127:1395-1406.

Petronczki, M., P. Lenart, and J.M. Peters. 2008. Polo on the Rise-from Mitotic Entry to Cytokinesis with Plk1. *Dev Cell*. 14:646-659.

Pollard, T.D., and J.Q. Wu. 2010. Understanding cytokinesis: lessons from fission yeast. *Nat Rev Mol Cell Biol*. 11:149-155.

Pringle, J.R. 1991. Staining of bud scars and other cell wall chitin with calcofluor. *Methods Enzymol*. 194:732-735.

Ray, S., K. Kume, S. Gupta, W. Ge, M. Balasubramanian, D. Hirata, and D. McCollum. 2010. The mitosis-to-interphase transition is coordinated by cross talk between the SIN and MOR pathways in Schizosaccharomyces pombe. *J Cell Biol*. 190:793-805.

Sanchez-Diaz, A., V. Marchesi, S. Murray, R. Jones, G. Pereira, R. Edmondson, T. Allen, and K. Labib. 2008. Inn1 couples contraction of the actomyosin ring to membrane ingression during cytokinesis in budding yeast. *Nat Cell Biol*. 10:395-406.

Sanchez-Diaz, A., P.J. Nkosi, S. Murray, and K. Labib. 2012. The Mitotic Exit Network and Cdc14 phosphatase initiate cytokinesis by counteracting CDK phosphorylations and blocking polarised growth. *EMBO J*. 31:3620-3634.

Schmidt, A., T. Schmelzle, and M.N. Hall. 2002a. The RHO1-GAPs SAC7, BEM2 and BAG7 control distinct RHO1 functions in Saccharomyces cerevisiae. *Mol Microbiol*. 45:1433-1441.

Schmidt, M., B. Bowers, A. Varma, D.H. Roh, and E. Cabib. 2002b. In budding yeast, contraction of the actomyosin ring and formation of the primary septum at cytokinesis depend on each other. *J Cell Sci*. 115:293-302.

Shannon, K.B. 2012. IQGAP Family Members in Yeast, Dictyostelium, and Mammalian Cells. *International journal of cell biology*. 2012:894817.

Shannon, K.B., and R. Li. 1999. The multiple roles of Cyk1p in the assembly and function of the actomyosin ring in budding yeast. *Mol Biol Cell*. 10:283-296.

Shaw, J.A., P.C. Mol, B. Bowers, S.J. Silverman, M.H. Valdivieso, A. Duran, and E. Cabib. 1991. The function of chitin synthases 2 and 3 in the Saccharomyces cerevisiae cell cycle. *J Cell Biol*. 114:111-123.

Sherman, F. 1991. Getting started with yeast. *Methods Enzymol*. 194:3-21.

Tolliday, N., L. VerPlank, and R. Li. 2002. Rho1 directs formin-mediated actin ring assembly during budding yeast cytokinesis. *Curr Biol*. 12:1864-1870.

Tong, Z., X.D. Gao, A.S. Howell, I. Bose, D.J. Lew, and E. Bi. 2007. Adjacent positioning of cellular structures enabled by a Cdc42 GTPase-activating protein-mediated zone of inhibition. *J Cell Biol*. 179:1375-1384.

Tully, G.H., R. Nishihama, J.R. Pringle, and D.O. Morgan. 2009. The anaphase-promoting complex promotes actomyosin-ring disassembly during cytokinesis in yeast. *Mol Biol Cell*. 20:1201-1212.

Vallen, E.A., J. Caviston, and E. Bi. 2000. Roles of Hof1p, Bni1p, Bnr1p, and myo1p in cytokinesis in Saccharomyces cerevisiae. *Mol Biol Cell*. 11:593-611.

VerPlank, L., and R. Li. 2005. Cell cycle-regulated trafficking of Chs2 controls actomyosin ring stability during cytokinesis. *Mol Biol Cell*. 16:2529-2543.

Versele, M., and J. Thorner. 2004. Septin collar formation in budding yeast requires GTP binding and direct phosphorylation by the PAK, Cla4. *J Cell Biol*. 164:701-715.

Wang, T., and A. Bretscher. 1995. The rho-GAP encoded by BEM2 regulates cytoskeletal structure in budding yeast. *Mol Biol Cell*. 6:1011-1024.

Weiss, E.L. 2012. Mitotic exit and separation of mother and daughter cells. *Genetics*. 192:1165-1202.

Xu, X., and B.E. Vogel. 2011. A secreted protein promotes cleavage furrow maturation during cytokinesis. *Curr Biol*. 21:114-119.

Yoshida, S., S. Bartolini, and D. Pellman. 2009. Mechanisms for concentrating Rho1 during cytokinesis. *Genes Dev*. 23:810-823.

Yoshida, S., K. Kono, D.M. Lowery, S. Bartolini, M.B. Yaffe, Y. Ohya, and D. Pellman. 2006. Polo-like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. *Science*. 313:108-111.

Zhang, G., R. Kashimshetty, K.E. Ng, H.B. Tan, and F.M. Yeong. 2006. Exit from mitosis triggers Chs2p transport from the endoplasmic reticulum to mother-daughter neck via the secretory pathway in budding yeast. *J Cell Biol*. 174:207-220.

Chapter 4

Conclusions and future directions

4.1 Cdc42 activity changes across the cell cycle **123**
4.1.1 Upstream cell cycle regulation of Cdc42 GTP-loading at G_1/S *123*
4.1.2 What is the function of active Cdc42 during anaphase? *124*

4.2 Cdc5/Polo inhibits Cdc42 activity during mitotic exit **125**
4.2.1 Regulation of Cdc42-GAPs Bem2 and Bem3 *125*
4.2.2 Other potential targets of Cdc5/Polo kinase during mitotic exit *126*

4.3 Cdc42 inhibits cytokinesis and cell separation in *S. cerevisiae* **128**
4.3.1 How does Cdc42 affect Iqg1 during cytokinesis? *128*
4.3.2 Targets of Ste20 during cytokinesis *130*
4.3.3 Potential connection of the 'NoCut' pathway to Cdc42 *132*

4.4 Final Conclusions **132**

4.5 References **133**

4.1 Cdc42 activity changes across the cell cycle

In Chapter 2 I presented data that Cdc42 undergoes changes in GTP-loading and local activation as *Saccharomyces cerevisiae* cells traverse the cell division cycle, with peaks at G_1/S and at anaphase and troughs during G_2, cytokinesis, and early G_1 (Fig 2-2). Furthermore, and consistent with previous studies that Cdc42 activity needs to be suppressed at the bud neck to 'clear the slate' after cytokinesis for normal bud site selection (Meitinger et al., 2013; Tong et al., 2007), our data indicate that Cdc42 activity at the bud neck during cytokinesis is markedly reduced compared to at the budding site during polarization in the next cell cycle (Fig 2-3). Overall, these data indicate that Cdc42 activation is under tight control by the cell cycle machinery in order to promote actin polarization at the correct site and at the correct time. These changes in Cdc42 activity are functionally critical for normal cell division, as demonstrated by the cytokinesis defects caused by persistent and inappropriate Cdc42 activation during mitotic exit (Chapter 3). While the current work has identified the major changes in Cdc42 GTP-loading across the cell cycle, it also raises new and interesting questions about how Cdc42 activity is regulated in time and space.

4.1.1 Upstream cell cycle regulation of Cdc42 GTP-loading at G_1/S

The CRIB pulldown assay I developed to measure Cdc42 GTP-loading from budding yeast lysates could potentially shed light on several interesting questions. While it is widely assumed that late G_1 cyclin/Cdk1 activity controls Cdc42 activation and cell polarization, this could be formally tested by measuring Cdc42 GTP-loading in synchronized *cdk1-as* analog-sensitive mutants, where kinase activity is blocked by

addition of an ATP analog (Bishop et al., 2000). The CRIB pulldown assay could also be useful in future studies examining the effect of Cdk1-dependent phosphorylation of cell polarity regulators, in particular of the Cdc42-GEF Cdc24 and Cdc42-GAPs. So far no obvious phenotypes have been observed when known or predicted Cdk1 sites in these proteins (such as Cdc24, Rga2, and Bem2) have been mutated *in vivo*, likely due to redundancy, but perhaps combining these mutant alleles in a single strain would cause detectable differences in Cdc42-GTP loading at the G_1/S transition (Knaus et al., 2007; McCusker et al., 2007; Sopko et al., 2007; Wai et al., 2009). Overall, the GST-CRIB pulldown assay should prove to be a useful tool to assess how Cdc42 GTP-loading is regulated not only during cell polarization but also in other contexts, including mating and stress responses.

4.1.2 What is the function of active Cdc42 during anaphase?

Other questions raised by this work regard the Cdc42 activation that I observed during anaphase (Fig 2-2C). The first question is which cell cycle regulators are involved. Mitotic Clb/Cdk1 is one cell cycle regulator that retains activity at early anaphase (Rahal and Amon, 2008). Since mitotic Clb/Cdk1 complexes are generally thought to inhibit polarization, it is non-intuitive that they would promote Cdc42 GTP-loading (Lew and Reed, 1993). One possibility is that Clb/Cdk1 promotes a short burst of GTP-loading (or inhibition of GAP activity) across the entire cell cortex, which would be consistent with the complete depolarization of actin patches and cables that occurs prior to mitotic exit (Bi and Park, 2012). Alternatively, other cell cycle regulators or pathways, such as the

Cdc14 early anaphase release (FEAR) network, could promote Cdc42 activity during anaphase, either directly or indirectly.

The function of Cdc42 during anaphase remains to be elucidated. While genetic data indicate that Cdc42 promotes the Mitotic Exit Network (MEN) through several effector proteins, including Cla4 and Ste20, so far it is unclear whether Cdc42 activation during mitosis is required for normal functioning of this pathway. In support of an active role for Cdc42 during anaphase, the kinase activity of the Cdc42 effector PAK kinase Cla4 peaks during late mitosis (Benton et al., 1997). On the other hand, while cells that lack MEN components arrest in late anaphase, cells that lack the function of Cdc42 or the GEF Cdc24 arrest as unbudded cells, so it is likely that Cdc42 plays at most a contributory or fine-tuning role to promote MEN signaling during mitosis (Adams et al., 1990; Adams and Pringle, 1984; Iwase et al., 2006; Johnson and Pringle, 1990; Stegmeier and Amon, 2004; Tolliday et al., 2002). The role of Cdc42 in MEN signaling during mitosis could be tested using *GALL-CDC20* synchronization of tight *cdc42*-ts mutants or using the Cdc42 temperature-degron mutant and monitoring cell cycle progression and Cdc14 release from the nucleolus as readouts for the Mitotic Exit Network (Iwase et al., 2006; Lu and Cross, 2009).

4.2 Cdc5/Polo inhibits Cdc42 activity during mitotic exit

4.2.1 Regulation of Cdc42-GAPs Bem2 and Bem3

My data indicate that the conserved mitotic regulator Cdc5/Polo kinase is one upstream inhibitor of Cdc42 GTP-loading during mitotic exit, likely acting through the Cdc42-GAP proteins Bem2 and Bem3 (Fig 2-4). Understanding the exact mechanism by

which Cdc5/Polo controls Cdc42 inactivation will be an interesting topic. By analogy to previous work from the Pellman laboratory investigating the Cdc5/Polo-dependent regulation of Rho1-GEFs, mass spectrometry could identify the exact Polo-dependent phosphorylation sites in both of these proteins (Dephoure et al., 2012; Yoshida et al., 2006). The large (approximately 250 kD) size of Bem2 may impede this effort, but since Bem2 only has one canonical Polo-box binding motif (S[S/T]P), an alternative approach would be to construct a mutant that is defective for binding to Cdc5/Polo similar to the *bem3-ST* mutant described in Chapter 2 (Elia et al., 2003a; Elia et al., 2003b; Yoshida et al., 2006). Subsequent experiments could determine the localization, stability, and activity of phosphosite mutants, along with whether the mutants possess any polarization or cytokinesis defects. Overall, based on our model I predict that Cdc5/Polo-dependent phosphorylation will promote the function of Bem2 and Bem3 to downregulate Cdc42 during mitotic exit.

4.2.2 Other potential targets of Cdc5/Polo kinase during mitotic exit

While so far only the Rho-family GEFs and GAPs have been screened for binding to the Polo-box domain of Cdc5 (Yoshida et al., 2006), it is possible that other Cdc42 regulators or downstream effectors could be targets of Cdc5/Polo to inhibit Cdc42 activation and downstream signaling during mitotic exit and cytokinesis. Bem1, the scaffold protein proposed to be involved in an autocatalytic positive feedback of Cdc42 activation during symmetry breaking (Kozubowski et al., 2008), is one attractive target that is known to undergo Cdk1-dependent phosphorylation *in vitro* and *in vivo*, which often primes for further phosphorylation by Cdc5/Polo (Elia et al., 2003a; Elia et al.,

2003b; Han et al., 2005; Ubersax et al., 2003). Bem1 contains three canonical Polo-box binding motifs in its N-terminus (Yeast Genome Database). In a simple scenario, Cdc5/Polo phosphorylation could lead to disruption of the tri-partite Bem1-PAK-GEF complex. Consistent with this notion, expression of an artificial fusion of Cdc24 to the PAK kinase Cla4 to bypass Bem1 led to hyperpolarized growth in approximately 10% of cells, suggesting that this complex could interfere with normal actin depolarization during mitosis (Kozubowski et al., 2008).

Another appealing potential target of Cdc5/Polo is Gps1, which sustains Rho1 localization but inhibits Cdc42-dependent polarization at the division site during the M-to-G_1 transition (Meitinger et al., 2013). Although the phosphorylation state of Gps1 is unknown, the protein does contain two canonical Polo-box binding motifs (Yeast Genome Database). The exact function of Gps1 is unclear, but it appears to interact directly with Cdc42, and in the absence of Gps1 cells polarize Cdc42 and rebud within the old division site following cytokinesis, leading to cell lysis (Meitinger et al., 2013). Therefore, the hypothetical Cdc5/Polo-dependent regulation may affect the ability of Gps1 to interact with Cdc42 or otherwise promote its function.

Another plausible approach to inactivate Cdc42 during mitotic exit is to increase the activity of the Rho-GDI Rdi1, which is expected to reduce the amount of Cdc42 able to be activated at the plasma membrane *via* sequestration in the cytoplasm. One way to test this idea is to determine whether the membrane association of Cdc42 in lysates from synchronized cell populations changes during mitotic exit and cytokinesis and then determining whether any changes depend on Cdc5/Polo (Tiedje et al., 2008). This experiment could also address whether Rdi1 activity changes during polarization at G_1/S,

where Rdi1 has been proposed to play a role in Cdc42 clustering and recycling at the presumptive bud site (Freisinger et al., 2013; Slaughter et al., 2009).

If none of these proteins are involved in inhibition of Cdc42 activity during mitotic exit, another way to address this question is to take an unbiased approach, such as by comparing the phospho-proteomes of *cdc5-2* cells to *cdc15-2* cells that contain high Cdc5/Polo activity by stable isotope labeling by amino acids in cell culture (SILAC) or other mass spectrometry-based approaches (Wu et al., 2011). Since both mutants arrest at a similar point in the cell cycle, proteins that exhibit decreased phosphorylation in *cdc5-2* mutants are potential Cdc5 substrates. Proteins involved in cell polarization or Rho GTPase regulation identified by this approach would be strong candidates for further testing.

4.3 Cdc42 inhibits cytokinesis and cell separation in *S. cerevisiae*

As presented in Chapter 3, I found that Cdc42 activation impairs the growth of cytokinesis mutants, especially in cells that lack a contractile actin ring and thus rely on septum formation for survival (Fig 3-1). Importantly, failure to inactivate Cdc42 during cytokinesis causes a defect in septum formation (Fig 3-7) and cell separation (Fig 3-2, 3-3) that requires the PAK kinase Ste20 (Fig 3-9B) and appears to involve mislocalization of the septum formation proteins Iqg1 and Inn1 (Fig 3-8). These findings open up a number of future avenues for research to understand how Cdc42 interferes with cytokinesis.

4.3.1 How does Cdc42 affect Iqg1 during cytokinesis?

I found that the IQGAP homologue Iqg1 had a partial localization defect during cytokinesis in *bem2-ts* cells (Fig 3-8A,C). Several important outstanding questions remain regarding how Cdc42 affects Iqg1 during cytokinesis. The first question is the nature of the localization defect. Because my experiment analyzed fixed cells released from a metaphase arrest, it is not clear if the localization defect and decreased fluorescence intensity during cytokinesis is an issue of mislocalization to a cytoplasmic pool, premature contraction of the ring, or premature degradation of Iqg1 (Ko et al., 2007). These questions could be addressed by live-cell fluorescence microscopy of synchronized *MET3-CDC20 SHS1-mCherry IQG1-GFP bem2-ts* cells using a microfluidic chamber that can maintain the cells at the restrictive temperature of 37°C, along with Western blotting to assess Iqg1 protein levels.

Another question is whether active Cdc42 directly interacts with Iqg1 to cause the inefficient localization during cytokinesis *via* sequestration. At present the physical interaction between GTP-Cdc42 and Iqg1 is not clear; by yeast two-hybrid Iqg1 interacts with GTP-Cdc42 but not GDP-Cdc42, while so far only very weak protein binding interactions have been observed (Osman and Cerione, 1998; Shannon and Li, 1999). A first step would be to test whether GTP-locked GST-Cdc42^{Q61L} is able to pull down Iqg1 from yeast cell lysates to a greater extent than GDP-locked GST-Cdc42^{T17N}. If an interaction were detected, then it would be interesting to test whether mutational disruption of the interaction with Cdc42 can suppress the cell separation defect of *bem2-ts* cells. Consistent with a possible sequestration of Iqg1 by active GTP-Cdc42, my preliminary unpublished data suggests that Iqg1-GFP can localize ectopically to the tips of polarized bud projections that form after extended incubation of *cdc5-2* mutants at the

restrictive temperature. These polarized projections depend on Cdc42, and GTP-Cdc42 is enriched at their tips (S. Yoshida and D. Pellman, unpublished data).

4.3.2 Targets of Ste20 during cytokinesis

The PAK kinase Ste20 is required for the cell separation defect caused by Cdc42 activation in *bem2-ts* cells (Fig 3-9B). Taken together with previous work in fission yeast and mammalian cells, this finding strongly suggests that PAK kinases are evolutionarily conserved negative regulators of cytokinesis (Bastos et al., 2012; Loo and Balasubramanian, 2008). In fission yeast, the PAK homolog Pak1/Orb2 phosphorylates the myosin II regulatory light chain Rlc1 at two residues, which appears to inhibit premature contractile actin ring (CAR) constriction especially in conditions where anaphase is delayed (Loo and Balasubramanian, 2008). The budding yeast homolog Mlc2 does not play a major role in CAR contraction but instead functions in CAR disassembly, so it is unclear if a similar mechanism would operate in budding yeast (Luo et al., 2004). In mammalian cells, inhibition of PAK1 is proposed to limit inappropriate adhesion at the cell division site, although the exact target is not clear (Bastos et al., 2012). Therefore, understanding how Ste20 interferes with cytokinesis will be an interesting future direction of research.

Despite many years of study, only a few *bona fide* Ste20 substrates are known. Ste20 has a well-known role as an upstream activating kinase for several MAPK pathways, including the mating, pseudohyphal growth, and high osmolarity glycerol (HOG) pathways (Cullen and Sprague, 2012; Dohlman and Slessareva, 2006; Saito and Posas, 2012). While these pathways are unlikely to inhibit cytokinesis, deletion of

downstream kinases could be tested for suppression of the cell separation defect of *bem2-ts* cells. The HOG pathway is especially unlikely to inhibit cytokinesis, as the upstream osmosensor Sho1 was recently reported to be involved in normal septum formation (Labedzka et al., 2012). Another clear substrate is the type I myosin Myo3, which activates the Arp2/3 complex that is required for actin nucleation in actin patches (Lechler et al., 2000; Wu et al., 1997). The non-phosphorylatable *myo3-S357A* mutant could be tested for suppression of the *bem2-ts* cell separation defect.

A targeted *in vitro* screen identified a modest number of candidate Ste20 substrates, including the formin Bni1 and the polarisome component Bud6, and Ste20 is partially required for Bni1 phosphorylation *in vivo* (Annan et al., 2009; Goehring et al., 2003). Only a few proteins related to polarization (Bem1, Boi1), exocytosis (Sec2, Exo84, Sro7), and cell wall integrity (Ssd1) were identified as potential Ste20 targets in a large scale *in vitro* screen for kinase substrates (Ptacek et al., 2005). While these proteins are candidates to be involved in Ste20's inhibition of cytokinesis, it would also be interesting to define novel *in vivo* substrates of Ste20, perhaps by constructing an analog-sensitive allele of Ste20 and performing quantitative phosphoproteomic analysis after inhibition, similar to the approach taken by the Morgan lab to identify *in vivo* Cdk1 phosphorylation sites (Holt et al., 2009). A parallel screen using the analog-sensitive *cla4-as* mutant could be used to subtract out targets of the partially overlapping PAK kinase Cla4 (Weiss et al., 2000). This screen could identify Ste20 targets involved in cytokinesis, but could also identify targets involved in mitotic exit, which remain unknown (Hofken and Schiebel, 2002; Reiser et al., 2006). Overall, understanding the role of Ste20 in cytokinesis may require broadening our list of Ste20 substrates.

4.3.3 Potential connection of the 'NoCut' pathway to Cdc42

The 'NoCut' pathway is a signaling network involving the Ipl1/Aurora B kinase that is proposed to inhibit abscission in response to spindle defects or the presence of chromatin across the bud neck (Mendoza et al., 2009; Norden et al., 2006). Interestingly, the most downstream effectors implicated in the process are the polarity scaffolds Boi1 and Boi2, which were compared by the authors to anillin due to the presence of a PH-domain and the fact that they have multiple binding partners (Norden et al., 2006).

Based on these reports, and the fact that Boi1/Boi2 are considered effectors of Cdc42 during cell polarization (Bender et al., 1996; Matsui et al., 1996; McCusker et al., 2007), it would be interesting to test whether manipulations that cause the NoCut-dependent abscission delay affect Cdc42 activation. Future experiments could also test whether Boi1/2 are required for the cell separation defect of *bem2-ts* cells. Since a similar Aurora B-dependent pathway operates in mammalian cells to delay abscission, understanding whether the ability of Cdc42 to interfere with or delay cytokinesis is exploited by cells under conditions where cytokinesis could be deleterious would be of broad interest (Steigemann et al., 2009).

4.4 Final Conclusions

Overall, this work shows that Cdc42 activity changes across the *Saccharomyces cerevisiae* cell cycle, with peaks at G_1/S and anaphase, and troughs during G_2 and cytokinesis/early G_1. In particular, Cdc42 activity is suppressed at the bud neck during cytokinesis compared to at the polarization site during G_1/S. The conserved mitotic

regulator Cdc5/Polo kinase is one upstream negative regulator of Cdc42 activity, which likely functions through Cdc42-GAPs. Failure to inactivate Cdc42 during cytokinesis causes septum formation and cell separation defects, likely due to partial mislocalization of two proteins required for normal septum formation, the IQGAP homolog Iqg1 and Inn1. The PAK kinase Ste20 is required to mediate Cdc42's inhibitory effect on cytokinesis. Overall, this work not only identified the major changes in Cdc42 activity across the cell cycle but also identified a novel inhibitory effect of Cdc42 during cytokinesis in *S. cerevisiae*. I anticipate that many of the elements of regulation described in this work could be conserved in other systems.

4.5 References

Adams, A.E., D.I. Johnson, R.M. Longnecker, B.F. Sloat, and J.R. Pringle. 1990. CDC42 and CDC43, two additional genes involved in budding and the establishment of cell polarity in the yeast Saccharomyces cerevisiae. *J Cell Biol*. 111:131-142.

Adams, A.E., and J.R. Pringle. 1984. Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant Saccharomyces cerevisiae. *J Cell Biol*. 98:934-945.

Annan, R.B., A.Y. Lee, I.D. Reid, A. Sayad, M. Whiteway, M. Hallett, and D.Y. Thomas. 2009. A biochemical genomics screen for substrates of Ste20p kinase enables the in silico prediction of novel substrates. *PLoS One*. 4:e8279.

Bastos, R.N., X. Penate, M. Bates, D. Hammond, and F.A. Barr. 2012. CYK4 inhibits Rac1-dependent PAK1 and ARHGEF7 effector pathways during cytokinesis. *J Cell Biol*. 198:865-880.

Bender, L., H.S. Lo, H. Lee, V. Kokojan, V. Peterson, and A. Bender. 1996. Associations among PH and SH3 domain-containing proteins and Rho-type GTPases in Yeast. *J Cell Biol*. 133:879-894.

Benton, B.K., A. Tinkelenberg, I. Gonzalez, and F.R. Cross. 1997. Cla4p, a Saccharomyces cerevisiae Cdc42p-activated kinase involved in cytokinesis, is activated at mitosis. *Mol Cell Biol*. 17:5067-5076.

Bi, E., and H.O. Park. 2012. Cell polarization and cytokinesis in budding yeast. *Genetics*. 191:347-387.

Bishop, A.C., J.A. Ubersax, D.T. Petsch, D.P. Matheos, N.S. Gray, J. Blethrow, E. Shimizu, J.Z. Tsien, P.G. Schultz, M.D. Rose, J.L. Wood, D.O. Morgan, and K.M. Shokat. 2000. A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature*. 407:395-401.

Cullen, P.J., and G.F. Sprague, Jr. 2012. The regulation of filamentous growth in yeast. *Genetics*. 190:23-49.

Dephoure, N., K.L. Gould, S.P. Gygi, and D.R. Kellogg. 2012. Mapping and analysis of phosphorylation sites: a quick guide for cell biologists. *Mol Biol Cell*. 24:535-542.

Dohlman, H.G., and J.E. Slessareva. 2006. Pheromone signaling pathways in yeast. *Sci STKE*. 2006:cm6.

Elia, A.E., L.C. Cantley, and M.B. Yaffe. 2003a. Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science*. 299:1228-1231.

Elia, A.E., P. Rellos, L.F. Haire, J.W. Chao, F.J. Ivins, K. Hoepker, D. Mohammad, L.C. Cantley, S.J. Smerdon, and M.B. Yaffe. 2003b. The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. *Cell*. 115:83-95.

Freisinger, T., B. Klunder, J. Johnson, N. Muller, G. Pichler, G. Beck, M. Costanzo, C. Boone, R.A. Cerione, E. Frey, and R. Wedlich-Soldner. 2013. Establishment of a robust single axis of cell polarity by coupling multiple positive feedback loops. *Nat Commun*. 4:1807.

Goehring, A.S., D.A. Mitchell, A.H. Tong, M.E. Keniry, C. Boone, and G.F. Sprague, Jr. 2003. Synthetic lethal analysis implicates Ste20p, a p21-activated potein kinase, in polarisome activation. *Mol Biol Cell*. 14:1501-1516.

Han, B.K., L.M. Bogomolnaya, J.M. Totten, H.M. Blank, L.J. Dangott, and M. Polymenis. 2005. Bem1p, a scaffold signaling protein, mediates cyclin-dependent control of vacuolar homeostasis in Saccharomyces cerevisiae. *Genes Dev*. 19:2606-2618.

Hofken, T., and E. Schiebel. 2002. A role for cell polarity proteins in mitotic exit. *EMBO J*. 21:4851-4862.

Holt, L.J., B.B. Tuch, J. Villen, A.D. Johnson, S.P. Gygi, and D.O. Morgan. 2009. Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. *Science*. 325:1682-1686.

Iwase, M., J. Luo, S. Nagaraj, M. Longtine, H.B. Kim, B.K. Haarer, C. Caruso, Z. Tong, J.R. Pringle, and E. Bi. 2006. Role of a Cdc42p effector pathway in recruitment of the yeast septins to the presumptive bud site. *Mol Biol Cell*. 17:1110-1125.

Johnson, D.I., and J.R. Pringle. 1990. Molecular characterization of CDC42, a Saccharomyces cerevisiae gene involved in the development of cell polarity. *J Cell Biol*. 111:143-152.

Knaus, M., M.P. Pelli-Gulli, F. van Drogen, S. Springer, M. Jaquenoud, and M. Peter. 2007. Phosphorylation of Bem2p and Bem3p may contribute to local activation of Cdc42p at bud emergence. *EMBO J*. 26:4501-4513.

Ko, N., R. Nishihama, G.H. Tully, D. Ostapenko, M.J. Solomon, D.O. Morgan, and J.R. Pringle. 2007. Identification of yeast IQGAP (Iqg1p) as an anaphase-promoting-complex substrate and its role in actomyosin-ring-independent cytokinesis. *Mol Biol Cell*. 18:5139-5153.

Kozubowski, L., K. Saito, J.M. Johnson, A.S. Howell, T.R. Zyla, and D.J. Lew. 2008. Symmetry-breaking polarization driven by a Cdc42p GEF-PAK complex. *Curr Biol*. 18:1719-1726.

Labedzka, K., C. Tian, U. Nussbaumer, S. Timmermann, P. Walther, J. Muller, and N. Johnsson. 2012. Sho1p connects the plasma membrane with proteins of the cytokinesis network through multiple isomeric interaction states. *J Cell Sci*. 125:4103-4113.

Lechler, T., A. Shevchenko, and R. Li. 2000. Direct involvement of yeast type I myosins in Cdc42-dependent actin polymerization. *J Cell Biol*. 148:363-373.

Lew, D.J., and S.I. Reed. 1993. Morphogenesis in the yeast cell cycle: regulation by Cdc28 and cyclins. *J Cell Biol*. 120:1305-1320.

Loo, T.H., and M. Balasubramanian. 2008. Schizosaccharomyces pombe Pak-related protein, Pak1p/Orb2p, phosphorylates myosin regulatory light chain to inhibit cytokinesis. *J Cell Biol*. 183:785-793.

Lu, Y., and F. Cross. 2009. Mitotic exit in the absence of separase activity. *Mol Biol Cell*. 20:1576-1591.

Luo, J., E.A. Vallen, C. Dravis, S.E. Tcheperegine, B. Drees, and E. Bi. 2004. Identification and functional analysis of the essential and regulatory light chains of the only type II myosin Myo1p in Saccharomyces cerevisiae. *J Cell Biol*. 165:843-855.

Matsui, Y., R. Matsui, R. Akada, and A. Toh-e. 1996. Yeast src homology region 3 domain-binding proteins involved in bud formation. *J Cell Biol*. 133:865-878.

McCusker, D., C. Denison, S. Anderson, T.A. Egelhofer, J.R. Yates, 3rd, S.P. Gygi, and D.R. Kellogg. 2007. Cdk1 coordinates cell-surface growth with the cell cycle. *Nat Cell Biol*. 9:506-515.

Meitinger, F., H. Richter, S. Heisel, B. Hub, W. Seufert, and G. Pereira. 2013. A safeguard mechanism regulates Rho GTPases to coordinate cytokinesis with the establishment of cell polarity. *PLoS Biol*. 11:e1001495.

Mendoza, M., C. Norden, K. Durrer, H. Rauter, F. Uhlmann, and Y. Barral. 2009. A mechanism for chromosome segregation sensing by the NoCut checkpoint. *Nat Cell Biol*. 11:477-483.

Norden, C., M. Mendoza, J. Dobbelaere, C.V. Kotwaliwale, S. Biggins, and Y. Barral. 2006. The NoCut pathway links completion of cytokinesis to spindle midzone function to prevent chromosome breakage. *Cell*. 125:85-98.

Osman, M.A., and R.A. Cerione. 1998. Iqg1p, a yeast homologue of the mammalian IQGAPs, mediates cdc42p effects on the actin cytoskeleton. *J Cell Biol*. 142:443-455.

Ptacek, J., G. Devgan, G. Michaud, H. Zhu, X. Zhu, J. Fasolo, H. Guo, G. Jona, A. Breitkreutz, R. Sopko, R.R. McCartney, M.C. Schmidt, N. Rachidi, S.J. Lee, A.S. Mah, L. Meng, M.J. Stark, D.F. Stern, C. De Virgilio, M. Tyers, B. Andrews, M. Gerstein, B. Schweitzer, P.F. Predki, and M. Snyder. 2005. Global analysis of protein phosphorylation in yeast. *Nature*. 438:679-684.

Rahal, R., and A. Amon. 2008. Mitotic CDKs control the metaphase-anaphase transition and trigger spindle elongation. *Genes Dev*. 22:1534-1548.

Reiser, V., K.E. D'Aquino, L.S. Ee, and A. Amon. 2006. The stress-activated mitogen-activated protein kinase signaling cascade promotes exit from mitosis. *Mol Biol Cell*. 17:3136-3146.

Saito, H., and F. Posas. 2012. Response to hyperosmotic stress. *Genetics*. 192:289-318.

Shannon, K.B., and R. Li. 1999. The multiple roles of Cyk1p in the assembly and function of the actomyosin ring in budding yeast. *Mol Biol Cell*. 10:283-296.

Slaughter, B.D., A. Das, J.W. Schwartz, B. Rubinstein, and R. Li. 2009. Dual modes of cdc42 recycling fine-tune polarized morphogenesis. *Dev Cell*. 17:823-835.

Sopko, R., D. Huang, J.C. Smith, D. Figeys, and B.J. Andrews. 2007. Activation of the Cdc42p GTPase by cyclin-dependent protein kinases in budding yeast. *EMBO J*. 26:4487-4500.

Stegmeier, F., and A. Amon. 2004. Closing mitosis: the functions of the Cdc14 phosphatase and its regulation. *Annu Rev Genet*. 38:203-232.

Steigemann, P., C. Wurzenberger, M.H. Schmitz, M. Held, J. Guizetti, S. Maar, and D.W. Gerlich. 2009. Aurora B-mediated abscission checkpoint protects against tetraploidization. *Cell*. 136:473-484.

Tiedje, C., I. Sakwa, U. Just, and T. Hofken. 2008. The Rho GDI Rdi1 regulates Rho GTPases by distinct mechanisms. *Mol Biol Cell*. 19:2885-2896.

Tolliday, N., L. VerPlank, and R. Li. 2002. Rho1 directs formin-mediated actin ring assembly during budding yeast cytokinesis. *Curr Biol*. 12:1864-1870.

Tong, Z., X.D. Gao, A.S. Howell, I. Bose, D.J. Lew, and E. Bi. 2007. Adjacent positioning of cellular structures enabled by a Cdc42 GTPase-activating protein-mediated zone of inhibition. *J Cell Biol*. 179:1375-1384.

Ubersax, J.A., E.L. Woodbury, P.N. Quang, M. Paraz, J.D. Blethrow, K. Shah, K.M. Shokat, and D.O. Morgan. 2003. Targets of the cyclin-dependent kinase Cdk1. *Nature*. 425:859-864.

Wai, S.C., S.A. Gerber, and R. Li. 2009. Multisite phosphorylation of the guanine nucleotide exchange factor Cdc24 during yeast cell polarization. *PLoS One*. 4:e6563.

Weiss, E.L., A.C. Bishop, K.M. Shokat, and D.G. Drubin. 2000. Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. *Nat Cell Biol*. 2:677-685.

Wu, C., V. Lytvyn, D.Y. Thomas, and E. Leberer. 1997. The phosphorylation site for Ste20p-like protein kinases is essential for the function of myosin-I in yeast. *J Biol Chem*. 272:30623-30626.

Wu, R., W. Haas, N. Dephoure, E.L. Huttlin, B. Zhai, M.E. Sowa, and S.P. Gygi. 2011. A large-scale method to measure absolute protein phosphorylation stoichiometries. *Nature methods*. 8:677-683.

Yoshida, S., K. Kono, D.M. Lowery, S. Bartolini, M.B. Yaffe, Y. Ohya, and D. Pellman. 2006. Polo-like kinase Cdc5 controls the local activation of Rho1 to promote cytokinesis. *Science*. 313:108-111.